Control of Calcium Carbonate Nucleation and Crystal Growth by Soluble Matrix of Oyster Shell

Abstract. *A calcium-binding soluble protein extracted from oyster shell suppresses calcium carbonate nucleation and decreases the rate of crystal growth in vitro. These findings suggest that soluble matrix may regulate shell growth.*

Part of the organic matrix of molluscan shell is rendered soluble after the mineral is dissolved with either HCl or EDTA. Protein or glycoprotein components of this soluble matrix (SM) have calcium-binding properties (*1, 2*). Two functions have been suggested for such proteins: (i) the calcium associated with them may be involved in the initiation of crystal nucleation (*1–3*), and (ii) the protein may inhibit and thus regulate crystal growth, (*3, 4*), possibly by binding to the growing centers of mineral (*4*). Although both suggestions are plausible, no direct evidence has been provided to support either idea for carbonate mineralizing systems. The studies presented here demonstrate an effect of SM on the rate of $CaCO_3$ precipitation from model solutions.

Soluble matrix was obtained from shells of freshly shucked oysters (*Crassostrea virginica*). Shells were dipped in 5 percent NaOH, lightly scrubbed, rinsed with distilled water, ground to powder with a porcelain mortar, and mixed with a small quantity of distilled water. The slurry was decalcified either by dialysis against a 10 percent EDTA, 50 m*M* sodium phosphate solution (*p*H 7.5) at 4°C, or by gradual addition of concentrated HCl at 20°C with the *p*H maintained at 3 to 5. After the mineral was dissolved, the resulting solution was centrifuged at 30,000*g* for 30 minutes to sediment undissolved shell and insoluble matrix. Routinely, the supernatant, containing the SM, was dialyzed against distilled water. For molecular weight determinations, the matrix extract was concentrated by dialysis against dry Sephadex. The protein content of the extracts was determined by the Miller modification of the Lowry technique (*5*).

The effect of oyster SM on the rate of precipitation of $CaCO_3$ was determined by recording the decrease in *p*H of a solution containing 3 ml of 20 m*M* $NaHCO_3$ and 0.3 ml of H_2O or redialyzed matrix protein when 3 ml of 20 m*M* $CaCl_2$ was added rapidly (*6*). The initial *p*H (and thus carbonate concentration) of the bicarbonate solution was adjusted to approximately 8.7, giving precipitation with 0.3 ml of H_2O added at 23°C in less than 5 minutes.

Recordings of a series of precipitation experiments are shown in Fig. 1. There was a nearly instantaneous downward shift when $CaCl_2$ was added to the bicarbonate solution. The cause of this sudden change is unknown, for it occurred even if the $CaCl_2$ portion was adjusted to *p*H 8.7 or buffered (1 m*M* tris-HCl, *p*H 8.7). The change may have resulted from complex formation among the ionic species, distinct from nucleation events. After this sudden shift, the *p*H was relatively stable until nucleation occurred, and it again declined as visible precipitate formed.

The duration of the stable period was increased markedly over the control by the addition of SM at 2.65 μg/ml (a total of 16.7 μg; Fig. 1A). In addition, once nucleation had occurred, the rate of net precipitation or crystal growth ($\Delta pH/\Delta t$) was less for reactions with protein than for controls. At 7.94 μg/ml (a total of 50 μg; Fig. 1A), the decrease in *p*H was delayed for hours, suggesting inhibition of nucleation. However, observations of *p*H changes for such long-term incubations were complicated by loss of CO_2 to the atmosphere, accompanied by an upward drift in *p*H. A direct effect of SM on crystal growth as opposed to nucleation was demonstrated by the sudden cessation of the decline in *p*H on addition of protein after nucleation had occurred and precipitation was in progress (Fig. 1B).

