Roles of larval sea urchin spicule SM50 domains in organic matrix self-assembly and calcium carbonate mineralization

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A B S T R A C T

The larval spicule matrix protein SM50 is the most abundant occluded matrix protein present in the mineralized larval sea urchin spicule. Recent evidence implicates SM50 in the stabilization of amorphous calcium carbonate (ACC). Here, we investigate the molecular interactions of SM50 and CaCO₃ by investigating the function of three major domains of SM50 as small ubiquitin like modifier (SUMO) fusion proteins – a C type lectin domain (CTL), a glycine rich region (GRR) and a proline rich region (PRR). Under various mineralization conditions, we find that SUMO CTL is monomeric and influences CaCO₃ mineralization, SUMO GRR aggregates into large protein superstructures and SUMO PRR modifies the early CaCO₃ mineralization stages as well as growth. The combination of these mineralization and self assembly properties of the major domains synergistically enable the full length SM50 to fulfill functions of constructing the organic spicule matrix as well as performing necessary mineralization activities such as Ca²⁺ ion recruitment and organization to allow for proper growth and development of the mineralized larval sea urchin spicule.

Keywords:

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1. Introduction

An underlying organic matrix can be found in all biominerals ranging from silica to calcium as well as iron carbonates, just to name a few varieties (Falini et al., 1996; Metzler et al., 2010; Wang and Li, 2002; Gur et al., 2013; Sel et al., 2012). And in each case, the organic component varies widely from one mineral to another, as well as within the mineral system, from species to species. The role of this organic component in biominerals has been explored by many groups (Belcher et al., 1996; Fratzl and Weinkamer, 2007; Hao et al., 2007; Weiner and Wagner, 1998; Amos et al., 2011; Evans, 2012; Fang et al., 2011; Metzler et al., 2010). These roles include functional requirements such as enhancement of mechanical performance and materials properties elasticity, strength, toughness (Fratzl and Weinkamer, 2007; Weiner and Wagner, 1998). In the endeavor to understand what makes these materials ‘strong’ and ‘high performance’, several groups have looked to the use of in situ mutagenesis in these mineral systems as well as

creating in vitro studies that mimic the conditions in which mineralization occurs (Amos et al., 2011; Ndao et al., 2010). Some have ascertained the roles of these organic components to include the templating for the respective mineral components, stabilization of amorphous mineral phases, as well as inhibition of mineralization (Falini et al., 1996; Foo et al., 2004; Gong et al., 2012; Metzler et al., 2010; Radha et al., 2010; Wang and Li, 2002). In complementing these studies, we investigate the well established developmental model system of the larval sea urchin *Strongylocentrotus purpuratus* and study the mineral organic interactions of the spicule matrix protein (SMP) SM50 found in larval spicule formation.

The diversity of the organic matrix found in the larval sea urchin spicule is surprising. Over 200 proteins are found in the larval spicule (Mann et al., 2010), which make up less than 0.1% w/v of the entire spicule (Wilt and Killian, 2008). The interest in examining these proteins in the sea urchin spicule can be observed from the single crystalline properties of the spicule, while the superstructure of a single spicule is observed to be smooth and curved, without providing evidence of step edges nor clues of its single crystalline properties. Politi et al. (2007) showed that adult sea urchins have the ability to regenerate their spines through an amorphous mineral precursor phase. Overgrowth experiments of the larval spicule in supersaturated solutions of calcium carbonate, reveal the preferential C axis alignment of the individual calcite

Abbreviations: SM50, spicule matrix protein 50; CTL, C-type lectin; GRR, gly-rich region; PRR, pro-rich region; SUMO, small ubiquitin-like modifier; IDP, intrinsically disordered protein.

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crystals that form the larval spicule (Beniash et al., 1997). In both of these studies, observations of a component which can stabilize amorphous mineral phases as well as organize mineral at the nanoscale level requirements which lead finally to the formation of a mesocrystalline structure of both calcite and amorphous calcium carbonate even in the adult spine (Seto et al., 2012). Wilt and coworkers have examined the organic matrix and revealed two large families of matrix proteins that are found during the first occurrence of mineral in the spicular mineralization space (Wilt and Killian, 2008; Katoh Fukui et al., 1991; Sucov et al., 1987). These protein families, SM30 and SM50, have common motifs, more specifically, a C type lectin domain, as well as regions with long amino acid repeats. Specifically, these long amino acid repeats have recently been implicated in various functions ranging from mediators of multiple binding interactions as well as chaperones in signaling (Uversky, 2002; Uversky et al., 2000; Uversky et al., 2008).

Specifically, the SM50 family is the most abundant occluded SMP found in the mineralized spicule. The primary sequences of both SM50 and SM30 families consist of homologous sequence motifs, mainly C type lectin domains and intrinsic disordered repeat domains (Livingston et al., 2006). These C type lectin domains have been implicated in many activities including complex macro molecule assembly, carbohydrate complexation, as well as receptor signaling processes. Interestingly, C type lectin domains in the genus *Strongylocentrotus* have been implicated in immunity responses and suggest that these domains have been co opted for biomineralization purposes through evolution (Gross et al., 1999; Silva, 2000; Smith, 2005). As is often the case, the C type lectins discussed here require proper amounts of Ca^{2+} ions to fold properly for correct structural conformations (Diao and Tajkhorshid, 2008; Fuerst et al., 2013). In contrast, numerous intrinsically disordered repeat (IDP) domains are found in the SMPs and have no such necessity of conformation for proper function. IDP domains are natively structurally flexible due to their composition of random repeats of various single amino acids, often charged in nature including aspartic acid (D) (Ndao et al., 2010) and glutamic acid (E) (Nonoyama et al., 2012), non charged in the case of serine (Ser), leucine (Leu), asparagine (Asp), or just glycine (Gly) and proline (Pro). The relevance of these IDPs have been underestimated and recently, their impact in understanding the regulation and processing of the proteome has been found to be essential (Uversky, 2002; Uversky et al., 2000; Kalmar et al., 2012). Functionally, IDPs have been connected to mis folding and signaling of proteins that are involved in cardiovascular and neurodegenerative diseases (Uversky et al., 2008). Our study will focus on the major structural domains found in SM50 as well as IDP domains, in order to delineate their contributions to the proper function of the full length SM50 in the biomineralization of the larval sea urchin spicule.

