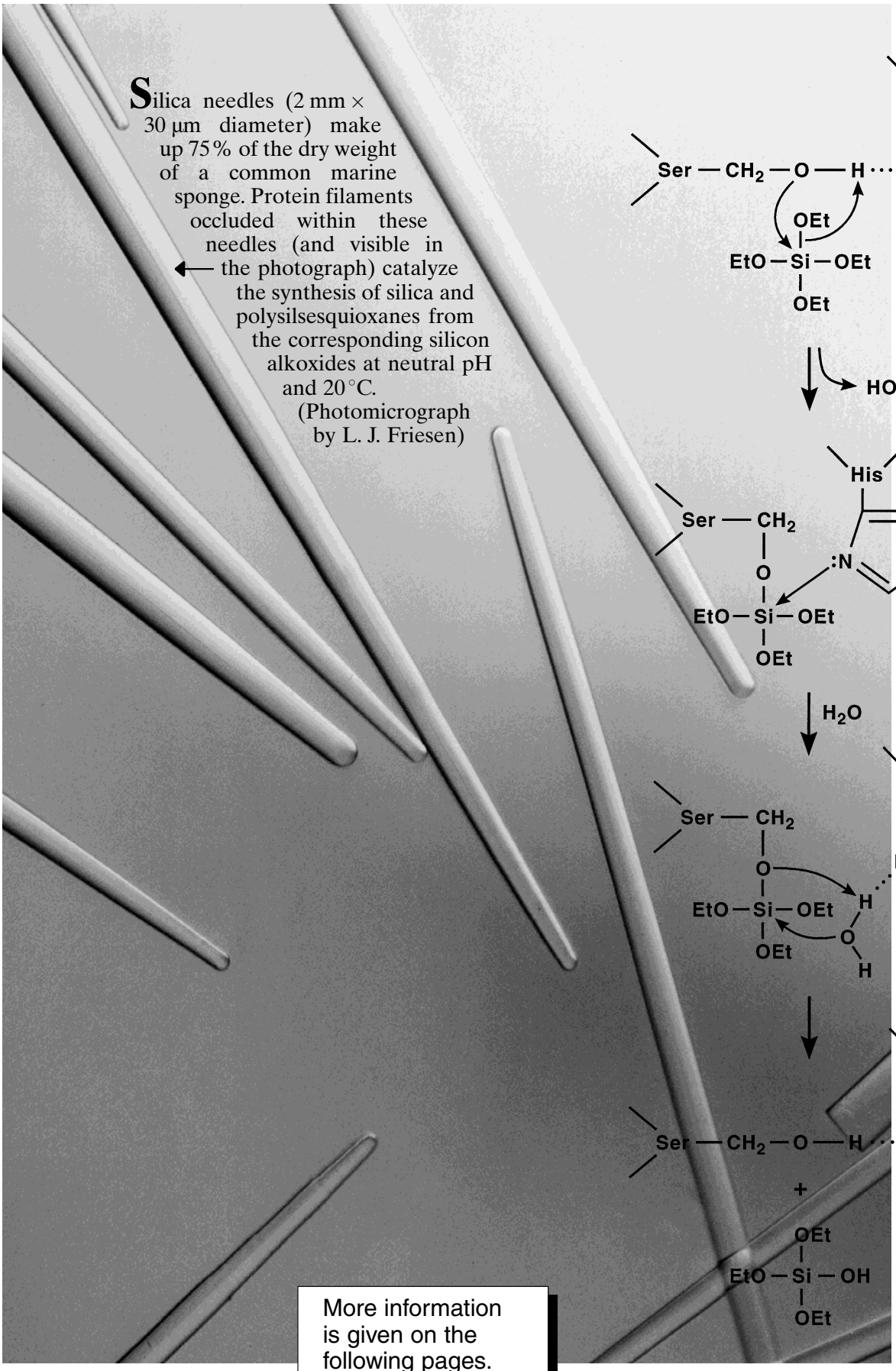

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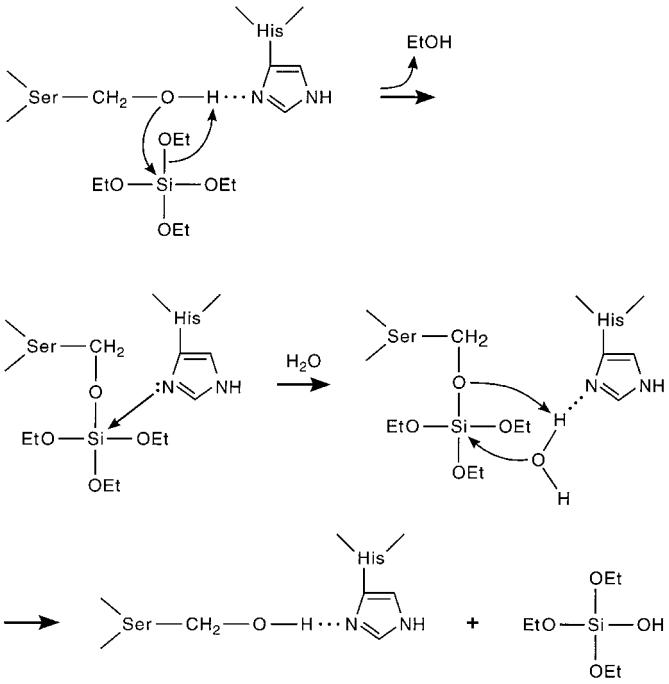
Efficient Catalysis of Polysiloxane Synthesis by Silicatein α Requires Specific Hydroxy and Imidazole Functionalities**

Yan Zhou, Katsuhiko Shimizu, Jennifer N. Cha, Galen D. Stucky, and Daniel E. Morse*

Many living marine organisms synthesize large quantities of shape-controlled silica under mild physiological conditions and at temperatures close to 0 °C.[1] This is in marked contrast to the conditions of industrial and geochemical synthesis of silica and other polysiloxanes that typically require extremes of pH, elevated temperatures, or both. We recently found that the proteins occluded within the macroscopic silica needles made by a marine sponge can catalyze the synthesis of silica and organically modified silsesquioxanes from the corresponding silicon ethoxides at neutral pH and low temperature.[2, 3] In addition to this catalytic activity, we observed that when the proteins (that we named "silicateins", for silica proteins) are assembled into macroscopic filaments (2 mm × approx. 2 μm diameter), they serve as scaffolds that spatially direct the synthesis of the polysiloxanes over the surface of the protein filaments. These observations suggested that the biochemical mechanism of catalysis of polysiloxane synthesis mediated by the silicateins might be useful as a model for new routes to synthesis of silicon-based polymers and materials under environmentally benign conditions.

Silicatein α, the principal subunit comprising about 70% of the mass of the silicatein filaments, exhibits a previously unexpected high structural homology to the well-known hydrolytic enzyme, cathepsin L (a protease), suggesting a possible mechanism for its catalysis of polysiloxane synthesis.[2, 3] The other two silicatein subunits comprising the protein filaments also are similar.