The molecular weight of SM was estimated by sodium dodecyl sulfate–acrylamide disc gel electrophoresis, using the periodic acid–Schiff (PAS) reaction to stain SM and Coomassie blue to stain cross-linked serum albumin standards (Sigma) (*7*). A single discernible band with an estimated molecular weight of 170,000 was obtained from matrix preparations. Accordingly, the regulatory effects of the protein occur at concentrations estimated as 1.56×10^{-8} *M* or less under the conditions reported here (*8*). Coomassie blue failed to stain the matrix in gel preparations or in solution (*9*). This lack of staining in contrast to the relatively intense staining by PAS suggests that a significant portion of the SM might be carbohydrate or that the carbohydrate portion blocks the staining reactions by Coomassie. Alternatively, if the SM has few basic residues, a weak reaction with Coomassie might be expected (*10*). The molecular weight, homogeneity, and carbohydrate content reported here for oyster SM are comparable to those observed for SM from clams (*1*). In that case, a single matrix protein of molecular weight 160,000 was found that was 10.2 percent carbohydrate by weight. However, the present findings are different from those

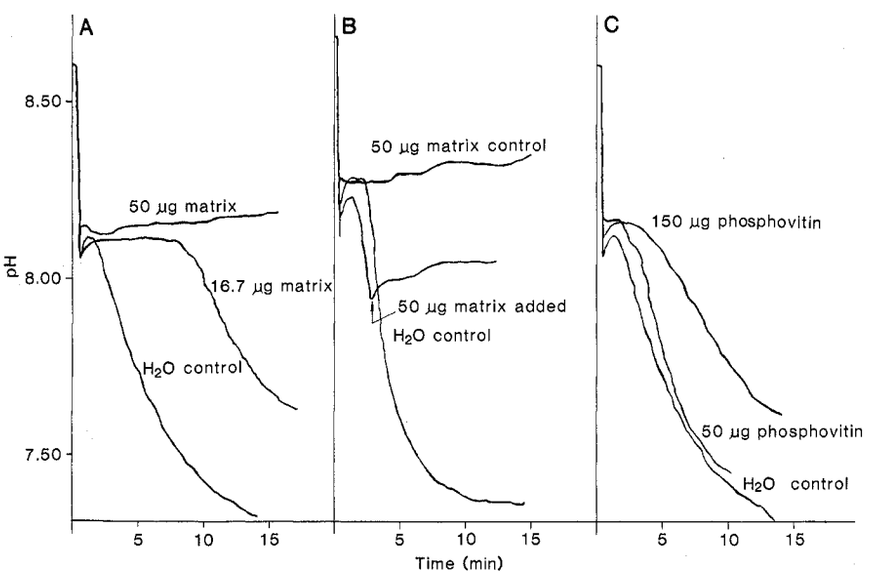

Fig. 1. Recordings of $CaCO_3$ precipitation in solutions containing 3 ml of 20 m*M* $NaHCO_3$ (*p*H approximately 8.7) after the addition of 3 ml of 20 m*M* $CaCl_2$ at time 0. (A). Effect of matrix protein on precipitation. The protein was introduced in 0.3 ml of water before the introduction of $CaCl_2$. The H_2O control had 0.3 ml of H_2O added before the $CaCl_2$ solution. (B) Effect of matrix protein addition after onset of $CaCO_3$ formation. Protein and water controls were treated in the manner described in (A). (C) Effect of phosphovitin on $CaCO_3$ precipitation. The procedure was as described in (A).

0036-8075/81/0619-1397$00.50/0

reported for a variety of gastropod shells, whose SM's were resolved as several proteins, all with molecular weights lower than that reported here (*2*).

Calcium-binding studies were performed on matrix solutions that had been redialyzed, first against 1 m*M* EDTA (*p*H 7.5) and then against water. Redialysis removed residual calcium from matrix preparations. The binding capacity of the protein was determined by measuring the decrease in free calcium in 10 ml of 10^{-3} to 10^{-4} *M* $CaCl_2$ (*p*H 6.0) when matrix protein (3 to 150 μg) was added (*11*). For all preparations, the quantity of calcium removed from solution was directly proportional to the quantity of protein added and was independent of calcium concentration in the range of 10^{-3} to 10^{-4} *M*, indicating that the protein was saturated with calcium during the experiments. The mean binding capacity for four preparations (two EDTA-extracted and two HCl-extracted) was 23.2 ± 2.92 μmole of calcium per milligram of protein (mean ± standard error). This is considerably higher than the 0.65 μmole/mg obtained for SM from clams (*12*). The difference may result in part from estimation of SM on the basis of protein content, since protein constitutes an unknown portion of the matrix molecule, or it may be related to differences in techniques for estimating amounts of matrix and protein. That the SM of oyster acts as an effective calcium-binding protein, though, is in general agreement with the findings for clam SM. The inhibiting effect on crystal nucleation and growth is not simply due to removal of calcium, however, because at an SM concentration of 7.94 μg/ml (Fig. 1) less than 2 percent of calcium in the medium is bound.