We describe here the expression of major SM50 domains (C type lectin, glycine rich repeat, and proline rich repeat domains) as small ubiquitin like modifier (SUMO) fusion proteins. In order to better understand the function of the full length SM50 in the biomineralization of the larval sea urchin spicule, we investigate the solution behaviors as well as the effects on the early stages of mineralization by vapor diffusion mediated growth of calcium carbonate of the major domains of SM50 as SUMO fusion proteins.

2. Materials and methods

2.1. Cloning and protein purification

Phusion Hi Fidelity Taq polymerase, T4 DNA ligase, restriction enzymes (New England Biolabs, Ipswich, MA, USA) and NTA Agarose (Qiagen GmbH, Hilden, Germany) were used for cloning and

protein purification. L Arginine (reagent grade, $\geq 98\%$) was purchased from Sigma Aldrich.

2.2. Mineralization experiments

Calcium chloride (Fluka, 1 M volumetric solution), sodium hydroxide (Alfa Aesar, 0.01 M standard solution), hydrochloric acid (Merck, 1 M standard solution), sodium carbonate (Aldrich, anhydrous, A.C.S grade), sodium bicarbonate (Riedel de Haën, A.C.S) were used for gas diffusion and titration experiments. For potentiometric titration measurements, a carbonate buffer composed of a mixture of 10 mM sodium carbonate and 10 mM sodium bicarbonate mixed such that the pH = 9.75 was used.

2.3. Bioinformatics analysis

The prediction of protein disorder in SM50 (*S. purpuratus*, NCBI accession No. NM_214610.2) was accomplished by using DISOPRED, IUPRED and RONN algorithms (Dosztányi et al., 2005; Wang et al., 2009; Yang et al., 2005). And the biochemical properties such as isoelectric point and amino acid composition were calculated using PROTPARAM (Gasteiger et al., 2005). The putative domains for proteins were predicted using the SMART server (Schultz et al., 1998). SignalP4.1 was used to identify the signal peptide sequences (Petersen et al., 2011).

2.4. Cloning and protein expression

Based on the results of disorder prediction, three regions were identified, namely a 13.6 kDa C type lectin domain (CTL), a 27.2 kDa glycine rich region (GRR) and a 3.9 kDa proline rich region (PRR). These domains were intracellularly expressed as 6XHis SUMO fusion products using *Escherichia coli*. These are appropriately referred to SUMO CTL, SUMO GRR and SUMO PRR, respectively. Cloning of SM50 cDNA templates was performed in the Center for Regulatory Genomics, Beckman Institute and the Eric Davidson Lab, Division of Biology at Caltech. The pET24a backbone based plasmid for expression of SUMO fusion proteins was provided by Prof. Elke Deuerling (Department of Biology, University of Konstanz, Konstanz, Germany). Primers were designed as per the gene sequence of SM50 (NM_214610.2) using OligoExplorer 1.5 and OligoAnalyzer 1.5. The restriction sites for enzymes *BsaI* and *XhoI* were used in the forward and reverse primers, respectively (Table 1). Initial constructs were made in *E. coli* DH5 α . Standard molecular biology procedures were followed (Sambrook et al., 1989). The transformants were selected by plating on Luria agar ampicillin plates. The sequences of the constructs obtained were verified by capillary sequencing (GATC Biotech GmbH, Konstanz, Germany). The expression plasmids were transformed into competent *E. coli* BL21 CodonPlusRIL (Stratagene, Agilent Technologies Inc., Santa Clara, USA) cells and grown in Luria broth supplemented with ampicillin (100 $\mu\text{g}/\text{mL}$) and chloramphenicol (35 $\mu\text{g}/\text{mL}$). After attaining an absorbance of 0.6 O.D., induction of cell culture was done by using isopropyl β -D-thiogalactopyranoside (IPTG, 0.5 mM) at 20 °C.

Table 1

Primers used for cloning of SM50 fragments. Restriction sites *BsaI* and *XhoI* are indicated in italics.

Primer name	Sequence
FP-CTL	CCAGTGGGTCTCAGGTGGTACGGGTCAAGAC
RP-CTL	CCAGCTCGAGTCAGGGCCAGCTACG
FP-GRR	CCAGTGGGTCTCAGGTGGTGTCAACCTCAGAACCCAT
RP-GRR	CCAGCTCGAGTCATTGCTGGCCACCCAT
FP-PRR	CCAGTGGGTCTCAGGTGGTATGGGTGGCCAGCAA
RP-PRR	CCAGCTCGAGTCACTCTTGAAGCATACG

2.5. Protein purification

In the purification of both SUMO CTL and SUMO PRR, cell lysis was achieved by lysozyme treatment in HEPES/sodium chloride (NaCl)/ β mercaptoethanol (β ME) buffer containing HEPES (20 mM), NaCl (50 mM) and β ME (10 mM). The lysis buffer also contained pepstatin A (10 μ g/ml) and phenylmethylsulfonyl fluoride (0.5 mM) to inhibit proteolysis. The lysate was centrifuged at 16,000 rpm (30,000g) for 30 min and the supernatant was loaded on a Ni^{2+} charged NTA (nitrilotriacetic acid) agarose column for immobilized metal affinity chromatography (IMAC). After an initial wash with 0.5 mM imidazole, the protein of interest was eluted by using 20 mM imidazole prepared in HEPES/NaCl/ β ME buffer. Final purification was accomplished by size exclusion chromatography using a Superdex 75 column (HiLoad 16/60, Amersham Biosciences, Pittsburgh PA USA) on an Akta FPLC system.

