Cloning and Characterization of Four Novel Coral Acid-Rich Proteins that Precipitate Carbonates In Vitro

Tali Mass,1 Jeana L. Drake,1 Liti Haramaty,1 J. Dongun Kim,1,3 Ehud Zelzion,2 Debashish Bhattacharya,2 and Paul G. Falkowski1,3,4,*

1Environmental Biophysics and Molecular Ecology Program, Institute of Marine and Coastal Sciences
2Department of Ecology, Evolution, and Natural Resources
Rutgers University, New Brunswick, NJ 08901, USA
3Department of Chemistry and Chemical Biology
4Department of Earth and Planetary Sciences
Rutgers University, Piscataway, NJ 08854, USA

Summary

Biomineralization is a widely dispersed and highly regulated but poorly understood process by which organisms precipitate minerals from a wide variety of elements [1]. For many years, it has been hypothesized that the biological precipitation of carbonates is catalyzed by and organized on an extracellular organic matrix containing a suite of proteins, lipids, and polysaccharides [2, 3]. The structures of these molecules, their evolutionary history, and the biophysical mechanisms responsible for calcification remain enigmatic. Despite the recognition that mineralized tissues contain proteins that are unusually rich in aspartic and glutamic acids [4–6], the role of these proteins in biomineralization remains elusive [5, 6]. Here we report, for the first time, the identification, cloning, amino acid sequence, and characterization of four highly acidic proteins, derived from expression of genes obtained from the common stony coral, Stylophora pistillata. Each of these four proteins can spontaneously catalyze the precipitation of calcium carbonate in vitro. Our results demonstrate that coral acid-rich proteins (CARPs) not only bind Ca\(^{2+}\) stoichiometrically but also precipitate aragonite in vitro in seawater at pH 8.2 and 7.6, via an electrostatic interaction with protons on bicarbonate anions. Phylogenetic analysis suggests that at least one of the CARPs arose from a gene fusion. Similar, highly acidic proteins appear to have evolved several times independently in metazoans through convergence. Based purely on thermodynamic grounds, the predicted change in surface ocean pH in the next decades would appear to have minimal effect on the capacity of these acid-rich proteins to precipitate carbonates.

Results and Discussion

Despite the broad interest in coral calcification and the potential for climate-driven adverse effects, the molecules and biophysical mechanism responsible for the precipitation of carbonates in corals are poorly understood. To date, we lack both a characterization of molecules involved in calcification and a mechanistic understanding of processes that lead to and control calcification. This lack of knowledge limits our ability to predict the response of corals to increasing atmospheric CO\(_2\).

To date, the best-characterized highly acidic proteins that catalyze the precipitation of carbonates are from mollusk shells and an echinoderm [4, 7–9]. However, in spite of the fact that stony corals are among the most abundant biomineralizing metazoans on Earth, surprisingly little is known about their skeletal organic matrix (SOM) proteins or how they regulate crystal formation. Furthermore, despite the fact that the amino acid composition of coral SOM proteins is characterized by a predominance of acidic amino acids [6, 10, 11], the only coral SOM protein fully characterized, i.e., galaxin, which is isolated from the stony coral Galaxea fascicularis, possesses neither acidic regions nor obvious Ca\(^{2+}\)-binding domains [12]. Recently, Drake et al. [13] identified 36 SOM proteins from a stony coral, of which two are highly acidic. They suggested, however, that their approach, based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) protein sequencing, might not be the most efficient method for identifying these molecules, due to highly redundant sequences. Although a number of N-terminal SOM protein sequences have been reported both in scleractinian and acropod corals, none of these share strong sequence similarity to other known proteins or to each other, and only one exhibits a highly acidic sequence [14–16]. A group of hypothetical, soluble acidic proteins (SAPs) has been identified in the Acropora dig- itifera genome, a transcriptome from A. millepora, and in expressed sequence tag (EST) libraries [17, 18]; however, the role of these hypothetical proteins in calcification was not examined, and the structure of the encoded genes was not validated using RT-PCR.

