A Novel Extrapallial Fluid Protein Controls the Morphology of Nacre Lamellae in the Pearl Oyster, *Pinctada fucata*

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Mollusk shell nacre is known for its superior mechanical properties and precisely controlled biomineralization process. However, the question of how mollusks control the morphology of nacre lamellae remains unresolved. Here, a novel 38-kDa extrapallial fluid (EPF) protein, named amorphous calcium carbonate-binding protein (ACCBP), may partially answer this question. Although sequence analysis indicated ACCBP is a member of the acetylcholine-binding protein family, it is actively involved in the shell mineralization process. In vitro, ACCBP can inhibit the growth of calcite and induce the formation of amorphous calcium carbonate. When ACCBP functions were restrained in vivo, the nacre lamellae grew in a screw-dislocation pattern, and low crystallinity CaCO3 precipitated from the EPF. Crystal binding experiments further revealed that ACCBP could recognize different CaCO3 crystal phases and crystal faces. With this capacity, ACCBP could modify the morphology of nacre lamellae by inhibiting the growth of undesired aragonite crystal faces and meanwhile maintain the stability of CaCO3-supersaturated body fluid by ceasing the nucleation and growth of calcite. Furthermore, the crystal growth inhibition capacity of ACCBP was proved to be directly related to its acetylcholine-binding site. Our results suggest that a “safeguard mechanism” of undesired crystal growth is necessary for shell microstructure formation.

Many living things have the ability to convert inorganic ions into solid minerals through a process termed biomineralization (1, 2). The biogenetic solid minerals, also known as biominerals, form external and internal hard tissues (e.g. otoliths, shells, and bones) that carry out diverse functions. Among these biomineralization products, the aragonite layer of molluscan shell (also called nacre or mother of pearl) is remarkable for its unique microstructure and superior mechanical properties (3, 4). Unlike other biogenetic mineral structures, nacre is not embedded in a tissue or layers of cells. Instead, a thin layer of fluid filling the space between the mantle epithelium and the shell, namely the extrapallial fluid (EPF), is employed as the final environment and achieve an appropriate supersaturation of CaCO3 necessary for shell crystal growth (5, 6). Meanwhile, various organic macromolecules, including proteins, polysaccharides, and lipids, are secreted by mantle cells or transported from elsewhere to the EPF (7–9). These macromolecules are thought to also be responsible for transporting the inorganic ions and stabilizing this CaCO3-supersaturated fluid (10, 11). The shell matrix proteins (the proteins found in the shell) are also in the EPF and self- or co-assemble to form the crystal deposition framework and initiate crystallization (4, 12, 13). Because all of these complex processes occur in an extracellular liquid, nacre mineralization provides a useful model of formation of a material with intricate nano-architectures in a mild condition.

For a long time, it was believed that the matrix proteins played a dominant role in directing the highly organized nanocomposites of CaCO3 crystals to form the layered structure of nacre (2, 14, 15). However, although matrix proteins extracted from nacre can induce the formation of aragonite in vitro like that in vivo (16, 17), the exceedingly orderly morphology of nacre lamellae cannot be reproduced in vitro. In fact, previous research revealed that once nucleation took place, the growth of the nacre lamellae would start, even in an inorganic environment (18). In the absence of the macromolecules of the EPF, nacreous lamellae grow by following the pattern of inorganic aragonite instead of forming the layered structure of nacre. In this case, it is reasonable to predict that besides keeping the stability of the CaCO3-supersaturated microenvironment, some macromolecules of the EPF are also directly involved in modifying the morphology of shell crystals. However, because of the difficulties in protein purification and in vivo functional identification, although some calcium-binding proteins were
found in the EPF (11, 19), how the macromolecules maintain the stability of the EPF and affect nacre lamellae growth was unknown. In the present study, a protein was extracted from the EPF of Pinctada fucata through a new method. A series of in vitro and in vivo experiments showed that this protein can inhibit undesired crystal growth and play a key role in stabilizing the CaCO₃-supersaturated body fluid and forming the exceedingly orderly microstructure of nacre.

**EXPERIMENTAL PROCEDURES**

**Animals**—The oyster, *P. fucata*, was purchased from Guofa Pearl Farm, Beihai, Guangxi Province, China. Animals were maintained in glass aquariums filled with aerated artificial seawater (Sude Instant Sea Salt, 3% at 20 °C) for 3 days prior to experimentation.

