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Calmodulin-Like Calcium-Binding Protein Identified in Calcium-Rich Mineral Deposits From Freshwater Mussel Gills

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ABSTRACT Extracellular calcium concretions found in the gills of freshwater unionids are largely inorganic, containing calcium and phosphate. The organic fraction of the isolated concretions accounts for 25% of their dry weight as determined by ashing. The organic fraction of isolated concretions was examined using SDS-polyacrylamide electrophoresis. The concretion fraction is separated into 13–14 protein bands. These bands can be further differentiated into EDTA soluble proteins and insoluble core proteins. One of the soluble proteins has a molecular weight of approximately 17,000 daltons (Da) and comigrates with authentic vertebrate calmodulin. The 17,000-Da concretion protein cross-reacts with sheep IgG prepared against bovine brain calmodulin as shown by immunoblot. Rabbits and mice, challenged with the entire concretion organic fraction, produce an IgG fraction which always recognizes an epitope of the 17,000-Da protein as the major antigenic determinant. This same protein is also a calcium binding protein as shown by ^{45}Ca autoradiography even after heat and SDS/mercaptoethanol treatment. To determine that this protein was not bound to the concretions as an artifact of the isolation procedures, immunocytochemical procedures were used to localize the antigenicity to 17,000-Da and vertebrate calmodulin in situ. Immunocytochemical localization indicates the protein is present on the concretions in situ but documents that the majority of this protein is located in concretion-forming cells. Electron microscopic immunocytochemistry demonstrates that the protein is found largely in early concretion-forming cells in protein granules formed before concretion architecture is observed. This may imply that the protein is likely important in the initiation of concretion formation, and is only a residual protein as found on the extracellular concretions.

The gills of freshwater mussels have extensive accumulations of calcium-rich mineralized concretions (CC) which can account for 50% of the gill's dry weight in some species (Silverman et al., '83, '85; Pynnonen et al., '87). These CC are mobilized during reproduction, and apparently act as a calcium source for larvae which develop a complete calcium carbonate-based shell while still enclosed within the water channels of the maternal gill (Silverman et al., '85; '87a). Following the reproductive period, the concretions are replaced beginning the storage of calcium for the next reproductive season.

The inorganic constituents of the concretions account for 75% of the concretions by dry ash weight (Silverman et al., '83), and are composed primarily of inorganic Ca and phosphates either as pyro- or ortho- forms. Iron and manganese are also present in minor amounts, while no other divalent cations are detected above 0.1% by weight (Silverman et al., '87b). The organic fraction of the concretions accounts for 25% by weight of which less than 3% is accounted for by carbonates, the remainder being complex organic molecules. The lack of appreciable sulfur as assayed using energy dispersive X-ray microanalysis indicates that sulfated polysaccharides are not major components of the system. Some oxidizable polysaccharide is present as revealed by periodic acid-Schiff and silver methinamine histochemistry (Silverman et al., '83). The present paper reports the partial solubilization and characterization of the organic matrix of these concretions, based on their ease of solubility and SDS-PAGE analysis.

Among the easily solubilized CC proteins a 17-kilodalton (kDa) protein was identified by its capacity to bind ^{45}Ca and the immunoreactivity of polyclonal antisera against bovine brain calmodulin. Additionally, immunohistochemical reactivity of anticalmodulin sera was localized primarily within CC-forming cells in frozen sections of mussel gills. This

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17-kDa CC protein may play a role in Ca regulation during the annual cycle of Ca mineralization/ mobilization within the mussel gill.

MATERIALS AND METHODS

Isolation of concretions for study

Two species of unionid, *Anodonta grandis* and *Ligumia subrostrata,* were collected from ponds in Livingston Parish, Louisiana, and kept in artificial pondwater (Silverman et al., '83) for several weeks before use. The concretions in the two species are similar to each other in ionic analysis and polyclonal antibodies made to CC material from either species specifically cross-reacts with CCs in tissue sections from the other species (Steffens et al., '85). Concretions were isolated for protein analysis by removal of the gill and dicing into cold dH_2O containing TLCK (N-a-p-tosyl-L-lysine chloromethyl ketone) (10 μg/ml) and PMSF (phenyl methyl sulfonyl fluoride) (1 ng/ml). The tissue was briefly homogenized using a Tekmar (Cincinnati, OH) tissue homogenizer, filtered through cheesecloth, and layered into 2.5 M sucrose. Concretions which settled through sucrose in 2–3 hours, leaving gill tissue on the surface, were collected and washed 4× by centrifugation at 5,400*g*, lyophilized, and stored at −20°C.