The purification of SUMO GRR was performed from the lysate pellet. Initially, the pellet was dispersed in Triton X PBS (phosphate buffered saline) by sonication and the mixture was centrifuged at 16,000 rpm (30,000g) for 20 min. This procedure was repeated three times with the pellet to remove residual soluble proteins. The resulting pellet was dispersed in Arg (0.5 M) solution and incubated for 2 h at 4 °C. Arg is known to improve protein solubility and inhibit protein aggregation (Golovanov et al., 2004; Tsumoto et al., 2004). The protein mixture was then centrifuged at 5000 rpm (3000g) for 15 min and the solubilized protein was further purified by IMAC as described above. The elution buffer contained imidazole dissolved in Arg (0.5 M). The fraction containing the protein of interest was dialyzed against carbonate buffer (50 mM, pH 9.75) for 16 h at 4 °C.

Protein estimation was performed by using the Bradford assay (Bradford, 1976). SDS polyacrylamide gel electrophoresis (SDS PAGE) of the protein fractions were performed using 14% acrylamide gels followed by staining with Coomassie Blue. The protein samples were desalted and concentrated by using 3000 MWCO Vivaspinn centrifugal concentrators (Sartorius Stedim Biotech GmbH, Göttingen, Germany). This was followed by solvent exchange by prolonged dialysis in either Milli Q (EMD Millipore Inc., Billerica, MA USA) water or carbonate buffer (10 mM, pH 9.75). These protein samples in water were used for gas diffusion assays and those prepared in carbonate buffer were used for circular dichroism, dynamic light scattering and titration studies.

During purification, SUMO CTL and SUMO PRR were soluble and were purified by conventional IMAC and SEC steps (SI Fig. 1). Whereas, SUMO GRR was initially insoluble; however, in the presence of Arg (0.5 M), it displayed improved solubility. A proposed mechanism for Arg induced solubility is the binding of the amino acid methylene groups to hydrophobic surfaces on the protein. This results in masking the exposed hydrophobic surfaces and inhibiting protein aggregation (Cantaert et al., 2012). It is reasonable that similar hydrophobic interactions exist between the

glycine rich regions of SM50 monomers resulting in the aggregation and poor solubility of the native protein. After purification, the SDS PAGE profiles of the proteins displayed single bands at the expected molecular masses (Fig. 1C). Bands were observed at approximately 26, 41 and 19 kDa for the SUMO CTL, SUMO GRR and SUMO PRR samples, respectively.

2.6. Vapor/gas diffusion

Calcium carbonate (CaCO_3) was precipitated from 0.2 mL drop lets of mixtures in 96 microwell plates; containing calcium chloride (CaCl_2) and protein (0.01 mg and 0.1 mg) using a gas diffusion set up. The droplets were exposed to CO_2 generated by decomposition of ammonium carbonate ($(\text{NH}_4)_2\text{CO}_3$) in a sealed desiccator. After 48 h of incubation, the calcium carbonate particles were analyzed by a desktop SEM (Hitachi TM 3000 SEM, Hitachi High Technologies Europe GmbH, Krefeld, Germany) equipped with an energy dispersive detector (Xcite, Bruker AXS GmbH, Berlin, Germany). During the gas diffusion experiments, the initial and final pH were 6.0 and 9.6, respectively.

2.7. Dynamic light scattering

Dynamic light scattering (DLS) experiments were performed with a custom built setup equipped with a goniometer, temperature control (0.05 K step control), an attached single photon detector ALV 6000/E, and a multiple tau digital correlator ALV 5000/FAST from ALV (ALV GmbH, Langen, Germany) as described in previous investigations (Cölfen, 2001). The protein solutions with different concentrations of calcium chloride were syringe filtered through 0.2 μ m pore size membrane filters (Whatman GmbH, Dassel, Germany) and their particle size distributions were determined.

2.8. Analytical ultracentrifugation

Sedimentation velocity experiments were performed using a Beckman XL I ultracentrifuge (Beckman Coulter Inc., Palo Alto, CA USA). The moving boundary was monitored by repetitive radial scanning at 280 nm using a UV absorption optical system. The sedimentation velocity datasets were analyzed using the software program SEDFIT (P. Schuck, NIH, Bethesda, Md USA) to generate sedimentation coefficient and molar mass distributions (Schuck, 2000). In addition, 2 dimensional spectrum analysis (2 DSA, Monte Carlo) with 20 iterations and grid resolution of 3600 was performed by using the UltraScan III software (B. Demeler, UltraScan Version 3.0, University of Texas Health Science Center at San Antonio, Department of Biochemistry) (Brookes et al., 2010). The solvent density, solvent viscosity and protein partial specific volumes were also calculated from the amino acid respective solvent composition with UltraScan. Axial ratios of prolate

Fig. 1. Primary and secondary structures of the domains in SM50. (A) Local disorder in SM50 predicted using DISOPRED (green), IUPRED (red) and RONN (blue) algorithms. (B) Schematic representation of the SM50 domains: secretion signal (1–17), C-type lectin domain (18–138, green), glycine rich region (139–410, blue) and proline rich region (411–440, grey). (C) Representative SDS-PAGE profile of purified proteins: Lane 1 standard molecular weight marker Lane 2 SUMO-CTL Lane 3 SUMO-GRR and Lane 4 SUMO-PRR proteins.

ellipsoids were predicted for given frictional ratios f/f_0 using the simulation option of Ultrascan.

2.9. Transmission electron microscopy

For sample preparation, an aliquot (5 μ L) of the protein solution was spotted on a TEM grid (Au, Formvar coated; Ted Pella, Inc., Redding, CA USA). Any excess liquid was blotted off and the grid was allowed to air dry for 12 h. TEM imaging was performed using a Libra 120 (Zeiss SMT GmbH, Oberkochen, Germany) transmission electron microscope at 120 kV with a beam current of 4 μ A. TEM micrographs were analyzed with the Olympus Soft Imaging Viewer (Olympus Soft Imaging Solutions GmbH, Muenster, Germany).