**Extrapallial Fluid Extraction and Protein Purification**—The extrapallial fluid (EPF) was extracted by inserting a 0.8 mm/11003 g needle into the central extrapallial space and sucking out the fluid gently into a sterile syringe. The needle tip was carefully kept in contact with the inner shell surface to avoid contamination by extraneous water or other body fluids. Samples collected (~50–100 µl per oyster) were immediately transferred to 15 ml of sterile centrifuge tubes and held on ice. After extraction, fluids were centrifuged for 20 min at 1500 × g and 4 °C.

Amorphous calcium carbonate (ACC) was synthesized according to the method of Koga et al. (20) with some modifications. Equimolar sodium carbonate (0.1 M) and calcium chloride (0.1 M) solutions were mixed with vigorous stirring at 0–4 °C. Sodium hydroxide (1 M) was added to obtain mixed solutions within pH 11.2–11.4. Then the precipitated colloidal phase was centrifuged for 10 min at 2000 × g and 4 °C to separate the ACC. To verify that only pure ACC existed, the precipitate was washed with acetone, dried in a vacuum desiccator for 1 day, and identified via x-ray diffraction. The separated ACC was collected (~50–100 µg per day), and identified via x-ray diffraction. The yield was about 3 mg/liter.

**Polyclonal Antibody Production**—Polyclonal antibody was prepared by immunizing rabbits with rACCBP and purified with HiTrap Protein A HP by following the Amersham Biosciences manual. Tissues, i.e. mantle, viscous, adductor muscle, and gill, were homogenized and the total soluble proteins were extracted with 50 mM Tris–HCl buffer, 5 mM EDTA, 150 mM NaCl, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40. Hemolymph and extrapallial fluid were extracted directly from the pearl oyster *P. fucata*. Shell powder was treated with 0.5M EDTA for 3 days and the EDTA soluble and insoluble shell matrix was separated by centrifugation. Protein concentrations were measured with the BCA protein assay kit (Pierce). Protein samples with an equal quantity (50 µg) were run on a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked overnight and treated with a 1:2000 dilution of antibody against ACCBP. It was then washed exhaustively four times, treated with a 1:500 dilution of alkaline phosphatase-labeled goat anti-rabbit antibody, washed exhaustively four times, and finally visualized with NBT/BCIP.

**Chemical Deglycosylation**—ACCBP and rACCBP were lyophilized overnight. Deglycosylation was performed on the freeze-dried sample with a GlycoProfile IV chemical deglyco-
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sylation kit (Sigma) according to the manufacturer’s instructions. The samples were then extensively dialyzed against 20 mM Tris-HCl buffer, pH 7.5, in dialysis tubing (GeBA) with a molecular size cutoff of 3,500 Da.

Western Blotting with Concanavalin A Probe—ACCBP and rACCBP were run on a 12% gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked overnight and treated with 10 μg/ml Biotin-Concanavalin A (Sigma). It was then washed exhaustively four times, treated with a 1:100 dilution of avidin-HRP, washed exhaustively four times, and finally visualized with an enhanced DAB kit.

 Protein Labeling with Rhodamine-α-bungarotoxin (RA-α-Bgt)—ACCBP or rACCBP was mixed with an equimolar amount of RA-α-Bgt (Sigma) in 10 mM Tris-HCl buffer, pH 7.5. For rACCBP, the mixture was held at 4 °C for more than 1 h. For ACCBP, the mixture was held at 4 °C for over 24 h.

 Protein Labeling with Rhodamine—1 mg of rACCBP was dia-lyzed against 0.1 M sodium bicarbonate (pH 8.3) and concentrated to 10 mg/ml. 0.5 mg of 5-TAMRA, S.E. (Promega) was dissolved in 0.05 ml of Me2SO immediately before the reaction. The calcium concentration of the solution was about 8.5 mM, as determined by EDTA titration, was brought to 10 mM HEPES (pH 7.5) was used to separate the large volume of 10 mM HEPES (pH 7.5) was used to separate the conjugate from the unreacted labeling reagent. 50 μg of ACCBP was labeled as above. The dosage of other reagents was reduced accordingly.