Amino acid analysis of the concretion organic fraction

Intact isolated concretions were hydrolyzed by treatment in 6 N boiling HCl for 24 hours. Amino acids present in the sample were analyzed using an automated amino acid analyzer (Model 470A Gas-Phase Protein Sequencer, Applied Biosystems) following the general methodology for amino acid analysis as in Parekh et al. ('85).

Isolation of protein fractions

Concretions were analyzed for superficial versus core proteins based on their ease of solubilization. Concretions treated with 25 mM EDTA, containing the protease inhibitors TLCK and PMSF, released a number of superficial proteins that were concentrated using Centricon-10 filter units (Millipore). The concretions cores which remain intact were further treated by boiling 2 minutes in 1% SDS-2% mercaptoethanol. Analysis of the superficial and the core proteins was carried out by SDS-PAGE using the discontinuous buffer system of Lammeli ('70). Gradient gels (6–14%) were loaded with 3 μg protein/lane (Biorad assay), and included a molecular weight standards (BRL) lane for comparison. Gels were stained initially with Coomassie blue followed by silver staining as developed by G.M.W. Adams (Sibley et al., '86).

Assays of calcium-binding capability of CC proteins

The capacity of CC proteins to bind calcium was evaluated by "dot blot" techniques modified from Maruyama et al. ('84). Briefly, solubilized CC protein fractions, bovine serum albumin, and bovine brain calmodulin were applied to 0.1, μm nitrocellulose paper (Schliecher and Scheuel) in 50 μl of buffer containing 1, 5, 10, or 20 μg/dot. Air-dried strips were incubated 10 min in 10 mM Tris-HCl, 60 mM KCl, 5 mM $MgCl_2$, pH 6.8, containing 2×10^6 CPM/ml ^{45}Ca. Strips were then washed, 3×5 minutes in dH_2O pH 6.8, air-dried, and autoradiographed at −70°C for 48 hours.

To examine calcium binding proteins contained in the CC further, proteins were separated by SDS-PAGE and transferred electrophoretically (50 V, 1 hour) onto nitrocellulose paper using the buffer system of Towbin et al. ('79), and exposed to ^{45}Ca as described above.

Immunoblot preparation

CC proteins separated by SDS-PAGE and blotted onto nitrocellulose paper (NCP) were blocked for 1 hour with Tris-saline, 25 mM Tris-HCl, 0.9% NaCl, pH 7.5, containing 1% TWEEN-80 and 3% BSA. NCP blots were incubated with IgG fraction antibodies prepared against concretion antigens in mice (Steffens et al., '85), and rabbits (1 : 100), or with sheep anticalmodulin (1 : 250) (bovine brain, Cabco and Medical Diagnostics, Cambridge, MA). Antigens recognized by 1° antisera were visualized by ^{125}I protein A binding (1×10^6CPM/ml, 2×10^6 CPM/ug), followed by autoradiography.

Alternatively, some immunoblots were screened using a second antibody conjugated to peroxidase. NCP strips were blocked and exposed to rabbit anti-CC or sheep anticalmodulin at 1 : 250 in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin. NCP strips incubated in anticalmodulin antibody were washed extensively and incubated in rabbit antisheep IgG (US Biochemicals, Cleveland, OH) diluted 1 : 200, in PBS, 0.5% BSA for 2 hours. Strips were washed 5× (10 min) in PBS, and exposed to peroxidase-conjugated antirabbit IgG, diluted 1 : 200 in PBS, 0.5% BSA for 3 hours, after which the strips were washed 5× (5 min) in PBS. Peroxidase activity was developed for 10 minutes using a reaction mixture containing 7.5% H_2O_2, 37.5% (wt/vol) 4-chloronaphthol, 12.5% methanol in PBS.

Calmodulin antibody binding to sections of mussel gills

Gills of *Ligumia subrostrata* were excised and frozen in liquid nitrogen, thawed to −20 °C, and sectioned at 20 μm. These sections were air-dried and then exposed to calmodulin antibody. Alternatively, gills were excised and placed in cold 0.1% glutaraldehyde containing 50 mM Tris for 2 days and then embedded in JB-4 plastic. The plastic resin was infiltrated without dehydration, and polymerized in the freezer.