2.10. Circular dichroism

Circular dichroism (CD) experiments were performed on a Jasco J 715 Spectropolarimeter with a Peltier temperature control unit (Jasco International Co. Ltd., Tokyo, Japan) and using 1 cm quartz cuvettes. The spectra were recorded at 0.5 nm intervals between 190 and 240 nm at 20 °C, at 20 nm/s scan rates. The secondary structure conformations were predicted from the measured spectra with the application K2D3 (Greenfield, 2006; Louis Jeune et al., 2012).

2.11. Potentiometric titration

Potentiometric titration experiments were performed by using a computer controlled titration system (Metrohm GmbH, Filderstadt, Germany) and Tiamo software (Tiamo v2.2, Metrohm GmbH, Filderstadt, Germany). During the titration measurements, the pH and free Ca^{2+} concentrations were monitored by utilizing a flat membrane glass electrode (for pH) and a polymer based ion selective electrode (for Ca^{2+} selection), respectively. The CaCl_2 (10 mM) solution was added at a constant rate of 0.01 mL/min to 10 mL of the protein solution in carbonate buffer (10 mM), which was under constant stirring with a magnetic stir bar at 900 rpm. A constant

pH of 9.75 was maintained by automatic counter titration of NaOH (10 mM). Calibration and reference experiments were performed by dosing CaCl_2 (10 mM) into water (pH 9.75) and carbonate buffer (10 mM, pH 9.75), respectively. All titration experiments were performed at room temperature.

2.12. Fourier transformed infrared spectroscopy (FTIR)

The titration mixtures were quenched in pure ethanol as described previously (Gebauer et al., 2010). Pellets were collected by centrifugation at 5000 rpm (3000g) for 30 min. FTIR analysis of the vacuum dried pellets was done using a Perkin Elmer Spectrum 100 spectrometer (Perkin Elmer Inc., Waltham, MA USA) using an attenuated total reflection (ATR) configuration.

3. Results and discussion

3.1. Bioinformatics analysis

In order to rationally define the major functional regions of SM50, bioinformatics tools were applied to identify truncated signal sequences as well as local regions of the full length protein for protein disorder. The first 17 amino acids were identified to be a secretion signal by using SignalP 4.1 Server (Petersen et al., 2011) to predict the presence and location of signal peptide cleavage sites in a given sequence. The analysis of the SM50 sequence using disorder prediction tools showed lack of structure after the first 100 amino acids of the protein (Fig. 1A). This is in agreement with previous studies where after removal of the repeat region sequences from the full length SM50, the residual sequence displayed about 35% identity with a family of C type lectins (Harkey et al., 1995). This C type lectin domain in SM50 also harbors a proline rich site towards its C terminal.

Within the region predicted to be disordered, the C terminal contains a Pro and Asn rich region that is 20 residues in length. This short domain contains tandem repeats and adopts an

Fig. 2. Continuous sedimentation coefficient and molar mass distribution from sedimentation velocity experiments of (A and D) SUMO-CTL (B and E) SUMO-GRR and (C and F) SUMO-PRR, respectively from SEDFIT analysis.

extended conformation in solution as shown by solution state NMR studies (Zhang et al., 2000). Recent mass spectrometry based analyses of whole organic extracts from sea urchin spicules reported a protein (NCBI Accession XP_786023) which contains a SH3 domain (predicted using SMART, E value 7.34×10^{-15}) (Mann et al., 2010). SH3 domains are known for their regulatory activity by binding to proline rich sites (Aitio et al., 2010; Alexandropoulos et al., 1995; Ren et al., 1993). Hence, it is possible that the proline rich region of SM50 is involved in such protein protein interactions during protein transport. The third region of SM50 lie between residues 139 and 410. It is marked by Gly and Gln rich repeats and its secondary structure is expected to have high β turn content (Xu and Evans, 1999). Thus, these three regions of interest were expressed as SUMO fusion proteins include (i) the C type lectin (CTL) domain, (ii) Gly rich region (GRR) and (iii) Pro rich region (PRR) as shown in Fig. 1B. Although bioinformatics based glycosylation prediction indicates O linked glycosylation at residues 122, 123, 124, 127, 136 and 138 and no N linked glycosylation (SI Fig. 2), previous studies have indicated SM50 to be non glycosylated protein at the repeat regions (Killian and Wilt, 1996) (Livingston et al., 2006). Hence, a prokaryotic system was selected for protein expression. The SUMO fusion system was selected due to advantages in expression and purification such as improved protein folding, enhanced expression and having flexible N and C termini (Butt et al., 2005).

