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Mollusk Shell Formation: Isolation of Two Organic Matrix Proteins Associated with Calcite Deposition in the Bivalve *Mytilus californianus*†

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ABSTRACT: The role of the organic matrix during shell formation is investigated by comparing the soluble protein constituents in two shell layers of the bivalve *Mytilus californianus*. The two shell layers differ primarily with respect to ultrastructure and mineralogy. The proteins are separated by using ion-exchange chromatography followed by high-performance liquid chromatography. They are further characterized by an analysis of their mild acid hydrolysis cleavage products which reveals information on aspartic acid containing amino acid sequences present in the proteins. Of the approximately 40 matrix constituents separated, only two proteins present in the outer prismatic (calcite) layer contain the amino acid sequence -(Asp-Pro-Thr-Asp)-. These proteins, which have been purified to homogeneity, may in part be responsible for determining the particular type of calcium carbonate polymorph deposited in the outer shell layer.

Mollusk shells are among the many skeletal hard parts that are formed by the so-called "organic matrix mediated" process (Lowenstam, 1981). Cells of the mantle epithelium synthesize an extracellular structural framework composed primarily of proteins and carbohydrates. The mineral crystals, usually either aragonite or calcite, nucleate and grow within this preformed framework. The precise manner in which the organic matrix influences crystal growth is not known. X-ray diffraction studies of the septal nacreous layer of *Nautilus repertus* show that the bulk of the matrix protein polypeptide chains is aligned parallel to the *b* axis of the associated mineral, aragonite (Weiner & Traub, 1980). Similar results have been obtained for the nacreous layer of the gastropod shell by using electron diffraction (unpublished data). These observations are consistent with the notion that crystal growth in mollusks occurs by an epitaxial process upon a matrix template. Yet little is known about the macromolecules responsible for this process or the precise manner in which crystal growth occurs.

Many attempts have been made to study the functions of matrix molecules in vitro (Nawrot et al., 1976; Blumenthal et al., 1979; Termine et al., 1980; Krampitz et al., 1976; Wheeler et al., 1981). Although these experiments undoubtedly provide important guidelines as to possible functions that these macromolecules may perform, they differ significantly from the in vivo situation in which the matrix constituents together constitute a macro-three-dimensional framework which is assembled prior to the onset of crystal growth. One approach is to reintroduce various components of the framework into the in vitro system. This has been done successfully by Termine et al. (1981), who demonstrated that a particular acidic bone matrix protein, called osteonectin, when complexed with insolubilized type I collagen, is capable of binding synthetic apatite crystals and free calcium ions. Biosynthesis studies of mineralized tissues indicate that certain of the matrix molecules do migrate rapidly to the mineralization front (Weinstock & Leblond, 1973; Dimuzio & Veis, 1978) or are introduced into the extracellular space only after the onset of mineralization (Price et al., 1981). These experiments, together with the large body of biochemical in-

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formation that has accumulated on the organic matrix components of mineralized tissues, provide invaluable insights into the general functions of these macromolecules. The specific roles that they perform are, however, poorly understood.

The basic strategy adopted in this study is to compare the matrix constituents from the shell layers of the same mollusk which differ primarily with respect to ultrastructure and mineralogy. Constituents common to both shell layers are assumed to perform common functions, whereas those specific to one shell layer are responsible for determining some unique property of that shell layer. As the shell layers differ in their mineralogy, some of the constituents specific to one layer should be responsible for this difference. Preliminary candidates for this role are then selected based on a partial characterization of Asp-containing amino acid sequences which may match certain Ca–Ca distances in the associated mineral phase (Weiner, 1981).

The study is limited to the matrix constituents which are soluble after decalcification in ethylenediaminetetraacetic acid (EDTA) at neutral pH. Transmission electron microscope investigations of sections cut through individual matrix layers indicate that the soluble matrix constituents are located primarily on the outer surface of the matrix (Iwata, 1975; Nakahara, 1979; Nakahara et al., 1980) and hence are in direct contact with the mineral (Weiner et al., 1983). The matrix core in gastropods is itself a layered structure with a thin sheet of β-chitin sandwiched between two thicker layers (Nakahara, 1983) of presumably silk-fibroin-like protein (Weiner et al., 1983).