Exposure to antibodies was as follows. All sections were exposed 10 minutes to nonimmune rabbit serum, 1 : 10 dilution with 0.1 M phosphate-buffered saline, pH 7.0 (150 mM Na, 5 mM K, 2 mM Ca). Following incubation, sections were washed for 20 minutes in PBS. Sections were then treated with the Cabco sheep antibody to calmodulin diluted 1 : 50 and washed. Next the sections were incubated with goat anti-sheep IgG conjugated to peroxidase (Dako). The sections were then washed for a final 20 minutes in PBS and then washed for 10 minutes in 0.1M Tris-HCl, pH 7.3. Peroxidase was localized using 0.1% diaminobenzidine and 1% H_2O_2 in Tris buffer for 3 minutes followed by washing with excess Tris-HCl for an additional 10 minutes and mounting in glycerol.

Controls for the immunocytochemical reaction described included reacting some sections for peroxidase after no antibody treatment, omission of the calmodulin antibody from the treatment, and omission of the peroxidase-conjugated antibody from the treatment.

Ultrastructural localization of calcium-binding protein

Standard methods of fixation for electron microscopy of gill tissue have been described in detail elsewhere (Silverman et al., '83; Steffens et al., '85; Dietz et al., '85).

For immunoelectron microscopy, gills of *L. subrostrata* were excised and placed directly into a dish containing ice-cold 0.5% glutaraldehyde in distilled water with the pH adjusted to 7.3 with NaOH. The tissues were fixed for 15 min at 4°C. Following fixation, the desired areas were dissected out, minced, rapidly dehydrated to 95% ethanol, and embedded in L.R. White resin with a thermal cure.

The primary antibodies utilized were mouse anticoncretion, rabbit anticoncretion, and sheep antibovine brain calmodulin. For labelling with the rabbit and mouse-derived antibodies (against whole concretions), sections mounted on Formvar-coated nickel grids were processed for indirect immunogold labelling utilizing optimally diluted primary antibodies and goat-antimouse IgG or goat antirabbit IgG secondary antibodies conjugated to 20 nm gold in a method similar to that described by De May et al. ('81) with some modifications. Controls were performed by utilizing nonimmune or irrelevant primary antiserum and by using nonspecific gold-labelled secondary antiserum. The sheep-derived anticalmodulin antiserum was utilized in a direct immunolabelling method as described by Roth ('83). The antiserum was conjugated to 20 nm colloidal gold using the technique of Roth ('83). Controls were performed utilizing gold-labelled sheep antiserum of irrelevant specificity. Following immunolabelling by either method, the sections were poststained with uranyl acetate and lead citrate.

TABLE 1. Amino acid content of isolated concretions from the gill of Anodonta grandis[1]

Amino acid	Content (μM/g)
GLU	99.2
ASP	89.4
GLY	79.6
ALA	59.3
PRO	54.1
SER	50.5
THR	47.8
LEU	46.6
ARG	42.1
LYS	38.8
ILE	35.3
PHE	23.2
TYR	20.8
HIS	15.7
VAL	5.0
MET	2.7
CYS	0.5

[1]Values represent the means from two concretion preparations.

RESULTS

Protein composition of the calcium-rich concretions

SDS-PAGE gels of the concretion protein components from *Anodonta grandis* are shown in Figure 1. Some of the CC proteins are easily solubilized by treatment with 25 mM EDTA (lane 4) for short periods. Other proteins were solubilized only by boiling in the running buffer for SDS-PAGE containing 2% SDS and 1% mercaptoethanol (lane 3). After this rigorous treatment some concretion core material remained insoluble. Our only successful attempts to solubilize this portion of the CC involves boiling in high concentrations of mercaptoethanol and the resultant suspension contains no intact peptides. The easily soluble and less soluble proteins associated with the concretions include 12–15 major proteins, which account for all peptides seen in the total CC