To better characterize the proteins responsible for coral calcification, we generated a draft genome assembly from the cnidarian host cells of the Indo-Pacific stony coral, Stylophora pistillata (see Supplemental Experimental Procedures available online). In combination with transcriptomic data from EST libraries and RT-PCR, we identified and characterized four coral acid-rich proteins (CARPs 1–4) [13]; GenBank accession numbers KC148537–KC148539 and KC493647 (Figure S1 and Table S1). Three of these, CARPs 1–3, contain a secretory signal peptide, suggesting that they are discharged from the cells. CARP1 also contains an EF hand Ca\(^{2+}\)-binding domain. CARPs 2 and 3 contain an isoleucine-proline-valine (IPV)-like motif following the signal peptide that has previously been suggested to assist in the trafficking of secreted, acidic, calcium-binding proteins out of the rough endoplasmic reticulum in metazoans [19] (Figure S1). An additional gene encodes a protein, CARP4, which lacks a targeting sequence but contains regions that have previously been identified in proteins extracted from the S. pistillata aragonite skeletal matrix [13, 14] and is therefore highly likely to be a component of the SOM. The calculated isoelectric points (pI) of CARPs 1–4 are 4.23, 4.78, 3.04, and 3.99, respectively (Table S1). Isolated SOM proteins from corals and other marine invertebrates are often posttranslationally modified [20, 21]; analysis of the amino acid sequence of the four CARPs reveals several potential sites of posttranslational modification, including phosphorylation and O- and N-glycosylation sites (Figure S1). Indeed, glycoproteins were recently reported to be present in the SOM of S. pistillata [13].
To examine the effect of CARPs on CaCO₃ precipitation, we expressed and purified the acidic domain (excluding the putative signal peptide) from CARPs 1 and 2 and the entirety of CARPs 3 and 4. The recombinant His-tagged proteins were visualized, and their identities were verified by western blot analysis using polyclonal antibodies generated against each recombinant protein (Figure S2). In addition, the predicted amino acid composition of the pure proteins was verified by high performance liquid chromatography (HPLC). The molecular masses, estimated from SDS-PAGE for these proteins, are slightly higher than predicted from their sequences (14, 20, 18, and 37 kDa, respectively), probably because of the high levels of negatively charged amino acids [8].

To determine the stoichiometry of acidic residues involved in binding calcium ions, we developed a ⁴⁵Ca²⁺ assay, in which 20 μCi of the chloride salt of the radiotracer, diluted in 20 mM ⁴⁰CaCl₂, was overlaid on a polyvinylidene fluoride membrane containing various dilutions of pure CARPs 1 and 3 (Supplemental Experimental Procedures). Ca²⁺ binds to CARPs 1–4 (Figure S2). Based on the binding affinity, we calculated the molar ratio between acidic amino acid residues and Ca²⁺ in...
Coral Biomineralization

Figure 1 shows Scanning electron microscope (SEM) images of primary cell culture in enriched seawater [11]. In vitro aragonite precipitation has been previously reported in the presence of organic remains surrounding the crystallized typical crystalline organization. This structure occurs due to the mineral phases of biominerals, regardless of their origin, CaCO₃, we conducted in vitro experiments in which the four mineralizing metazoans that are not in RefSeq (listed in Table 152x96). In addition, given the measured alkalinity of 2,221 and 2,233 m·mol L⁻¹, suggest that the crystals are indeed aragonite, similar to the results of Helman et al. [24]. Although the specific form of the carbonate mineral is not critical to the role of CARPs in the precipitation process, the thermodynamically favorable form is aragonite in modern seawater [25]. The wide distribution of calcification in protists and metazoans [26] leads to the question of the evolutionary history of this process. To address this question, we looked for putative homologs of CARPs 1–4 in our comprehensive local database derived from NCBI Reference Sequences (RefSeq) v51. This database also included 13 genomes or EST data sets from biomineralizing metazoans that are not in RefSeq (listed in Table S2). The sequence comparisons showed that CARP1 is closely related to two calumenin-related proteins widely distributed in metazoans (Figure 2).