3.2. Analytical ultracentrifugation

The sedimentation coefficient and molar mass distributions of the SUMO fusion proteins were obtained by fitting the experimental data to the Lamm equation model using SEDFIT (Fig. 2) (assuming partial specific volumes of 0.718, 0.714 and 0.718 ml/g for SUMO CTL, SUMO GRR and SUMO PRR, respectively as calculated from the primary amino acid sequence). The sedimentation coefficient distribution for SUMO CTL revealed a major peak at 2.5 ± 0.4 S (27.3 ± 2.8 kDa) (all errors are $\pm$ standard deviations of Gaussian fits to the experimental distributions) (Fig. 2A and B). This corresponds to a pure monomer taking the molar mass of 12.9 kDa for SUMO and 13.6 kDa for the CTL domain in account. The distributions for SUMO GRR presented a peak at 3.9 ± 0.2 S (39.0 ± 4.1 kDa) as well as peaks at 7.7 ± 3.2 S, 10.7 ± 2.7 S, 13.4 ± 2.9 S, 17.6 ± 2.5 S, 21.6 ± 3.1 S, 29.5 ± 3.2 S and 35.7 ± 3.8 . (Errors are given as standard deviations of a Gaussian fit of the distribution). By using Gaussian multiple peak fitting, the relative concentrations were found to be 22.1%, 21.3%, 11.7%, 10.1%, 9.9%, 6.6%, 5.3% and 4.6% for the monomer and oligomers, respectively (see inset Fig. 2B and E). Furthermore, the data indicated that the glycine rich protein can oligomerize to form large protein assemblies. These exhibited molar masses of the oligomers were 130 ± 55 kDa, 214 ± 57 kDa, 300 ± 62 kDa, 460 ± 57 kDa; 601 ± 107 kDa, 975 ± 103 kDa and 1282 ± 214 kDa. The defined molar mass of the assemblies indicates an irreversible interaction because a reversible interaction on a time scale faster than the duration of the ultracentrifuge experiment of a few hours would result in a single peak comprising the time average of the different oligomers. The protein SUMO PRR displayed a 16.8 ± 3.8 kDa peak at 2.5 ± 0.0 S corresponding to the monomer (3.9 kDa for the PRR domain + 12.9 kDa for SUMO) and the peak at 5.3 ± 0.7 S (85.1 ± 14.3 kDa) likely indicates the pentamer (46%). Self assembly of the proline rich region was possibly due to hydrophobic interactions between the pyrrolidine rings and hydrogen bonding interactions between the Asn residues (Zhang et al., 2000). It must be noted that the AUC data confirms the SDS PAGE results for the monomers shown in Fig. 1C.

To determine the protein domain solution structure via the frictional ratio (f/f_0) of these protein species, the sedimentation velocity data was analyzed by the 2 DSA Monte Carlo method

(Ultrascan, Fig. 3 and SI Fig. 3). As SUMO is known to have a well folded, globular structure (Mueller et al., 2001), a f/f_0 value close to unity can be assumed for the fusion tag. The SUMO CTL fusion protein displayed a peak at about 27 kDa corresponding to the monomer species. This species had a f/f_0 value of 1.1 indicating that the CTL domain had a spherical hydrodynamic shape and globular structure with $a = 1.94$ nm and $b = 1.00$ nm (Fig. 3A). The 2 DSA analysis showed that SUMO GRR and SUMO PRR monomers sediment as single species. While SUMO GRR has a globular structure with $f/f_0 = 1.1$ ($a = 2.1$ nm, $b = 1.9$ nm), the SUMO PRR has a high f/f_0 value of 2.8 (Fig. 3B and C). By approximating the hydrodynamic shape to a prolate ellipsoid of revolution, the axial ratios a/b for SUMO PRR was calculated. These corresponded to axial dimensions of 21.1 and 0.5 nm (a and b values, respectively) for SUMO PRR. This indicated an extended conformation for these monomer species, which is in agreement with previous (Zhang et al., 2000). However, the f/f_0 of 2.8 for SUMO PRR cannot simply be translated into the dimensions of an extended chain via a prolate ellipsoid of revolution since it extends the length of the stretched molecule (10.5 nm for 35 amino acids).

For SUMO GRR, relatively large oligomers (120, 240 and 840 kDa) were observed corresponding to f/f_0 values of 1.6, 1.7 and 3.6, respectively. The aggregates at 120 and 240 kDa have a

Fig. 3. Pseudo 3-dimensional plots for the 2-DSA analysis for (A) SUMO-CTL, (B) SUMO-GRR and (C) SUMO-PRR. Schematic representation of stages in SUMO-GRR assembly is depicted (B, inset).

rod like shape corresponding to an axial ratio of 12.3 and 15.3, respectively. Moreover, the prolate ellipsoid axial ratio for the larger protein assembly (840 kDa) was 80.5. While the smaller oligomers have an extended shape, larger oligomers become more compact and sheet like. Therefore, self assembly of SUMO GRR appears to involve the association of rod like assemblies into sheet like superstructures (Fig. 3B).

DLS of the SUMO fusion proteins was performed to check whether Ca^{2+} ions induce protein oligomerization. The size distributions are presented in Fig. 4A. There was no observable aggregation for the SUMO protein (SI Fig. 4D), SUMO CTL and SUMO PRR proteins in the presence of Ca^{2+} ions. However, SUMO GRR formed particles about 27 ± 8 nm in average size, which increased proportionally with Ca^{2+} ion concentration (95 ± 16 nm in presence of 5 mM CaCl_2). It must be noted that DLS is not a fractionating technique and thus, an average of all oligomers sizes is detected. Previous studies have reported a similar effect for certain biomimetic proteins such as amelogenin and Asprich 3 (Bromley et al., 2011; Ndao et al., 2012). The glycine rich region of SM50 is devoid of highly charged, polar residues such as Asp and Glu. Hence, the charge neutralization based protein oligomers proposed for Asprich 3 (Ndao et al., 2012) may not be true for SUMO GRR. In contrast, the growth of amelogenin oligomers has been attributed to hydrophobic interactions as well as protein disorder (Bromley et al., 2011). Hence, a similar mechanism may exist for the self assembly of the glycine rich region. TEM image of these roughly spherical protein aggregates is depicted in Fig. 5, confirming the aggregate size range as determined by DLS. In addition, taking the high frictional ratio of the oligomers without Ca^{2+} ions into account, TEM micrographs can also be construed as an almost round sheet instead of a sphere. As DLS was performed after filtration of the sample, larger protein aggregates were possibly excluded during these measurements,