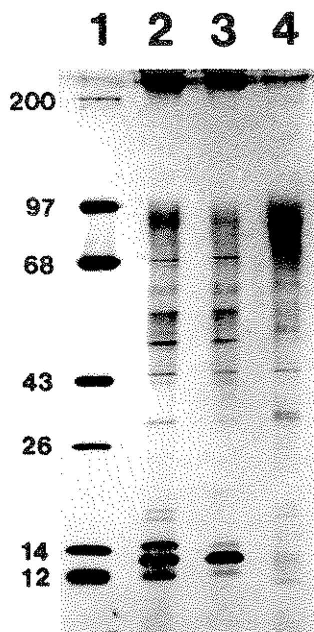

Fig. 1. SDS-PAGE gel of concretion proteins. **Lane 1:** Contains molecular weight standards (BRL), with the molecular weights of each of the standard indicated in (kDa). **Lane 2:** The total proteins from EDTA-untreated concretions. **Lane 3:** The EDTA-insoluble core proteins. **Lane 4:** The EDTA-soluble "surface proteins." The lanes have been stained initially with Coomasie blue followed by silver staining for the detection of proteins. The most abundant of the isolable proteins in the complete concretions appear as a triplet of bands between 12,000 and 14,500 Da. There are an additional 9 to 10 lesser bands of higher molecular weight appearing in the EDTA-untreated lane. Of interest is the complementary pattern of the two lanes (3, 4) derived by EDTA treatment. The three low molecular weight bands present in the whole concretion (lane 2) are also present in lane 3, while barely represented in lane 4 indicating that these major components are not solubilized by EDTA, and remain with the core. Note also that the next three higher molecular weight bands, a doublet around 17,000 Da and a band at 32,000 Da, in the whole concretions are not present or much reduced in lane 3, indicating that these components are extracted by EDTA treatment. Lane 4 confirms this result. There are several additional bands (49,000, 55,000, and 68,000 Da) that also appear more associated with the core than with surface components.

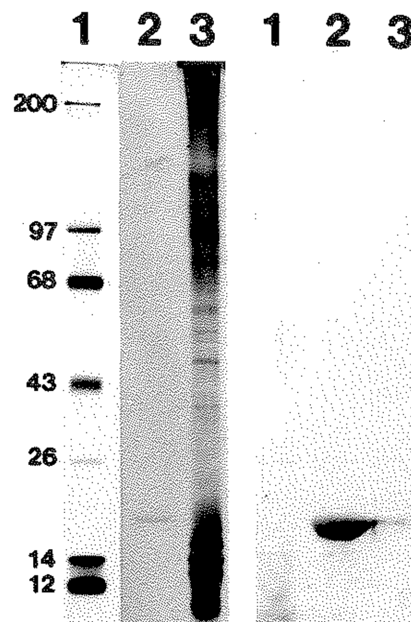

Fig. 2. A complementary SDS-PAGE gel **(left)**, and nitrocellulose paper **(right)** to which the proteins on the gel have been electrophoretically eluted. In both, **lane 1** contains molecular weight standards, **lane 2** has been loaded with authentic bovine brain calmodulin (17,000 Da), and **lane 3** has been loaded with total concretion protein. The gels in this figure have been overloaded as compared to the gels seen in Figure 1. The gels (left) are shown after silver staining. After protein elution, the nitrocellulose was exposed to ^{45}Ca after Maruyama et al. ('84) and the autoradiograph is shown (right). Lane 1 shows no proteins with calcium binding activity under these conditions. Vertebrate calmodulin binds calcium as does a 17,000 molecular weight protein from the concretions. By comparison with Figure 1, it is clear that this protein is one of the EDTA-soluble components of the concretion.

protein SDS-PAGE gels (Fig. 1, lane 2).

Amino acid composition of the protein components of the concretions is shown in Table 1. The acidic amino acids glutamic acid and aspartic acid are by far the most abundant amino acids in the total protein pool and the amino acids containing sulfur, methionine, and cysteine represent the least abundant amino acids.

The identified fractions of solubilized proteins were assayed for calcium binding using ^{45}Ca to overlay protein administered in dot blots to nitrocellulose paper. The total CC protein shows Ca-binding activity as does the easily solubilized fraction, while the less easily solubilized fraction shows no binding activity. Reaction was also seen in the whole CC fraction which was solubilized in this study by using SDS/mercaptoethanol, indicating that binding is not precluded by such treatment. The less easily solubilized fraction by itself showed no calcium binding using these techniques, further indicating that the protein(s) involved are only those of the easily solubilized fraction. Serum albumin shows no binding activity under these same conditions. Qualitative evaluation of dot blots indicates that calcium binding is a function of the concentration of CC protein loaded onto the blot. As a positive control calmodulin, provided by Dr. Larry Ruben, Southern Methodist University, shows binding in a concentration dependent manner using these techniques.