 Injection of Antibody against ACCBP into the Extrapallial Space—Oysters with shells of about 4–6 cm in diameter and 30–40 g in wet weight were held in a tank for 7 days prior to use. The purified antibody against ACCBP (anti-ACCBP) was injected into the center of the extrapallial space through the mantle with a microsyringe at dosages of 0.5 mg/ml of Me2SO immediately before the reaction. While the protein solution was being stirred, 0.01 ml of the reactive dye solution was added slowly. Then the reaction was incubated for 1 h at room temperature with continuous stirring, and the reaction was stopped by adding 0.01 ml of freshly prepared 1.5 M hydroxylamine, pH 8.5. Finally, dialysis against a large volume of 10 mM HEPES (pH 7.5) was used to separate the conjugate from the unreacted labeling reagent. 50 μg of ACCBP was labeled as above. The dosage of other reagents was reduced accordingly.

 In Vitro Crystal Growth Experiments—Saturated Ca(HCO3)2 solution was prepared by following the method of Xu et al. (21) with some modification. CO2 gas was bubbled into the mixture of CaCO3 and Milli-Q deionized water for 4 h. Excess solid CaCO3 was removed via filtration, and the filtrate was aerated with CO2 for another hour. The calcium concentration of the solution was about 8.5 mM, as determined by EDTA titration, and the pH 6.2.

 The experiments were carried out in 96-well COSMO multidishes with cleaned glass slides or silicon wafers on the bottom of each well. Saturated Ca(HCO3)2 solution and protein solution were mixed in each well to a final volume of 100 μl with a calcium concentration of 8 mM. 100 μl of Milli-Q water was introduced to the wells next to the samples to trap the CO2 that diffused from the bicarbonate solution. Water or the same concentation of BSA was chosen as negative controls. The multidishes were placed in a sealed system at room temperature. After 48 h, the glass slides or silicon wafers were lightly rinsed with Milli-Q water, dried, and observed by S.E. and Raman spectrum. For the FTIR spectrum, the precipitate of each well was collected and dried under vacuum. For the crystal growth experiment performed with the mixture of ACCBP and α-Bgt (molar ratio: 1:2), the proteins were incubated 24 h in advance. The mixture of BSA and ACCBP at the same concentration was used as a control.

 Crystal Growth Inhibition Experiments—The saturated Ca(HCO3)2 solution was prepared as above. The solution, about 200 μl per well, was introduced into a 96-well COSMO multidish with glass slides on the bottom, and 100 μl of Milli-Q water was introduced into the wells next to the samples to trap CO2. After 30 min, the wells were inspected under an optical microscope (Leica DMRB). When rhombohedral crystal seeds became visible, pictures were taken, and the relative positions were marked. Then 5 μl of protein solution was added to give a final concentration of 0.2 μM, and the multidish was placed into a sealed system at room temperature. Pictures of the crystals were taken at the marked place after 24 h.

 Crystal Binding Experiments—The CaCO3 crystals were synthesized as in the crystal growth inhibition experiments, except that the multidish was placed in a sealed system at 40 °C to induce the formation of both calcite and aragonite. 24 h later, the glass slides were lightly rinsed with Milli-Q water and air-dried. The crystal polymorphs were determined by Renishaw Raman Spectroscopy. The labeled proteins were diluted with CaCO3-saturated Tris-HCl buffer (10 mM pH 7.5, prepared by mixing 1 g of reagent grade calcite with 100 ml of Tris-HCl buffer and stirring for 24 h) to a final concentration of 0.1 μM and incubated with the glass slides for 1 h at room temperature. Then the glass slides were rinsed with CaCO3-saturated Tris-HCl buffer and Milli-Q water and air-dried. Slides were observed under a fluorescence microscope (Leica DMR). Images were collected with a Leica CCD 300F camera. The exposure time for the fluorescence images was set to 6.5 s. To confirm the binding patterns of different proteins, the crystal binding experiments were repeated at least three times for each kind of protein.

When the interaction of shell nacre and rhodamine-labeled ACCBP (RA-ACCBP) was tested, the inner surface of the shell nacre was mechanically cleaved into small pieces (approx. 8 × 8 × 1 mm). In the group of normal nacre, the nacre pieces were incubated in the RA-ACCBP solution together with some synthesized calcite (Sigma). The incubation lasted until fluorescence appeared on all the synthesized calcite. Before observing the inner surface of the nacre under the fluorescence microscope, some of the calcite crystals were carefully dropped on it as positive controls. The nacre pieces from all the specimens of each group were tested. The total examined surface area of normal nacre (and other groups) is more than 6 cm2. The exposure time for these fluorescence images was set to 600 ms.