[2] The close relationship between silicatein α and the hydrolytic enzyme includes high similarities between their amino acid sequences, three-dimensional structures, and membrane-enclosed intracellular localizations. Because the rate of condensation of the silicon alkoxides is limited by the hydrolysis of the alkoxide (usually catalyzed by acid or base),[4] we proposed that silicatein α catalyzes this reaction at neutral pH through the activity of the serine and histidine side chains that occupy positions corresponding to the catalytically active, functionally related side chains in the proteolytic enzymes of both the cathepsin L (cysteine – histidine) and trypsin/chymotrypsin (serine – histidine) types.[3] This suggested mechanism of catalysis (Scheme 1), in which the putative active site serine-26 and histidine-165 side chains interact across the substrate binding cleft to form a general acid/base catalyst, is based on the high structural homology between silicatein and the corresponding proteases, the well-known mechanism of hydrolysis of the peptide bond by the corresponding side chains of the proteolytic enzymes (which also act as general acid/base catalysts),[5] and the requirement for silicon alkoxide hydrolysis to initiate polysiloxane condensation. We present here direct experimental evidence supporting the proposed role of the specific serine and histidine side chains in the mechanism of catalysis of polysiloxane synthesis by silicatein α.



Scheme 1. Key role of the hydroxy group of serine-26 and the imidazole side chain of histidine-165 in the mechanism proposed for silicatein-mediated catalysis. Catalysis of the rate-limiting step in alkoxysilane polycondensation at neutral pH is proposed analogous to the mechanism of peptide bond hydrolysis by the homologous protease.[3] Hydrogen bonding between the hydroxymethyl and imidazole side chains of serine-26 and histidine-165 increases nucleophilicity of the serine oxygen atom; attack on the silicon atom of the tetraethoxysilane is suggested to form a transitory protein-substrate intermediate that potentially would be stabilized as a pentavalent silicon species through a donor bond from the imidazole nitrogen atom. Hydrolysis of this intermediate releases the silanol product and regenerates the serine – histidine pair.