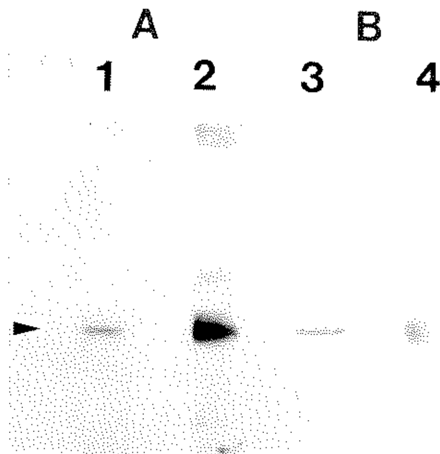

Fig. 3. Immunoblots directed against concretion proteins and calmodulin. Proteins isolated by SDS gel electrophoresis were transferred to NCP. The arrowhead denotes 17 kDa. **Lanes 1–3:** Isolated concretion protein. **Lane 4:** Bovine brain calmodulin. Lane 1 was reacted with iodinated rabbit IgG prepared against total concretion proteins. Lane 2 was reacted with iodinated mouse IgG prepared against total concretion protein. Lane 3 was reacted against iodinated sheep antibovine brain calmodulin IgG. Lane 4 was reacted against sheep antibovine brain calmodulin.

Following these preliminary experiments, total CC proteins were separated by SDS-PAGE and transferred to NCP. These NCP blots were analyzed for ^{45}Ca binding (Fig. 2). A single protein of 17 kDa found in the CC bound calcium after ^{45}Ca overlay. Bovine brain calmodulin also bound ^{45}Ca in blot overlays; however, none of the molecular weight standards demonstrated ^{45}Ca-binding. Figure 2 also demonstrates that the CC calcium-binding protein comigrates with calmodulin from cow brain.

To assay further the similarity of the two molecules, immunoblotting was performed using prepared calmodulin antibody produced in sheep to bovine brain calmodulin, and the previously described polyclonal antibodies to CC proteins (Steffens et al., '85). These polyclonal antibodies prepared against CC from *Ligumia subrostrata* were previously shown to cross-react against both CCs and CC-forming cells from several species of unionids. The mouse polyclonal IgG fraction labelled several CC proteins, but clearly labelled the 17-kDa protein as a major antigen (Fig. 3). The rabbit antibody reacts only with the 17-kDa protein, suggesting the high antigenicity of this protein when injected into mammals (Fig. 3). Anticalmodulin antibody reacted with both authentic bovine brain calmodulin (Fig. 3, lane 4) and the 17-kDa band in CC (Fig. 3, lane 3).

Immunocytochemical localization of the 17-kDa calcium-binding protein

With the aid of either frozen sections or sections which have been fixed in the cold and embedded in a methacrylate resin, antigenic sites identified by sheep antibovine brain calmodulin were localized by peroxidase immunolabelling (Figs. 4, 5). Peroxidase activity was localized in the CCs, but primarily it was localized to those cells which are thought to initiate production of the CCs (Silverman et al., '88). These cells are characteristically located in the connective tissue lying below the gill filaments, and also between the chitinous rods which support the gill filaments (Figs. 4, 5). A lesser amount of peroxidase activity was found associated with the lateral ciliated cells of the gill filament epithelium, and the epithelium lining the water channel (not shown) also shows some peroxidase localization. None of the controls—including a) no antibody treatment of the sections to test for endogenous peroxidase activity, b) only calmodulin antibody, and c) only the peroxidase-coupled antibody—showed any peroxidase localization.

The immunocytochemical studies were repeated at the electron microscopic level, and the location of colloidal gold conjugated to rabbit concretion antibody was evaluated. The localization studies suggest that the antibody is located over electron-dense granules known (Silverman et al., '88) to be the site of concretion initiation (Figs. 6, 7).

DISCUSSION

Mineralization in molluscan systems is similar to mineralization processes found in other animal phyla

Fig 4. Immunohistochemical localization of a polyclonal sheep antibody against bovine brain calmodulin on a freshwater mussel gill section. Visualization was indirect employing horseradish peroxidase conjugated to a secondary antibody. The section demonstrates concretion masses (open arrows) showing diffuse reaction product, and a substantial amount of reaction product occurs on concretion forming cells (filled arrows). For orientation the gill filaments appear at the top of the micrograph and the filaments are supported by chitinous rods (r). Bar = 250 μm.