 Raman Spectroscopy—The glass slides or silicon wafers were first observed under reflected white light with a Leica microscope at a magnification of ×500. After focusing on a single crystal, the light source of the microscope was transferred to
514-nm diode laser. The spectra were scanned 3 × 20 s in the range of 100–1200 cm⁻¹ with a Renishaw Raman imaging microscope.

Fourier Transform Infrared (FTIR) Spectroscopy Analysis—Precipitate was powdered and mixed with anhydrous KBr. The mixture was pressed into a 13-mm diameter pellet. FTIR spectra were obtained with a Nicolet560 Fourier transform infrared spectrometer.

Scanning Electron Microscopy—The silicon wafers or incised shells were sputter-coated with gold and analyzed with a FEI Sirion2000 scanning electron microscope, which was operated at 10 kV and equipped with a Kevek energy dispersive x-ray spectrometer for element analysis of crystals.

RESULTS

Extraction and Characterization of ACCBP—The stability of the EPF is demonstrated by its CaCO₃ crystalline inhibition ability. It has been shown that the organic components of the EPF, even at high dilution, can effectively decrease the rate of CaCO₃ crystallization (9). During our research on EPF, we found that EPF can retard further crystallization of the synthesized amorphous calcium carbonate (ACC), which is the most unstable form of CaCO₃ (22, 23). To investigate the abnormal stability of ACC, we separated ACC from the EPF by centrifugation and decalcified the ACC with EDTA. SDS-PAGE analysis of the EDTA-soluble component revealed an obvious ~38-kDa protein band that could not be observed in the SDS-PAGE of the EPF (Fig. 1). This specifically enriched protein was named ACCBP (amorphous calcium carbonate-binding protein). When the HPLC-purified ACCBP was run on a SDS-PAGE gel under unreduced conditions, a band with an apparent molecular mass of ~76 kDa could be observed (Fig. 1). This result suggested that under physiological conditions ACCBP might exist as a homodimer.

Partial N-terminal amino acid sequence of ACCBP (KCDY-PEAKLLKFLDLDDYKLRVPVP) was obtained by Western blotting and Edman degradation. On the basis of the N-terminal sequence, 3'-RACE and 5'-RACE were performed to reveal the cDNA sequence of ACCBP (GenBank™ accession no. DQ473430). The 930-base pair cDNA sequence encoded a single protein of 240 amino acids and a typical transcription termination signal (AATAAA), which confirmed the integrity of the cDNA sequence (Fig. 2). The mature protein starts with Lys at position 25 and has a predicted molecular weight of 24,389.

The sequence analysis indicated that ACCBP is an acidic protein with a theoretical pl of 4.56 and is rich in acidic residues (Asp + Glu nearly 16%). However, compared with many matrix proteins purified from the shell matrix of bivalves, such as caspartin (13), ACCBP is not a typical Asx-rich protein, and the mol percentage of positively charged residues is high (Arg + His + Lys nearly 11%). In fact, except for the most abundant residues (Asp and Leu, nearly 11%), the amino acid composition of ACCBP is not significantly biased to any residue. The BLAST search against GenBank™ revealed that ACCBP has 30% sequence identity with the extracellular ligand-binding domain of the α7 nicotinic acetylcholine receptors (nAChR) of Drosophila melanogaster (Fig. 2). ACCBP has the residues which
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This phenomenon might be explained in this way: immediately after ACCBP is synthesized in these tissues, it is secreted into the body fluid; this makes its content in these tissues too low to be detected on Western blots with equal quantities of total extract proteins.

The Effect of ACCBP on CaCO₃ Crystallization in Vitro and Its Interaction with Calcite—To examine whether ACCBP is responsible for the enhanced stability of ACC in the EPF, in vitro crystal growth experiments were performed. We found that just 0.2 μM ACCBP can totally inhibit the formation of calcite. The infrared spectra of all precipitants show broad v2 and v1 absorption bands of ACC at 864 and 1070 cm⁻¹, respectively, and the split of the v3 band around 1450 cm⁻¹, which indicates that only pure ACC is present (Fig. 5F). S.E. images showed that the ACC precipitate was dominated by uniform spherical particles with diameters of about 200–300 nm, though particles as small as 50 nm in diameter could also be found (Fig. 5, A and B). Under normal conditions, ACC will rapidly transform to more stable crystal phases in water through dissolution of itself and growth of the stable phase (22, 23). In an artificial system, the stability of ACC can be increased by retarding the dissolution of ACC or preventing the nucleation and growth of the crystalline polymorphs (20, 28–30). In this experiment, the concentration of Ca²⁺ was adjusted to 8 mM; this meant one ACCBP molecule would have to stabilize 40,000 CaCO₃ molecules if it stabilized ACC by coating the whole surface of ACC particles and inhibiting the dissolution as polycarboxylates do (28). The average molar mass of polycarboxylates is about 70 kDa, similar to the MW of ACCBP dimer. Obviously, in this case, compared with the ratio of polycarboxylates to Ca²⁺ (about 1:1750), the amount of ACCBP was far from enough. The ACC stabilization mechanism of ACCBP was further investigated with a calcite growth inhibition experiment. Fig. 6, A and B showed that ACCBP could rapidly inhibit the growth of newly emerging calcite. Therefore, ACCBP could arrest the growth of the more stable crystal form of CaCO₃ and consequently stabilize the intermediate form, ACC.