To assess whether the CARPs catalyze precipitation of CaCO₃, we conducted in vitro experiments in which the four purified proteins were incubated in artificial seawater adjusted to pH 8.2 and 7.6. Given the measured alkalinity of 2,221 and 2,114 μmol kg⁻¹, the calculated Ωarag for these conditions was 4.15 and 1.27, respectively. These experiments were designed to simulate contemporary and predicted pH conditions in the upper ocean in the coming century [22]. In addition, in vitro aragonite precipitation has been previously reported in the presence of primary cell culture in enriched seawater [11]. Figure 1 shows Scanning electron microscope (SEM) images of CaCO₃ crystals grown in the presence of CARPs 1–4, at pH 8.2 and 7.6. In all cases, a typical reticulate crystallization pattern is visible at nanometer length scales (Figure 1, insets). The mineral phases of biominerals, regardless of their origin, always show a reticulate structure that is different from the typical crystalline organization. This structure occurs due to the presence of organic remains surrounding the crystallized measured by ICP-MS, suggest that the crystals are indeed aragonite, similar to the results of Helman et al. [24]. Although the specific form of the carbonate mineral is not critical to the role of CARPs in the precipitation process, the thermodynamically favorable form is aragonite in modern seawater [25]. The wide distribution of calcification in protists and metazoans [26] leads to the question of the evolutionary history of this process. To address this question, we looked for putative homologs of CARPs 1–4 in our comprehensive local database derived from NCBI Reference Sequences (RefSeq) v51. This database also included 13 genomes or EST data sets from biomineralizing metazoans that are not in RefSeq (listed in Table S2). The sequence comparisons showed that CARP1 is closely related to two calumenin-related proteins widely distributed in metazoans (Figure 2).

Analysis of the (noncalcifying) sea anemone Anemonea viridis shows calumenin to be the most strongly upregulated protein when symbiotic dinoflagellates (i.e., Symbiodinium species) are present in animal cells [27]. In S. pistillata, CARP1 is comprised of five exons, with the N-terminal exon
containing the acid-rich domain described above that is absent in other calumenin-related proteins. The second and third exons encode the EF hand Ca$^{2+}$-binding domain (Figure 2A) and are similar to calumenin-related proteins. The exon-intron structure of CARP1 is strongly supported by mRNA-seq data, and independent assembly using transcriptome data recovered the intact cDNA that includes the five exons shown in Figure 2A. Phylogenetic analysis of CARP1 (Figure 2B) reveals that the N-terminal acid-rich domain is coral specific (Figures 2A and 3). The observation that neither calumenin nor other related nonacidic Ca$^{2+}$-binding proteins are known to take part in the calcifying process, together with the fact that CARP1 contains an N-terminal acid-rich domain capable of precipitating CaCO$_3$, is consistent with the idea that CARP1 results from a gene fusion. Fusion of EF hand domains to heterologous genes, leading to novel functions, has been previously observed (e.g., the NEFA and nucleobindin genes [28]) and, in general, allows the translation of a regulatory signal (i.e., Ca$^{2+}$-binding) to different functional responses [29]. These results demonstrate that CARP1, while widely dispersed among metazoans, evolved independently [26] but converged in function to other metazoan calcifying proteins. The search also revealed a high similarity of CARP2 (Figure 4A) and CARP4 (see Figure S3 in Drake et al. [13]) only to other acidic scleractinians. The fact that CARP2 and CARP4 exhibit a high similarity only to other acidic scleractinians proteins supports the argument suggested by Drake et al. [13], that CARP4 and potentially CARP2 belong to highly acidic subfamilies of proteins that are well conserved across order Scleractinia. Interestingly, CARP3 revealed high similarity both to acidic scleractinian proteins and to the prismatic shell matrix protein family of the bivalve *Atrina rigida*, Aspich [4] (Figure 4B).