Fig. 5. Immunohistochemical localization of a polyclonal sheep antibody against bovine brain calmodulin on a freshwater mussel gill section. Higher magnification of what was seen in Figure 4 confirms the diffuse localization of label over concretions masses (open arrow) and the heavy activity of concretion forming cells (closed arrows). Rods (4) support the filaments at the top of the section. Bar = 100 μm.

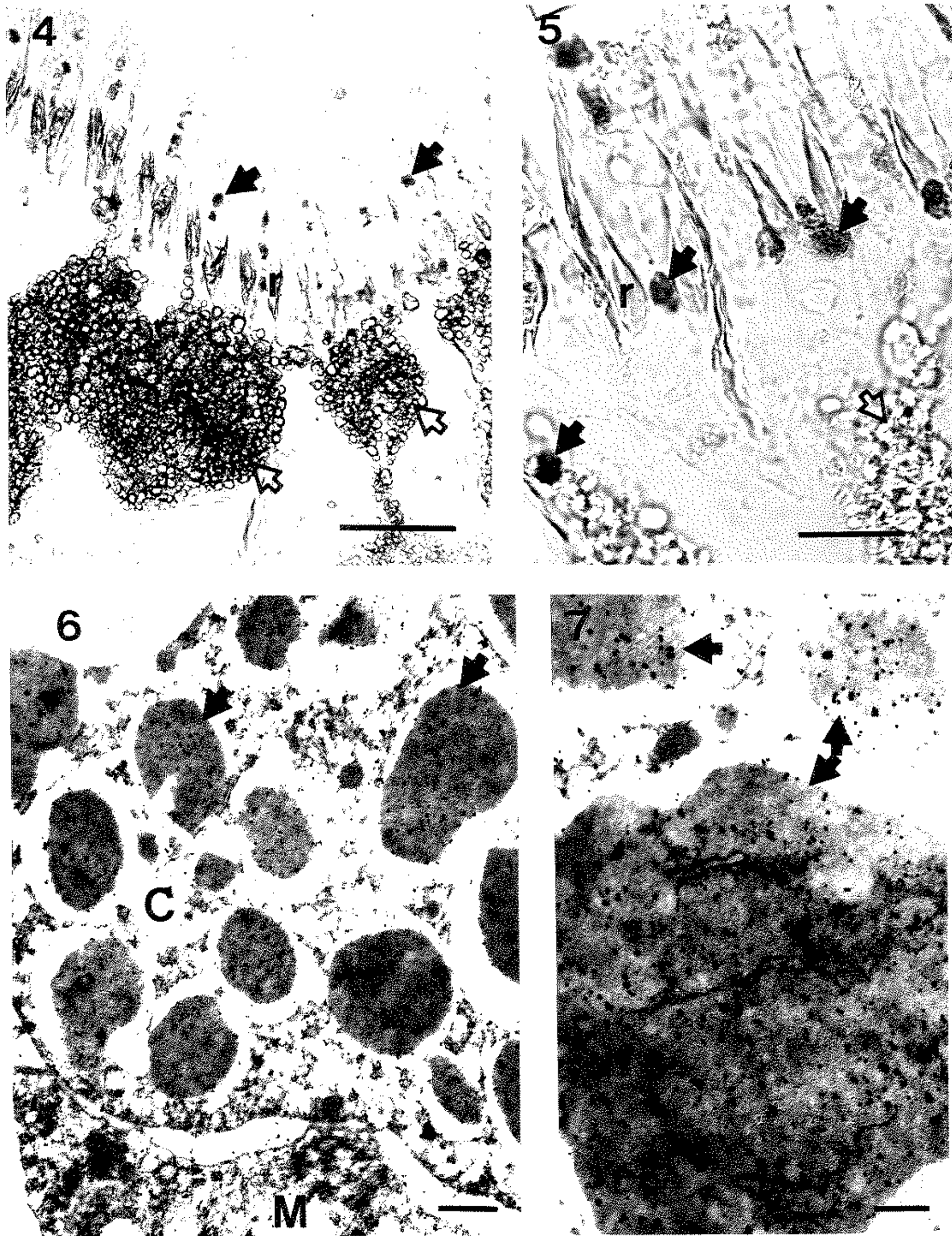

Fig. 6. Transmission electron micrograph of a lightly fixed concretion forming cell demonstrating that rabbit antibody to the 17-kDa concretion protein, conjugated to colloidal gold, is localized over a concretion-forming cell (C). A muscle cell (M) is located in the lower portion of the micrograph. Both the muscle cell and the connective tissue spaces are relatively devoid of gold particles. Most of the gold is located over electron dense cytoplasmic granules (arrows) in the concretion forming cell. Bar = 0.75 μm.