We produced structural variants of the silicatein α protein in which a) the serine residue (with a hydroxymethyl side chain) at position 26, and b) the histidine residue (with an imidazole side chain) at position 165 were specifically

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replaced with an alanine residue (bearing a methyl side chain). These variants were produced by the method known as site-directed mutagenesis, in which the cloned recombinant DNA coding for the silicatein α is modified in a site-specific manner in vitro, and the mutant DNAs then used as templates to direct the synthesis of the corresponding proteins in bacteria. Quantitative comparison of the catalytic activities of the resulting substituted proteins to the activity of the original protein supports the suggestion that both serine-26 and histidine-165 of the silicatein α are required for efficient catalysis of silica synthesis from silicon tetraethoxide at neutral pH (Figure 1). Additional data from these experiments verify the specificity of the catalysis of silica synthesis

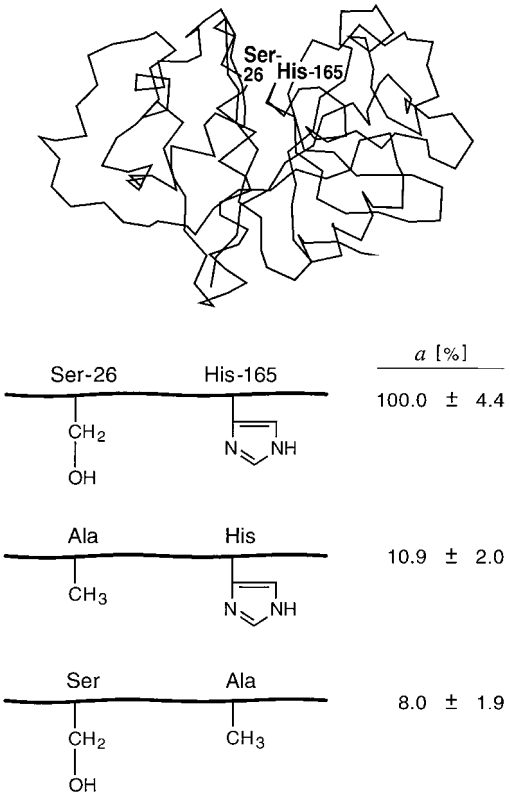

Figure 1. Relative catalytic specific activities (a) of the original and substituted silicatein α proteins. Pictured above is the topology of the protein, based on the three-dimensional structure of the highly homologous cathepsin L, and constrained by three intramolecular disulfide crosslinks at the same positions in the two proteins. Note the close juxtaposition of serine-26 and histidine-165 across the substrate-binding cleft. The three lines below illustrate schematically the protein backbones of the original protein and the two variants produced by site-directed mutagenesis to substitute the methyl side chain of an alanine (Ala) residue in place of the side chains of serine-26 (Ser-26) and histidine-165 (His-165), respectively. Relative catalytic specific activities were determined as described in the Experimental Section.

by silicatein α under the conditions employed. Thus, thermal denaturation, that collapses the three-dimensional structure of the protein, significantly reduces the activity of silicatein α (leaving only $6.3 \pm 1.4\,\%$ residual catalytic specific activity), proving that the efficient catalytic activity is dependent on the precise three-dimensional conformation of the native protein. Furthermore, the maltose binding protein, whose gene was used as a "carrier" for the silicatein α gene in the cloning and protein expression (cf. Experimental Section), after purification from the fusion protein, lacks significant activity in the catalysis of silica synthesis from the alkoxide under the conditions employed ($5.4 \pm 1.6\,\%$ catalytic specific activity relative to that of the native silicatein α). The catalytic activity of the mutant proteins is reduced by an order of magnitude (Figure 1), to a residual value somewhat higher than that of the heat-denatured protein. Similarly, site-directed mutagenesis replacing the active-site serine and histidine side chains of the proteases that have been shown to depend on these moieties for their acid–base catalysis also leaves residual activities higher than exhibited by the corresponding controls.[6] As shown to be the case with the proteases, this suggests that catalytic efficiency depends on the synergistic interaction between the active-site side chains (and an asparagine side chain that we have not altered),[6, 7] and that neither substitution alone is sufficient to remove all traces of activity.

The results reported here demonstrate that the functional hydroxy and imidazole side chains of serine-26 and histidine-165, which were identified from the structure of silicatein α as its putative active site, are required for the protein's efficient and specific catalysis of alkoxysilane polycondensation at neutral pH. These observations, and further definition of the structural determinants of the control of the polymerized product by the silicatein molecule, may prove useful in the design of synthetic catalysts for environmentally benign routes to the synthesis of silica and organically modified polysilsesquioxanes.