Furthermore, the interaction between ACCBP and calcite was analyzed with a series of crystal binding experiments. Because ACCBP has an ACh-binding site, RA-α-Bgt (α-Bgt, a specific blocker of nAChRs of vertebrate neuromuscular junctions (31)) was introduced to visualize ACCBP. RA-α-Bgt-labeled ACCBP was incubated with freshly synthesized calcite that was identified with Raman spectra, in a solution of saturated CaCO₃. BSA and RA-α-Bgt at the same concentrations were utilized as a control. After 1 h of incubation, fluorescence appeared on the whole surface of the calcite in the ACCBP-treated groups (Fig. 7A), but not on those of the controls (Fig.

FIGURE 3. Western blotting analysis of ACCBP & rACCBP. SDS-PAGE was done on 12% acrylamide gels. A, tissue distribution analysis of ACCBP. Lane 1, soluble extracts of the viscus; lane 2, soluble extracts of the mantle; lane 3, soluble extracts of the adductor muscle; lane 4, EDTA-soluble shell matrix; lane 5, EDTA-insoluble shell matrix; lane 6, total proteins of the EPF; lane 7, soluble extracts of the gill; lane 8, total proteins of the hemolymph. B, comparison of ACCBP and rACCBP. Lane 9, ACCBP. Lane 10, rACCBP; arrow, unreduced rACCBP homodimer. Lane 11, TFMS-treated ACCBP. Lane 12, TFMS-treated rACCBP. C, analysis of ACCBP and rACCBP with biotin-labeled Concanavalin A. Lane 13, ACCBP is not recognized by biotin-Concanavalin A; lane 14, rACCBP is recognized by biotin-Concanavalin A.

FIGURE 4. RT-PCR analysis of ACCBP in different tissues. RT-PCR was performed on RNA prepared from viscus, mantle, gill, adductor muscle, and hemocytes. GAPDH was used as the positive control. Specific primer sets for ACCBP and GAPDH are presented under "Experimental Procedures."
To confirm the above phenomena, the natural ACCBP was directly labeled with rhodamine and incubated with CaCO₃ crystals as above. Fig. 7B showed the same binding patterns as those of the RA-α-Bgt-labeled ACCBP. Hence, it appears that ACCBP can halt the growth of calcite by binding to its whole surface.

The Functions of ACCBP in Nacre Mineralization and Its Interaction with Aragonite—ACCBP is expressed in the mantle and exists mainly in the EPF (Figs. 3A and 4). Because nacre formation is a crystalline process, which occurs in the EPF, the physiological functions of ACCBP, a CaCO₃ crystalline inhibitor, during this process became an interesting question. To
explore this, the purified polyclonal antibody against ACCBP (anti-ACCBP) was injected into the extrapallial space of *P. fucata* at dosages of 0.5 μg and 1.5 μg of protein per gram wet weight of oyster per day; the same dosages of preimmune rabbit serum were used as controls. Five days later, the animals were sacrificed and the inner surfaces of the nacre were observed by S.E. In the low dosage group, the nacre lamellae were obviously enlarged and grew as a screw-dislocation pattern (Fig. 8, E and F). In the high dosage group, a large amount of low crystallinity precipitation appeared on the nacre lamellae (Fig. 8, I and J). Element analysis showed that these precipitations are CaCO$_3$ (data not shown). Although in the control groups, the lamellae surface appeared coarser (Fig. 8, M–P) and zigzag at the edges (in the high dosage group, Fig. 8, O and P) compared with the untreated group (Fig. 8, A and B), these changes were much smaller than in the antibody-treated groups (Fig. 8, E, F, I, and J). These results suggest that nacre lamella growth was perturbed to some extent when the function of ACCBP was inhibited. In addition, the CaCO$_3$-supersaturated EPF became so unstable that large amounts of low crystallinity CaCO$_3$ appeared.