The study reported here shows that about half the soluble matrix constituents are common to both the outer prismatic-calcite layer and the nacreous-aragonite layer of the bivalve *Mytilus californianus* and half are specific to one or the other layer. Among the latter are two proteins from the calcitic layer, which have been purified to homogeneity, and have an aspartic acid containing amino acid sequence not found in the aragonitic shell layer. These proteins may, in part, be responsible for determining the particular type of calcium carbonate polymorph deposited in the outer shell layer.

Experimental Procedures

Organic Matrix Preparation. Shells of *Mytilus californianus* Conrad (Los Angeles, CA; freshly collected) were air-dried and stored until use. The outer prismatic (calcite-bearing) layer was prepared from the portions of the shell outside the pallial line. The nacreous (aragonite-bearing) layer was obtained by grinding off the outer and inner prismatic layers. Care was taken to exclude the myostracal areas. All surfaces were carefully cleaned. The shell layers were decalcified according to the method of Weiner (1979), except that the desalting step at the end of the procedure was omitted. Instead, the sample was redialyzed exhaustively against double-distilled water. Note that in all the following experiments Spectrapore 1 dialysis tubing was used. Gel electrophoresis patterns of the soluble constituents of *M. mercenaria* decalcified in the presence and absence of protease inhibitors were identical, and hence the latter were not used in this study.

Ion-Exchange and High-Performance Liquid Chromatography (HPLC) of the Soluble Matrix Constituents. About 10 mg of the soluble fraction of each shell layer was chromatographed on a Whatman DE 52 ion-exchange column in 0.05 M tris(hydroxymethyl)aminomethane (Tris), pH 8.20, by using a linear 0–0.6 M NaCl gradient, as described earlier (Weiner, 1979). The eluant was pooled into 6 fractions, dialyzed against water and lyophilized. The fraction containing urea was treated separately. Each fraction was redissolved in 300 μL of 0.05 M sodium acetate, pH 6.5, containing 2.5% (v/v) acetonitrile and dialyzed against a liter of the same solution. After low-speed centrifugation, the samples were chromatographed on an HPLC by using a reversed-phase C_{18} (5 μm) column and a 2.5–40% gradient of acetonitrile in sodium acetate, pH 6.5. A steplike gradient composed of 10 equal increments of increasing acetonitrile concentration was used. This gradient form resulted in improved peak resolution when compared with a smooth linear gradient. The details of this method and a description of the chromatographic apparatus were given earlier (Weiner, 1982). The absorption of the eluant was monitored at both 229- and 254-nm wavelengths by using two Waters (Models 441 and 440, respectively) detectors in series. The peaks were collected and lyophilized. All the solvents and chemical reagents used for preparing buffers for the HPLC were spectral grade or analytical grade, respectively.

Analysis of Milk Acid Hydrolysates. Aliquots of the peaks obtained from the HPLC were dissolved in 0.5 mL of 0.25 M acetic acid and dialyzed overnight against the same solution. The contents of the dialysis tubing were sealed in vacuo and hydrolyzed at 110 °C for 24 h after flushing twice with nitrogen. These conditions preferentially cleave the peptide chains on both sides of aspartic acid (and not asparagine) (Ingram, 1963; Weiner & Hood, 1975). After being dried, the mixture of amino acids and peptides were reacted with dansyl chloride (Dns-Cl) (Sigma) according to the method of Gray (1967). Fifty microliters of 0.2 M sodium bicarbonate, pH 8.50, and 50 μL of 2.5 mg/mL Dns-Cl in acetone were mixed, reacted at 37 °C for 1 h, and dried down. The samples was redissolved in 25 μL of a 1:1 mixture of the two end-member buffers used for separation of the hydrolysate products (see below) and transferred to a small centrifuge tube. The procedure was repeated twice.