3.3. Circular dichroism spectroscopy

Circular dichroism (CD) measurements were performed to investigate the effect of Ca^{2+} ions on protein secondary structure. The far UV CD spectra of the proteins are shown in Fig. 3. Using K2D3, the secondary structure content percentages were predicted for SUMO CTL, SUMO GRR and SUMO PRR, respectively, with respect to different Ca^{2+} ion concentrations (Table 2). An increase in Ca^{2+} concentration led to a decrease in α helix content of SUMO CTL from 33.5% to 27.4%, while the β strand content increased from 19% to 27.4%. Thus, the Ca^{2+} ions induced a change in the secondary structure of SUMO CTL marked by an increase in the β strand content. For SUMO GRR, the α helix content decreased significantly from 17.19% to 5.32% at higher Ca^{2+} concentration, however the β strand content ($33.5 \pm 0.4\%$) did not change significantly. Contrary to a previous study (Xu and Evans, 1999), no bands typical of β turns were detected for the glycine rich domain. This can be an effect of the different solvents used in the studies or the presence of a 'hybrid' CD spectrum on account of different secondary structures that mask the β turn component (Feeney et al., 2003). In the present study, the buffer carbonate anions could have led to intermolecular β sheet formation at expense of the β turns. This effect has been reported in relation to water content for wheat glutenin, a protein containing Gln rich repeats (Feeney et al., 2003). The protein self assembly and enhanced β strand content observed for SUMO GRR are indicative of a similar phenomenon. Livingstone et al. (1991) also predicted a unique β spiral supersecondary structure for pLSM1 from *Lytechinus pictus*, a homologue of SM50. For SUMO PRR, the presence of Ca^{2+} (10 mM) in the protein mixture did not affect the CD spectra, although minor changes in intensity were observed. The spectra displayed a negative band at 206 nm as reported earlier for the CD spectra of a 20 residue peptide domain of the proline rich region (Zhang et al., 2000). This

Fig. 4. Solution studies of SUMO-CTL, SUMO-GRR, and SUMO-PRR (A) Hydrodynamic radii of proteins at different Ca^{2+} concentrations [SUMO-CTL (●), SUMO-GRR (■) and SUMO-PRR (▲)]. Representative CD spectra for (B) SUMO-CTL, (C) SUMO-GRR and (D) SUMO-PRR in presence of different Ca^{2+} concentrations [0 mM (---), 1 mM (—) and 10 mM (.....)].

Fig. 5. Representative TEM image of particles formed after SUMO-GRR self-assembly in the presence of Ca^{2+} ions (5 mM).

confirms that the proline rich domain does not adopt a poly L proline type II conformation. During the CD experiments, there was no visible aggregation in the SUMO CTL and SUMO PRR samples, however SUMO GRR samples displayed an aggregate formation in the presence of Ca^{2+} ions.

From these CD spectra, we conclude that interactions with Ca^{2+} ions lead to structural reordering of SUMO CTL and SUMO GRR. For the latter molecule, this can explain the suggested increased hydrophobic interactions leading to the aggregation upon Ca^{2+} ion addition as observed by DLS on top of the inherent aggregation tendency of this molecule already observed by AUC without the presence of Ca^{2+} ions. Similar conformational changes induced by Ca^{2+} ions have been reported earlier with biomineral associated proteins such as dentin matrix proteins (Hao et al., 2007). The effect of Ca^{2+} ions on the N terminal SUMO tag was also checked (SI Fig. 4E). At 1 mM, there was no significant change in the estimated secondary structure of the SUMO protein, whereas at 10 mM, the α helix content decreased by about 5%.

3.4. SM50 domain influence on CaCO_3 mineralization

The influence of proteins on the growth of calcium carbonate particles was evaluated by gas/vapor diffusion assays. Fig. 6 shows the different morphologies of calcium carbonate particles formed using (A) 0.01 and (B) 0.1 mg protein each at 1 mM and 100 mM CaCl_2 concentrations. At low concentration of SUMO CTL and 1 mM CaCl_2 , rhombohedral crystals typical of calcite were observed with few exhibiting a terraced morphology. This morphology was typical for crystals observed for 0.01 and 0.1 mg of SUMO PRR at 1 mM CaCl_2 as well. An increased concentration of SUMO CTL led to formation of crystals with rounded edges as well as irregular, dumbbell like particles (Fig. 6B(i)). The theoretical pl

of SUMO CTL, SUMO GRR and SUMO PRR are pH 5.37, 10.48, and 6.05, respectively. The absolute charge of these molecules at pH 9.75 is 19.1 for SUMO CTL, +6.7 for SUMO GRR and 7.2 for SUMO PRR. SUMO CTL has a relatively higher negative charge at the pH conditions during the mineralization experiment (pH 9.75). This indicates that electrostatic interactions between SUMO CTL residues and the crystal surface should be weak. However, a previous report also described the formation of calcite with distinct morphologies using positively charged polymers (Cantaert et al., 2012) like the GRR under the applied pH conditions.

With 1 mM CaCl_2 , at low concentrations of SUMO GRR, regular rhombohedral calcite crystals were observed. A higher amount of SUMO GRR led to truncated growth of large crystals as well as displaying step like features. Similar crystal morphology has been reported for calcite grown in the presence of polymers and small molecules such as poly Asp and lysozyme (Politi et al., 2007; Wang et al., 2009). On a few crystal surfaces, pores were also seen (Fig. 6A(iii) and B(iii)). Such features have been reported earlier for calcite crystals grown in the presence of Asprich Fragment 1 proteins and were attributed to irregular crystal growth rates (Delak et al., 2008). At 100 mM CaCl_2 , spheroid like and dumb bell shaped calcium carbonate particles were observed in the presence of both low and high concentrations of SUMO CTL. Few spindle shaped particles were also observed (Fig. 6A(ii)). These can be explained as an intermediate stage in growth of the dumbbell shaped particles (Kniep and Busch, 1996; Xiang et al., 2008).