To confirm that the abnormal nacre structures of the anti-ACCBP-injected groups were directly related to the interference of ACCBP functions, the nacre of the untreated specimens (N-nacre), the low dosage group (L-nacre), and the high dosage group (H-nacre) were incubated with RA-ACCBP. Fig. 8C shows that fluorescence cannot be observed on the inner surface of N-nacre. In contrast, strong fluorescence appeared on the L-nacre and H-nacre, but was restricted to the abnormal structures on the nacre surface (Fig. 8, G, H, K, and L). These results suggest that, in the EPF of an untreated pearl oyster, the abnormal structures are recognized and bound by ACCBP and their growth is stopped at an early stage. However, ACCBP does not affect the growth of normal nacre lamellae. This result also explains why the content of ACCBP in the shell matrix is so low that it cannot be detected on Western blots of total matrix proteins (Fig. 3A).

The nacre binding experiments suggest that ACCBP interacts with aragonite (the crystal phase of nacre) differently than calcite. Accordingly, the ACCBP was labeled as above and incubated with synthesized aragonite. Interestingly, unlike on the surface of calcite, the binding of ACCBP was mainly limited to the needlelike extensions of aragonite (Fig. 7, A–C). This means that ACCBP can recognize different CaCO$_3$ crystal phases and crystal faces. With this capacity, ACCBP might be able to eliminate undesired crystal growth and thereby modify the morphology of nacre lamellae.

ACCBP Crystal Growth Inhibition Capacity Is Directly Related to the Acetylcholine-binding Site—Although ACCBP has an ACh-binding site like that of α7 nAChRs and
AChBP, it functions in a totally different way from them. The role of the ACh-binding site in ACCBP function is of interest to explore. In the crystal binding assays, when ACCBP was mixed with calcite or aragonite 1 h before the RA-ACCBP was added, no fluorescence could be detected (data not shown). This observation suggests that, when ACCBP adsorbs onto a crystal, the ACh-binding site is not accessible to RA-ACCBP and, hence, the ACh-binding site might be important for ACCBP function. In further studies, when in vitro crystal growth experiments were performed with a mixture of ACCBP and RA-ACCBP (molar ratio of 1:2), S.E. images showed that the majority of CaCO₃ precipitated remained amorphous, but they were not as uniform in size as the crystals formed in the presence of ACCBP alone (Fig. 5, C and D). Many non-spherical crystals, which were much bigger than typical ACC, could be found. A few huge spherical crystals with diameters of about 50 μm are also observed. Raman spectra of these huge crystals exhibited peaks at 1084 cm⁻¹, 712 cm⁻¹, and 280 cm⁻¹, which are typical for calcite (Fig. 5E). Because of the low crystallinity, the base line is high and the peaks are weak and broad. FTIR spectrum of the precipitate displays the peculiar v2 absorption band of calcite at 875 cm⁻¹, but the remainder of the spectrum shows the typical peaks of ACC (Fig. 5F). This spectrum might indicate the presence of multiple crystal polymorphs. No peak shift was observed when RA-ACCBP was replaced with BSA (Fig. 5F). In a direct assay of crystal growth inhibition, RA-ACCBP interferes with the capacity of ACCBP to inhibit crystal growth (Fig. 6, C and D). Thus, RA-ACCBP has an effect on the capacity of ACCBP to affect crystal growth. In addition, RA-ACCBP has no obvious effect on the binding of RA-ACCBP or RA-rACCBP to crystals in an assay like that of Fig. 7 (data not shown). Taken together, these results indicate that the ACh-binding site