The chromatographic conditions used for separating the partial acid hydrolysate products are the same as described by Weiner & Tishbee (1981) for separating Dns-amino acids. The eluant was monitored for both fluorescence intensity (Waters Model 420 detector) and absorption at 254-nm wavelength (Waters Model 441 detector). The Dns-derivatized amino acids and peptides absorbed strongly at this wavelength. Amino acid peaks were identified by injecting appropriate standards (Sigma) and comparing their retention times, UV absorptions, and fluoresence intensities with the unknown peaks. Peptide peaks were collected, dried down, and hydrolyzed in vacuo for 20 min at 140 °C in 0.4 mL of 6 N HCl after flushing twice with nitrogen. Half of the hydrolyzed sample was rechromatographed on the HPLC under the conditions described above in order to identify the N terminus. The same procedure was used for determining the N terminus of the two proteins analyzed in this study. Note that Dns-Pro is destroyed after 20 min of hydrolysis at 140 °C. When a Pro N terminus is suspected, hydrolysis should be limited to 10 min. The second half of the sample was used for amino acid composition analysis. A Biotronik LC 6001 instrument was used with a fluorescent detector and *o*-phthalaldehyde as the derivatizing agent.

Amino acid compositions of the proteins were determined by using a Durrum D500 amino acid analyzer. The samples were hydrolyzed in vacuo in 0.5 mL of 6 N HCl at 110 °C for 20 h after flushing twice with nitrogen. The amount of protein in each sample was determined from the molar yields of amino acids.

Gel Filtration Chromatography. The proteins obtained from the HPLC were first radioactively labeled by reacting

Table I: Composition of the Prismatic-Calcite Proteins of Ion-Exchange Fraction 3 after Separation on HPLC As Shown in Figure 3A

component[a]	P4	P6	P9	P12	P16	P20	P25	P30
Asp + Asn	38.49	29.51	27.66	31.55	24.11	21.73	17.66	16.56
Thr	4.30	4.05	4.51	8.17	6.45	10.28	11.53	10.82
Ser	5.09	10.22	10.30	9.32	7.49	8.34	9.30	8.46
Glu + Gln	18.37	11.35	16.32	19.33	17.25	13.34	10.64	10.25
Pro	5.55	3.51	4.86	6.96	7.60	10.03	10.01	8.67
Gly	15.06	22.54	14.93	6.68	17.84	12.81	12.46	14.05
Ala	6.26	3.51	4.40	3.03	4.81	3.68	5.38	6.52
Val	1.87	2.27	2.55	2.57	3.07	4.57	3.97	5.44
Met	0.27	0.81	0.58	0.16			0.29	
Ile	0.12	1.57	3.13	3.11	3.36	3.05	3.54	3.37
Leu	0.15	1.14	4.75	1.26	1.74	3.07	4.34	4.59
Tyr		0.87		0.18		0.62	0.80	
Phe	0.10	0.32		0.19	0.76	1.42	2.03	2.58
His	1.75					0.27		0.29
Lys	2.60	7.24	5.09	4.66	3.16	3.83	5.04	5.59
Arg		1.08	1.04	2.89	3.00	2.92	2.96	2.79
proportion of total protein per fraction (%)	15.2	4.8	4.1	26.5	14.3	20.7	10.7	3.6
amount of protein per fraction (μg)	24	7	6	43	22	33	17	6

[a] Amino acids listed as mole percent. Cysteine absent or present in trace amounts.

10 μCi of [^{14}C]glycine ethyl ester (New England Nuclear) with the protein carboxyl groups according to the method of Hoare & Koshland (1967). After 30 min cold glycine methyl ester was added to the reaction mixture and the reaction allowed to continue for an additional 3.5 h. After exhaustive dialysis against 0.001 M HCl, the sample was dried down and dissolved in 1 mL of 4 M guanidine hydrochloride in 0.05 M Tris, pH 7.4. The sample was passed through a Bio-Gel A-5M (200–400 mesh) column (55 × 2 cm) in the same guanidine-containing buffer with a flow rate of 5 mL/h. Fractions of 0.82 mL were collected. The radioactivity of a third of every second sample was determined on a Packard Tri-carb 460 liquid scintillation counter. The following molecular weight standards were used: γ-globulin, bovine serum albumin, ovalbumin, chymotrypsinogen A, myoglobin, and ribonuclease A.