The SUMO GRR protein has a high theoretical isoelectric point (pl 10.48) and hence, was expected to affect crystal growth on account of its positive charge. The protein mediated the formation of crystals with rounded edges and spherulitic particles at 0.01 and 0.1 mg protein content, respectively, both at 100 mM CaCl_2 concentration. Some spherulites displayed a polycrystalline surface (Fig. 6B(iv)) and are similar to those reported for calcium carbonate precipitated in the presence of egg shell associated and mollusk shell prismatic associated proteins (Ajikumar et al., 2003; Delak et al., 2008; Kim et al., 2004; Pokroy et al., 2007). These results indicate that this protein affects crystal morphology by influencing the early stages of nucleation or directly influencing a growing crystal surface.

With 0.01 mg of SUMO PRR, the calcium carbonate particles displayed splayed surfaces (arrow, Fig. 6A(vi)). This can possibly be due to the protein molecules binding to certain sites on the crystal surface, thereby affecting the kinetics of crystal growth. This effect was more pronounced at higher protein concentration (Fig. 6B(vi)). In addition, these particles also exhibited an irregular dumbbell like shape. Hence, it was seen that all proteins containing domains of SM50 affected the morphology of calcium carbonate particles, especially at higher protein concentrations. The morphologies at high CaCO_3 and protein concentrations are typical morphologies which are expected for diffusion limited spherulitic growth at a high driving force for crystallization (Oaki and Imai, 2003). For these protein additives, aggregates were also observed, although there were no distinct interactions with the mineral particles (SI Fig. 5). The control experiments in the absence of proteins showed rhombohedral crystals and few spherulites (arrow, SI Fig. 4A). In presence of the SUMO protein, the crystals displayed a terraced morphology (SI Fig. 4B and C).

3.5. Potentiometric titration

To confirm whether a few of the SM50 SUMO fusion proteins maintain effects at early stages of mineralization, a technique to investigate processes before nucleation would provide insight into these early mineralization processes. We studied the effect of these proteins on early stages of calcium carbonate mineralization prior to nucleation processes through the use of potentiometric titration

Table 2
Secondary structure content of SUMO fusion proteins estimated using K2D3 analysis.

Ca^{2+} (mM)	SUMO-CTL		SUMO-GRR		SUMO-PRR	
	α helix	β strand	α helix	β strand	α helix	β strand
0	33.54	19	17.19	33.54	38.35	4.71
1	28.89	21.43	14.91	33.83	37.41	4.79
10	25.83	27.48	5.32	33.11	35.34	5.46

Fig.6. Representative SEM images of calcium carbonate particles from gas diffusion assays using 1 mM and 100 mM CaCl_2 in the presence of (A) 0.01 mg and (B) 0.1 mg protein, respectively. Bars indicate 50 μm (1200 $\times$, panel figures) and 20 μm (5000 $\times$, inset figures).

assays at constant pH. The slope of the initial increase in free Ca^{2+} ions prior to nucleation, the time of nucleation and the solubility of the nucleated precipitate were considered since it is known from prior studies that these may be significantly altered by soluble additives (Gebauer and Coelfen, 2011; Gebauer et al., 2010; Gebauer et al., 2008; Verch et al., 2011). The plots of free Ca^{2+} ion concentrations with respect to time are presented in Fig. 7(A C). At 0.1 mg/mL protein concentrations, the pre nucleation slope was not affected significantly. However, at higher concentrations, SUMO CTL displayed a minor increase in the slope, indicating a destabilizing effect on prenucleation clusters (Fig. 7A.) (Gebauer and Coelfen, 2011; Gebauer et al., 2008). This can be related to the characteristic Ca^{2+} ion binding properties of C type lectins (Drickamer and Dodd, 1999). On the other hand, SUMO GRR and SUMO PRR did not exhibit a significant change in the pre nucleation slope at 1 mg/mL (Fig. 7B and C).

A direct relation between the time of nucleation with the protein concentration for all proteins was observed. This effect is described by defining a normalized nucleation time, τ_{nuc} , as the quotient of the nucleation time in the presence of protein and that of the carbonate buffer reference (Picker et al., 2012). At 0.1 mg/mL, there was no significant difference among the time points of nucleation for SUMO CTL, SUMO GRR and SUMO PRR. However, at 1 mg/mL, the τ_{nuc} values were 2.55, 3.80 and 2.17 for SUMO CTL, SUMO GRR and SUMO PRR, respectively. This indicated that the glycine rich repeat region of SM50 was comparatively more

effective in stabilizing the prenucleation clusters of calcium carbonate, which delays their coalescence and nucleation of the resulting phase. The most abundant amino acids in this repeat region are glycine, glutamine, proline, arginine and asparagine. According to a previous report (Picker et al., 2012), Arg and Pro do not alter the nucleation time, whereas the other amino acids such as Asn ($\tau_{nuc} = 1.26$), Gly ($\tau_{nuc} = 1.2$), and Gln ($\tau_{nuc} = 1.19$) delay nucleation (at 10 mM CaCl_2 concentration). It can be concluded that in addition to amino acid composition, other factors governed by protein conformation such as solvent exposed amino acid residues and surface charge distribution are likely to play an important role in the stabilization of prenucleation clusters. An intriguing observation is that the glycine rich and proline rich regions are abundant in Asn residues each containing 15 and 13 Asn residues, respectively. Nevertheless, SUMO PRR was not as effective as SUMO GRR in stabilizing prenucleation clusters, emphasizing the role of protein structure in prenucleation cluster stabilization.

The measured solubility products of the nucleated phases are indicative of various amorphous or crystalline calcium carbonate polymorphs formed after nucleation (Fig. 7D F). It should be noted that in these experiments, the measured solubility is indicative of the most soluble phase present and therefore, phases with lower solubilities present in lower amounts are not identified. The reference with carbonate buffer (pH 9.75) yielded a solubility product ($K_{sp} \approx 3.7 \times 10^{-8} \text{ M}^2$) that was indicative of proto vaterite ACC. This was consistent with solubility reported by Gebauer and

Fig.7. Potentiometric titration assays in the presence of SUMO-CTL, SUMO-GRR, SUMO-PRR (10 mM Carbonate Buffer, pH 9.75) using (A and D) SUMO-CTL, (B and E) SUMO-GRR and (C and F) SUMO-PRR as additives at 0.1 mg/mL (—) and 1 mg/mL (•••••), respectively. The reference is represented by a solid black line. Plots represent free Ca^{2+} concentration (A–C) or solubility product (D–F) with respect to time.