Fig. 7. Transmission electron micrograph of a portion of a concretion-forming cell demonstrating the localization of colloidal gold over the electron-dense granules (arrows). Bar = 0.3 μm.

in that organic matrices are associated with inorganic mineral. It is generally thought that the matrix is involved in initiation of crystal formation, orientation or alignment of crystal planes, and/or regulation of the size of crystal or mineralized layer growth (e.g., Mann, '83; Sikes and Wheeler, '86).

Several calcium binding proteins associated with the mineralization fronts in bone and teeth have been identified. Perhaps the best known is osteonectin (Termine et al., '81a,b), the 43-kDa protein associated with bone and dentine matrix. This protein is a calcium binding protein of the troponin/calmodulin family being rich in acidic amino acids which are located in multiple precise conformations—E–F hands as defined by Kretsinger ('80) and his colleagues—for calcium binding. The exact function of these proteins in the regulation of mineralization is as yet unknown. It was initially thought that these proteins might be directly involved in the initiation step of mineralization, but the presence of the 43-kDa bone protein in nonmineralizing tissues (Wasi et al., '84; Tung et al., '85; Young et al., '86; Holland et al., '87) suggests a broader function, related to regulation of calcium content at the mineralization front (Romberg et al., '85). While there are several examples of similar related proteins in the different mineralization systems, the presence of calmodulin in such systems has only been suggested by indirect experimentation (Sasaki and Garant, '87). Their study demonstrates an inhibition of Ca-ATPase activity in secreting ameloblasts by the calmodulin antagonist trifluoperazine.

In this study, we have identified a calmodulin-like protein isolated from mineralized concretions, but which is present in more abundance in the cells which produce these concretions. Evidence suggesting the molecule isolated is a mussel calmodulin includes 1) the abundant amount of acidic protein associated with the CC; 2) comigration of the 17-kDa mussel protein with bovine brain calmodulin on SDS-PAGE; 3) calcium-binding ability of the 17-kDa protein following boiling in SDS-mercaptoethanol; 4) cross-reactivity of the mussel 17-kDa protein with sheep antibovine brain calmodulin; and 5) cross-reactivity of CC antibody with authentic calmodulin. The calmodulin found associated with the concretions is in the easily solubilized EDTA fraction and may be located superficially. The immunohistochemical studies also suggest that the presence of this protein on the CC is not an isolation artifact, because of its presence on the CC in situ and its localization on CC-forming cells.

The role of the calmodulin in the mineralization of the concretions is unknown. The immunocytochemical evidence suggests that the molecule is most closely associated with the electron-dense granules in which the concretions are initially formed (Silverman et al., '88). Such localization suggests the molecule may be present to enhance the amount of calcium present at concretion initiation sites. Initiation of mineralization in the concretion system differs from both bone and teeth in that it is intracellular instead of extracellular, and calcium must be accumulated and stored instead of accumulated and secreted.

The gill CC are an interesting model to study the regulatory events associated with mineralization because of their annual cycle of production and mobilization. The CC serve as a source of calcium to be used in the mineralization of the shells of embryos brooded in the gills of the mussels (Silverman et al., '85, '87a). The calcium in the CC is mobilized precisely before reproduction, but not during other times of the year even when the organism is subjected to lower blood pHs during experimental anoxia (Silverman et al., '83). The role of the identified calmodulin either within the CC-forming cells or on the concretions themselves remains undetermined. Calmodulin is a generalized regulatory protein modulating many cellular metabolic events based on small increases in cytoplasmic ionic calcium (Carafoli, '87 for review). As such, calmodulin is a high-affinity protein capable of binding calcium in the 0.1 μM range at four specific binding sites (Kretsinger, '80). The structure and properties of the protein thus are not advantageous for direct storage of high levels of calcium, i.e., the concretion calcium. On the other hand, calmodulin has been implicated in regulating calcium transport across cellular membranes of various organelles in many different cell types (see Klee and Vanaman, '82; Carafoli, '87) and may function similarly in the CC-forming cells to promote accumulation of calcium at the mineralization site within membrane bound intracellular granules. Further investigation of this system should demonstrate the possible role of calmodulin in regulating intracellular mineralization systems.

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