Results

The soluble fractions of the prismatic-calcite layer and the nacreous-aragonite layer were chromatographed separately under identical conditions on the ion-exchange column (Figure 1). The prismatic layer sample contained more material and possibly more EDTA contamination (EDTA elutes at about 0.2 M NaCl salt strength) than the nacreous layer sample. Although the relative proportions of the matrix constituents are very different in the two layers, the approximate retention times of the components eluting from the column are similar. The eluant from each run was pooled into six fractions as shown in Figure 1. Each of the 12 fractions thus obtained was rechromatographed on the HPLC under conditions in which the more acidic matrix constituents (primarily proteins) are well resolved (Weiner, 1982). An initial comparison of the matrix components in the two shell layers was performed at this stage based on their retention times from the HPLC and their relative absorbances at 229 and 254 nm. As proteins tend to absorb more at 229 than 254 nm, peaks with a 229/254 ratio of less than 1.5 were ignored in the survey. In addition, as some of the peaks eluting in the first 10 min after sample injection are due to low molecular weight contaminants such as Tris [see discussion in Weiner (1982)], the comparative survey included only peaks eluting after the onset of the gradient, i.e., 10 min or so after injection. The results of the survey are schematically shown in Figure 2. Forty-one components are identified. Six are found only in the prismatic-calcite layer, and 11 are specific to the nacreous-aragonite layer. These figures are only a rough approximation of the component diversity and distribution between shell layers as aggregates of the same component may chromatograph separately (Weiner, 1982)) and the same component may be in adjacent ion-exchange fractions.

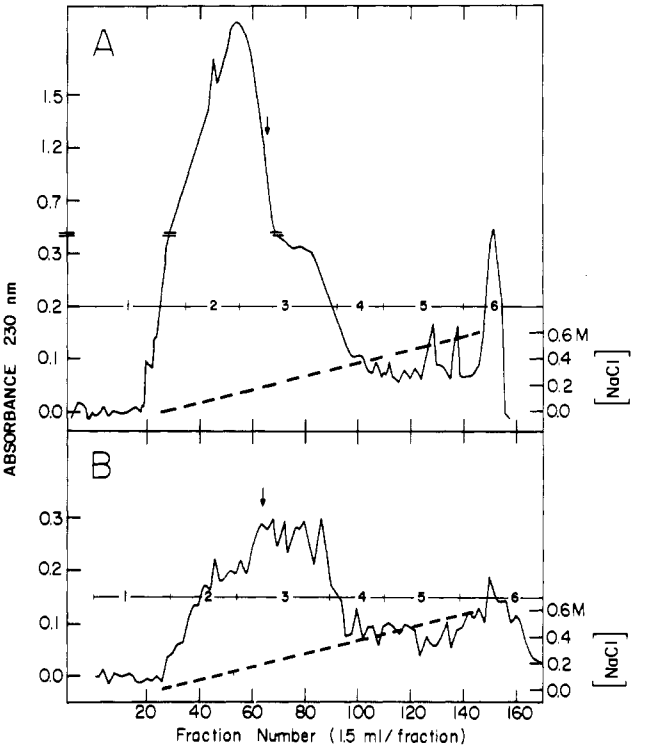

FIGURE 1: DEAE-cellulose ion-exchange chromatograms of the EDTA-soluble organic matrices of the outer prismatic-calcite layer (A) and nacreous-aragonite layer (B) of *M. californianus*. (↓) Elution position of EDTA not removed by dialysis. The collected fractions were pooled as designated. The salt gradient is marked by the dashed line.