Fig.8. Schematic representation of SM50 domains and their putative functions. A model of SM50 was predicted using DISOCLUST/nFOLD3 server (Jones et al, 2005; McGuffin, 2008). Although the quality-score (0.0728) was low, certain structural features are in agreement with the AUC results such as a globular CTL domain and the extended conformations of the GRR and PRR domains. A scheme has been proposed for the self-assembly of the GRR domain (right) that involves (i) secondary structure changes of monomers induced by Ca^{2+} ions and (ii) organization into spherical/sheet-like assemblies as observed in AUC and CD solution analyses.

coworkers (Gebauer et al., 2008). In the presence of the SUMO fusion CTL and PRR (0.1 mg/mL), the solubility products after nucleation were considerably higher than for the reference, specifically $K_{sp(\text{SUMO-CTL})} \approx 6.43 \times 10^{-8} \text{ M}^2$, $K_{sp(\text{SUMO-GRR})} \approx 6.48 \times 10^{-8} \text{ M}^2$ and $K_{sp(\text{SUMO-PRR})} \approx 5.73 \times 10^{-8} \text{ M}^2$. At increased protein

concentrations (1 mg/mL), the solubility increased up to four times that of the reference ($K_{sp(\text{SUMO-CTL})} \approx 11.60 \times 10^{-8} \text{ M}^2$; $K_{sp(\text{SUMO-GRR})} \approx 5.96 \times 10^{-8} \text{ M}^2$ and $K_{sp(\text{SUMO-PRR})} \approx 12.50 \times 10^{-8} \text{ M}^2$). Even higher solubilities have been reported in the literature for a polymer induced liquid precursor (PILP) phase in the case of

additives such as sodium triphosphate ($K_{sp} \approx 40 \times 10^{-8} \text{ M}^2$ at 0.1 mg/mL) and poly (aspartic acid) ($K_{sp} \approx 20 \times 10^{-8} \text{ M}^2$ at 0.1 mg/mL) for (Verch et al., 2011). This indicates that proteins SUMO CTL and SUMO PRR also modulate the formation of a PILP like phase (Gower, 2008) or a dense liquid phase (Bewernitz et al., 2012) after the nucleation event. The solubility products for SUMO GRR were about twice that of the reference in the presence of protein concentrations at 0.1 and 1 mg/mL. However, in contrast to SUMO CTL and SUMO PRR, the solubilities of SUMO GRR did not increase with respect to the protein content.

3.6. ATR FTIR

ATR FTIR analyses of the final titration products were performed in order to identify nucleated calcium carbonate phases (SI Fig. 6). For SUMO CTL and SUMO PRR, only crystalline polymorphs vaterite and calcite were detected, with a lack of bands unique to the amorphous precursors. Specifically, the crystalline polymorphs vaterite and calcite were detected at 0.1 and 1 mg/mL, respectively for both SUMO CTL and SUMO PRR (SI Fig. 6A and C). As vaterite is the preferential phase at pH 9.75, the preference for calcite for both proteins at higher concentration suggests an influence on polymorph selection. Alternatively, in the presence of SUMO GRR (SI Fig. 6B), ACC was formed after nucleation, as indicated by a peak at 1066 cm^{-1} (Anderson and Brečević, 1991). A shoulder peak at 1074 cm^{-1} was also observed, which can be attributed to proto calcite ACC (Gebauer et al., 2010). Our results indicate that the glycine rich repeat domain mediates ACC stabilization as well as directs the formation of the proto calcite polymorph.

In order to accurately consider the effect of the SUMO fusion tag on the titration results, experiments were performed using the purified 6(X) SUMO tag as an additive at 0.1 mg/mL (SI Fig. 7). The plot of free Ca^{2+} ions versus time displayed a minor decrease in slope in the pre nucleation regime. The time required for nucleation ($\tau_{nuc} = 1.2$) was slightly lower than those observed in the reference experiments and the solubility product after nucleation product did not change significantly (1.06 times the reference solubility). Although, the slight increase in solubility could be on account of a glutamate residue that constitute the SUMO protein (Smt3p, NCBI accession NP_010798), the increase is within statistical variation of the reference solubilities. Thus, the SUMO expression system appears ideal for the expression of biomineralization proteins especially for titration studies.

4. Conclusion

The present study demonstrates the distinct roles of major SM50 domains in terms of self assembly, protein secondary structure conformation and calcium carbonate mineralization. The globular SUMO CTL domain investigated here does not display self assembly, but does influence mineralization as demonstrated by gas diffusion and titration studies. Several proteins from the spicule matrix family contain CTL domains in their sequences, hinting at the possibility that this domain may be required for biomineralization processes. The SUMO glycine rich repeat domain of SM50 displayed aggregation and formed large protein superstructures. It is likely that during sea urchin spiculogenesis, such protein self assembly drives ACC stabilization and mineral organization in a manner analogous to amelogenin and calcium phosphate interactions (Fang et al., 2011). Whereas, the SUMO fusion proline rich repeat domain leads to the formation of calcium carbonate particles with distinct morphologies as well as affected the early stages of mineralization. Although this protein showed some aggregation behaviors, no change in secondary structure in response to an

increase presence of Ca^{2+} ions was observed. A scheme summarizing the diverse behavior of these domains in mineralization processes in relation to the full length SM50 protein is shown (Fig. 8). By examining these major constituents of SM50, we provide clues on the function of the full length SM50 protein as well as the assembly mechanism of SM50 with calcium carbonate mineral phases into the larval sea urchin spicule.

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