Experimental Section

All molecular genetic methods were standard.[8, 9] DNA coding for the silicatein α mature protein was obtained by selective enzymatic replication from its original cloning vector[2] and integrated into a recombinant bacterial plasmid DNA to produce a fusion protein in which the silicatein α protein is coupled to MBP (maltose binding protein). After verification of the sequence of the fusion protein, and of the ability of the recombinant DNA to direct the synthesis of high quantities of the fusion protein in cells of the bacterium, *Escherichia coli*, variants of the protein were produced by site-directed mutagenesis[10] to yield altered silicateins in which the serine at position 26 and the histidine at position 165 were specifically replaced with alanine residues (Figure 1). These specific replacements were accomplished by: a) synthesis of oligonucleotides which correspond exactly to the sequences flanking the sites to be replaced, and contain the oligonucleotides coding for alanine in place of the sequence coding for either the serine or histidine; b) molecular hybridization between the replacement oligonucleotides and the complementary single-strand DNA of the fusion-protein plasmid; c) enzymatic replication of the resulting double-stranded intermediate in vitro to produce both the mutant and "wild-type" (native, original) fusion-protein gene; and d) isolation of the sequence-replaced mutant DNA, and confirmation of its identity and structure by direct DNA sequence analysis. The two mutant DNAs and the original native DNA then were introduced separately into cultures of bacteria that were grown in parallel to overexpress the native or mutant silicatein proteins encoded by the recombinant DNAs. Controls verified that no silicatein (or silica-polymerizing activity) is produced from bacteria without the recombinant DNA. After lysis of the bacteria, the silicatein–MBP fusion proteins were purified by maltose-affinity chromatography;[11] the silicatein proteins were released from the carrier MBP by site-specific cleavage with a highly purified proteolytic enzyme, and the released silicateins and MBP individually purified by conventional means. The

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purified silicateins then were reconstituted to their native form by dialysis (at 2 °C) from buffered guanidinium hydrochloride and mercaptoethanol to permit proper refolding and formation of intramolecular disulfide bonds. The reconstituted proteins then were immediately adjusted to equivalent protein concentrations and assayed in triplicate for their catalysis of silica synthesis from tetraethoxysilane at neutral pH (1 h, 20 °C) under conditions described previously.[3] The silica product was washed by centrifugation, dried by evaporation and quantified colorimetrically with the molybdate reagent after hydrolysis with alkali. Results are expressed as relative specific activities of alkoxysilane polycondensation after correction for the yield of the uncatalyzed reaction. Under these conditions, the average specific activity of the native reconstituted silicatein α protein was 140.0 ± 6.2 nmol silica synthesized per hour per 60 μg protein; the control value obtained in the absence of any protein was 6.7 ± 2.1 nmol silica synthesized per hour.

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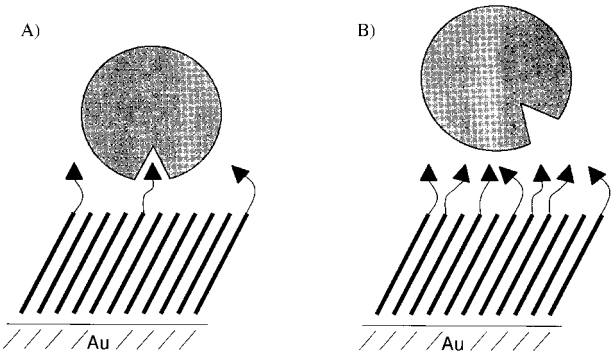
The Role of Ligand Density in the Enzymatic Glycosylation of Carbohydrates Presented on Self-Assembled Monolayers of Alkanethiolates on Gold**

Benjamin T. Houseman and Milan Mrksich*

The enzymatic modification of immobilized carbohydrates is important in protein trafficking,[1] viral and bacterial pathogenesis,[2] and cell migration.[3] Many model systems, including those that use polymers,[4] dendrimers,[5] and liposomes[6] to present carbohydrates, have provided information on the interactions between proteins and immobilized sugars. These systems, however, present carbohydrates in a heterogeneous environment and offer little flexibility in tailoring the structure of groups surrounding the ligands. These limitations make mechanistic studies of enzymatic processes at interfaces difficult.

Self-assembled monolayers (SAMs) of alkanethiolates on gold are structurally well defined substrates that represent an excellent model system for studies in bio-interfacial science.[7] Several reports have utilized SAMs to examine the interactions between immobilized ligands and proteins in solution. It is clear from much of this work that the accessibility of a ligand will influence the ability of a protein to bind it.[8a–d] Ligands immobilized at higher densities,[8e–f] for example, may have biological properties that are substantially different from those of the same ligand presented at a low density (Scheme 1). Here we report the use of mixed SAMs that present *N*-acetylglucosamine (GlcNAc) and tri(ethylene



Scheme 1. Ligands immobilized at low density (A) should be more accessible for binding to protein than are ligands immobilized at high density (B).

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