The convergent sequence evolution of highly acidic proteins in all calcium carbonate-precipitating organisms identified to date suggests a common underlying mechanism for biomineralization. Calcium is an s block element that forms a metal-ligand complex via coordinate bonds, typically with oxygen atoms from carboxylates in either a bidentate or syn/anti monodentate mode [30]. We propose that the acid-rich regions in CARPs effectively localize calcium ions, thereby increasing the ionic strength in the microenvironment and leading to an increased local dielectric constant, which, in turn, decreases the pKa of the microenvironment [31] (Figure S3A). The coordination bond strength of calcium with carboxylates is not as strong as for transition metals, such as zinc; the carboxylates are readily replaced by a stronger Lewis base [32]. The net negative charge on the oxygen atoms in the carboxyl groups leads to electrostatic displacement of protons from bicarbonate anions, thereby allowing the precipitation of inorganic carbonates on an organic scaffold [33] (Figure S3A).

A β sheet conformation has been shown to accelerate calcification [34], but acidic protein domains of CARPs 1–4 form neither α helices nor β sheets (Figure S3B). Indeed, aspartic acid favors neither α helices nor β sheets but has a relatively higher propensity to form $\gamma_L$ conformational states compared to other amino acid residues [35]. The electrostatic interactions between calcium and acidic residues may confer an $\gamma_L$ conformation in a number of residues of the experimentally determined acidic domains. For example, in a catgrip Ca$^{2+}$-binding motif, the backbone residues have alternating $\gamma_L$ and $\delta_R$ conformations, which is a characteristic of an $\alpha$ sheet structure [36]. We propose that calcium carbonate precipitation and its higher-order assembly might be a consequence of $\alpha$ sheet-mediated protein aggregation. This reaction is almost certainly controlled by other, nonmineralizing proteins, such as collagens [13], which help guide the arrangement of the crystals to achieve the
Conclusions

Our research has identified and characterized four unique, acidic proteins in corals that catalyze the precipitation of CaCO₃ in vitro and opens an approach not only for understanding how the process is controlled in vivo but also for in vitro applications in novel biomaterials. The precipitation reaction is likely to be driven by an electrostatic interaction of Ca²⁺ ions with the carboxylate groups on the proteins, followed by dehydration and precipitation of carbonate. Whereas calcification is widely dispersed across metazoan phyla [26], cnidarians (corals in particular) appear to be the most anciently diverged extant phylum possessing this trait [26]. To the extent that the animal can buffer the reaction from the external pH of the bulk fluid [37], the calculated pH values strongly suggest that these proteins will continue to catalyze calcification reactions at ocean pH values projected in the coming century, assuming that the animals can physiologically acclimate and/or genetically adapt to these changing conditions.

Experimental Procedures

DNA and RNA Purification and Complementary DNA Synthesis

Total genomic DNA that was free of cells from the dinoflagellate symbiont Symbiodinium species was extracted from harvested cells using a blood and cell culture DNA Mini Kit (QIAGEN) with small modifications. Total RNA was extracted using TRIzol Reagent (Life Technologies) following the manufacturer’s protocol with small modifications. Further details are described in Supplemental Experimental Procedures.

Stylophora pistillata Draft Genome and Gene Model

Genomic DNA that was free of cells from the dinoflagellate symbiont Symbiodinium species and total RNA were used to produce a draft genome as well as transcriptome data using single-read and paired-end protocols on an Illumina Genome Analyzer IIx. Further details are described in Supplemental Experimental Procedures.

4⁰Ca⁺ Overlay Assay

SDS-polyacrylamide gel was transferred to polyvinylidene fluoride (PVDF) membranes. CARPs 1–4 and control proteins (glycosyl hydroxide 2 [E. coli M863] and BSA) blotted on PVDF membrane were labeled with ⁴⁰Ca, as described previously [98]. Further details are described in Supplemental Experimental Procedures.

In Vitro CaCO₃ Precipitation

Calcium carbonate precipitation experiments were carried out by adding 0.1 μM of CARPs 1–4 to 1 ml of artificial seawater (Instant Ocean sea salt, Aquarium Systems; salinity = 34). Further details are described in Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.05.007.
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References