The effect of SM on $CaCO_3$ precipitation is apparently specific for the matrix protein. When phosphovitin (Sigma), a phosphoprotein that inhibits calcium phosphate crystal growth (*13*), was tested in this system (Fig. 1C) only slight inhibition of the rate and negligible inhibition of the initiation of precipitation were observed, and then only with quantities of phosphovitin greater than the quantities of matrix normally employed in these experiments. Similarly, up to 1.5 μmole (0.3 ml of 5.0 m*M*) EDTA had no effect on the rate of precipitation or the time required for initiation of precipitation (data not shown).

The results reported here directly demonstrate regulation of crystal nucleation and growth by a protein in a $CaCO_3$ system. Such regulation by calcium-binding proteins has been demonstrated for calcium phosphate systems and has been proposed for components of insoluble (*14–16*) and soluble (*4*) matrix of molluscan shell. The results do not favor a role for calcium-binding SM in initiating crystal growth, nor do they exclude it. However, one might have expected an effect on precipitation if initiation were the primary function of the protein.

Exactly how the matrix protein binds to a forming crystal is not known. However, it is tempting to speculate that the multiple binding sites for calcium present on these proteins might interact with the nucleation site or surface of the forming crystal in such a way as to prevent further accretion of mineral, presumably by raising the free energy for activation of this process. For this protein-crystal association to occur, the protein must have a much higher affinity for the crystal than for free calcium, as the binding sites on the protein would normally be saturated with calcium from solution.

In summary, SM from molluscan shells has the potential to regulate $CaCO_3$ deposition. Control of crystal growth thus seems to depend on control of SM secretion or activation, processes about which virtually nothing is known.

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12. This was calculated from data in (*1*). Binding capacity was expressed as micromoles of CA^{2+} bound per micromole of matrix sulfate, with sulfate and protein estimated as 9.9 and 79.5 percent of the matrix by weight, respectively.
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17. We thank D. Dillaman and S. Sikes for commenting on the manuscript. Supported by NSF grant PCM79-16587.

31 March 1981

Improved Detail in Biological Soft X-ray Microscopy: Study of Blood Platelets

Abstract. *Improved image quality in soft x-ray contact microscopy can be obtained by examining the resist with transmission rather than scanning electron microscopy. Application of the new technique to air-dried preparations of human blood platelets reveals structures not visible in the same cells with transmission electron microscopy or when the resist is examined by scanning electron microscopy. As seen by the new technique, platelet pseudopods contain a central structure connected to a network in the platelet and dense bodies exhibit a lamellar structure.*

Contact x-ray microscopy is an excellent imaging technique for use in biological studies (*1*). It has several advantages over other imaging techniques currently in use. Resolutions in the range of 5 nm can be achieved with relatively thick and hydrated specimens, and information about the subcellular distribution of light elements can be obtained from their absorbance or fluorescence properties. In addition, staining is not required to obtain relatively good contrast in biological structures, and imaging is possible at radiation doses somewhat lower than those needed for imaging with electrons. The technique has been limited by the necessity to metallize the relatively thick resist for examination in the scanning electron microscope (SEM) and by the limited resolution of commercially available SEM's. Valuable information contained in small topological variations in resist thickness is thus obscured and not quantifiable, and the best achievable resolution is limited by the necessity to distinguish between small differences in secondary electron emission. This report describes a modification of the x-ray resist technique which permits examination of thin x-ray resists directly in

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