FIGURE 8. The effect of antibody against ACCBP on the growth of nacre lamellae. A, S.E. image of the inner surface of normal shell nacre of the oyster P. fucata (N-nacre). The stair-like growth pattern of nacre can be seen. Bar, 50 μm. B, enlargement of the box in A. Bar, 5 μm. C, N-nacre and synthesized calcite were incubated with RA-ACCBP and observed under a fluorescence microscope. ACCBP cannot bind to the nacre surface, but can bind to the synthesized calcite (arrow). D, the corresponding image of C under white light. Bar, 50 μm. E, S.E. image of the inner nacre surface of the low dosage group (L-nacre). The lamellae are obviously longer than normal and screw-dislocation structures can be seen (arrow). Bar, 50 μm. F, enlargement of the box in E. Bar, 5 μm. G, L-nacre was incubated with RA-ACCBP. When excited at 535 nm, strong orange fluorescence appeared on the abnormal structures. H, the corresponding image of G under white light. Bar, 50 μm. I, S.E. image of the inner nacre surface of the high dosage group (H-nacre). Large amounts of low crystallinity CaCO₃ precipitated on the nacre lamellae. Bar, 50 μm. J, enlargement of the box in I. Bar, 5 μm. K, H-nacre was incubated with RA-ACCBP. When excited at 535 nm, fluorescence appeared on the low crystallinity CaCO₃ precipitate, while not appearing on the normal lamellae. L, the corresponding image of K under white light. Bar, 50 μm. M, S.E. image of the inner shell surface of the low dosage control group. N, enlargement of the box in M. Bar, 5 μm. O, S.E. image of the inner shell surface of the high dosage control group. The stair-like growth pattern was disturbed. Bar, 50 μm. P, enlargement of the box in O. Edges of the lamellae became zigzag. Bar, 5 μm.
does not mediate the binding of ACCBP to crystals, but plays a role in crystal growth inhibition.

Although rACCBP can bind CaCO₃ crystals in the same way as ACCBP does (Fig. 7C) and obviously reduce the calcite growth rate (Fig. 9, D and E), it cannot effectively inhibit the formation of calcite (Fig. 9, A and B). These results suggest that the function of the ACh-binding site of rACCBP may be impaired. In addition, in the crystal binding assays, we found that, even when mixed with RA-α-Bgt for as long as 24 h, the fluorescence of the RA-α-Bgt-labeled ACCBP group (Fig. 7A) was heterogeneous and much weaker than that of the rACCBP group (Fig. 7D), which was mixed with RA-α-Bgt for only 1 h at the same concentration. As mentioned above, α-Bgt has no significant effect on the crystal binding properties of ACCBP, so the weak and heterogeneous fluorescence of RA-α-Bgt-labeled ACCBP might be caused by less effective labeling of ACCBP with RA-α-Bgt relative to the labeling of rACCBP. To validate this prediction, the crystal binding experiments were performed with rACCBP labeled with both α-Bgt and RA-α-Bgt (molar ratio 9:1). The results showed that, for the partially labeled rACCBP group (Fig. 7E), the fluorescence was heterogeneous and much weaker than for the fully labeled group, but similar to RA-α-Bgt-labeled ACCBP group. Therefore, the weak fluorescence of the RA-α-Bgt-labeled ACCBP group might be due to lower binding affinity of RA-α-Bgt to the natural protein.

In addition, an obvious difference between ACCBP and rACCBP is their migration patterns on SDS-PAGE. Fig. 3B shows that the apparent molecular mass of rACCBP is about 34 kDa, obviously smaller than ACCBP. When natural ACCBP and rACCBP were treated with TFMS, the molecular weight of these molecules was reduced to the same size (Fig. 3B). Hence, the major post-translational modifications of natural ACCBP are carbohydrate. However, Concanavalin A only recognizes rACCBP, but not natural ACCBP (Fig. 3C). Also, the two bands of rACCBP were shown to come from the different extents of glycosylation on Asn₁₈₄ (data not shown). The results here indicate that both the size and the composition of the post-translational modifications of ACCBP and rACCBP are different. Perhaps, some modifications of the natural protein make the structure of the ACh-binding site of ACCBP different from that of the recombinant protein and less accessible for α-Bgt. The difference in the structure of the ACh-binding site might consequently lead to the differences in the crystal growth inhibitory activity of rACCBP and natural ACCBP.
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DISCUSSION

The solubility of biominerals is usually very low. When the mineral ions are transported in body fluid, the risk of undesired crystallization and precipitation exists. In mammals, two proteins, matrix GLA protein (MGP) (32) and fetuin-A (33), were found to function as calcification inhibitors in blood and soft tissues. The mutation or knock-out of these proteins induced abnormal calcium phosphate precipitation in arteries and cartilage. As for the mollusk, to support the growth of the shell, ions involved in shell formation (\(\text{Ca}^{2+}, \text{HCO}_3^-, \text{and CO}_3^{2-}\)) are actively exchanged between the EPF, the hemolymph, and the environment (10). Both the EPF and the hemolymph are thought to be a \(\text{CaCO}_3\)-saturated solution. Accordingly, some calcification inhibitors that inhibit abnormal \(\text{CaCO}_3\) precipitation in these body fluids might exist. When the function of ACCBP was perturbed with anti-ACCBP, a large amount of low crystallinity \(\text{CaCO}_3\) appeared on the nacre lamellae. Moreover, the tissue distribution analysis showed that ACCBP is present mainly in EPF and hemolymph (Fig. 3A). Considering the strong ability of ACCBP to inhibit calcite formation, we believe that ACCBP may function as an inhibitor of calcification inhibition capacity described above, this molecule also functions as a crystal morphology controller. When anti-ACCBP was injected at a low dosage which was far from enough to affect the stability of the EPF, many screw-dislocation structures and oversized lamellae appeared. The screw-dislocation is an inorganic crystal growth pattern, which occurs when supersaturation is low (37). It was also infrequently observed in newly deposited nacre lamellae of bivalves Pinctada sp. (38, 39). However, to our knowledge, no large scale screw-dislocations and overgrowth of mature nacre lamellae have been reported. These results indicate that, even with the involvement of matrix proteins, the nacre lamellae still tend to grow as they would in an inorganic environment. To strictly control the morphology of nacre microstructures, mollusks should have a safeguard mechanism to eliminate undesired crystal growth in addition to utilizing matrix proteins to initiate the desired one. ACCBP has been shown to be a powerful crystal growth inhibitor and to be able to recognize different \(\text{CaCO}_3\) crystal phases and bind to the undesired crystal faces with specific orientation. These capabilities make ACCBP an appropriate safeguard candidate.

One important common feature of the proteins involved in shell biomineralization is the existence of some functional domains in their structures. For example, carboxic anhydrase domains were found in nacrein (40) and N66 (41); a C-type lectin domain was detected in the abalone nacre protein perlwacin (42); and recently, a protein with whey acidic protein (WAP) domains, named perlwpain, was purified from abalone nacre and shown to be able to inhibit the growth of calcite (43). All these domains are believed to be involved in protein functions in the biomineralization process. ACCBP is the first ACh-binding protein family member that was found to participate in biomineralization. As detailed in this report, the ACh-binding site is the functional domain of the ACCBP crystal growth inhibition capacity. In addition, some post-translational modifications of natural ACCBP, in particular glycosylation, might also be necessary for the ACCBP crystal growth inhibition capacity. The ACh-binding site and the polysaccharides might form a unique structure that can fully prevent the addition of \(\text{Ca}^{2+}\) and \(\text{CO}_3^{2-}\) to the crystal. Any disturbance of this structure, such as the binding of \(\alpha\)-Bgt, will obviously affect the crystal growth inhibition capacity of ACCBP. Why these polysaccharides are necessary is an interesting question. The structural analysis of lithostathine (a well-studied calcite crystal growth inhibitor found in human pancreatic juice) indicated that the backbone of a crystal growth inhibitor must be flexible enough to spread onto the crystal surface and develop strong coulombic interactions with it (44). However, with the method of Schlessinger et al. (45), only 23% of the total residues of ACCBP are predicted to be flexible and are dispersed throughout the whole sequence (data not shown). Hence, it is difficult for ACCBP to have a flexible domain like lithostathine. It may be that there is another way for ACCBP to fulfill its inhibitory role in shell biomineralization, and perhaps some modifications are needed. Polysaccharides of intracrystalline glycoproteins were shown to be able to modulate the growth of calcite crystals in vitro (46). The polysaccharides of ACCBP may confer a flexible interface and thus lead to the inhibitory effect.

Biomineralization is a process in which inorganic crystallization occurs on self-assembled organic templates under the control of organic macromolecules (47). In this process, matrix proteins provide a clear example of the capacity of biomolecules to form the mineralization framework, initiate crystallization, and act as the crystal nucleus to induce the desired crystal polymorph and crystal face (14, 15). Accordingly, many studies have been performed to mimic the function of matrix proteins with synthetic self-assembled macromolecules and many exciting results have been obtained (21, 48). However, so far, it is still not possible to synthesize nacre in an artificial system. The discovery of ACCBP suggests that some indispensable components involved in shell calcification might be unknown, because these components have not been found in mature shells or their functions are difficult to identify. In this context, both the results and the methods of this work will help in understanding the shell mineralization process from a new point of view. By involving a safeguard mechanism, as occurs in the pearl oyster, a new methodology might be developed to produce novel materials, including nacre, by a biomimetic approach.

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