In order to confirm the designations of "specific" or "common" to matrix components of the two shell layers which were based only on retention times and relative UV absorptions at 229 and 254 nm, the amino acid compositions of the con-

Table II: Composition of the Nacreous-Aragonite Proteins of Ion-Exchange Fraction 3 after Separation on HPLC As Shown in Figure 3B

component[a]	N4	N6	N12	N15	N18	N20	N26	N30
Asp + Asn	28.32	20.11	26.71	20.39	21.86	21.33	20.62	15.40
Thr	7.38	5.18	7.24	8.83	9.71	11.15	9.70	9.34
Ser	9.39	4.81	7.47	6.57	5.92	6.92	6.93	9.84
Glu + Gln	14.52	9.28	17.17	13.93	13.42	13.20	15.60	12.19
Pro	3.30	5.88	8.46	12.23	12.94	13.92	7.80	7.56
Gly	12.42	8.43	8.20	9.85	11.68	10.62	13.52	11.19
Ala	9.76	5.21	4.29	3.62	5.68	4.23	5.55	6.63
Val	3.38	6.32	4.21		4.66	4.23	6.07	6.63
Met	0.47	0.48	0.53	5.21		0.12		
Ile	0.22	10.17	4.17	3.28	4.26	3.30	4.33	2.57
Leu	0.23	7.13	2.28	5.55	4.03	3.78	3.64	5.84
Tyr	0.17	0.33						1.50
Phe		0.19	1.49	7.59	2.05	2.25	2.77	2.07
His	0.19			0.45	0.24	0.68	0.69	0.36
Lys	9.54	11.50	4.60	2.49	2.53	3.02	2.77	7.13
Arg	0.66	4.92	3.15		0.95	1.25		1.78
proportion of total protein per fraction (%)	44.7	10.5	19.2	3.4	4.9	9.6	2.2	5.4
amount of protein per fraction (μg)	46	11	21	4	5	10	2	6

[a] Amino acids listed as mole percent. Cysteine absent or present in trace amounts.

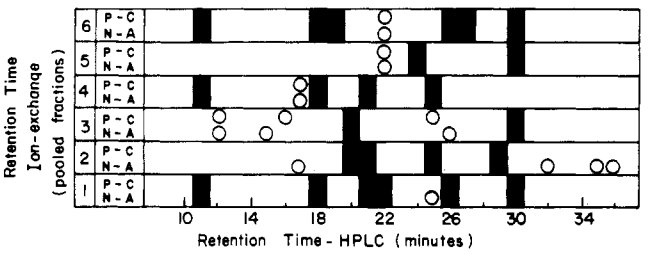

FIGURE 2: Schematic representation of the results of the comparative survey of matrix constituents in the outer prismatic-calcite layer (P-C) and nacreous-aragonite layer (N-A). Constituents specific to one or other shell layer are designated by circles. In some cases the latter have the same HPLC retention times but are clearly different based on their relative absorptions at 229 and 254 nm.

stituents eluted from the HPLC of the ion-exchange fraction 3 samples were also determined. Figure 3 shows the tracings of the original chromatograms monitored at 229 nm, and Tables I and II show the amino acid compositions of the peaks in the prismatic and nacreous layers, respectively, as designated in Figure 3. The only pairs of corresponding peaks which contain similar but not identical amino acid compositions are P20 and N20 and peaks P30 and N30. They also have similar retention times and absorption properties. All the other peaks are indeed different with respect to their amino acid compositions (except possibly for N18 and N20) and are presumably different proteins. Note also that some of the major protein-containing peaks eluted prior to the onset of the gradient. More protein can also be flushed from the column at the end of the run with dimethyl sulfoxide (Weiner, 1982). These initial and final fractions were also not included in the survey.

In order to select those matrix components potentially responsible for determining the mineral type deposited in a given shell layer, analyses of mild acid hydrolysate products from the various soluble matrix proteins were made. This provides indirect information on the arrangement of potential calcium binding sites along the polypeptide chain (Weiner & Hood, 1975; Weiner, 1981). The products were fractionated as their fluorescent Dns derivatives by using HPLC chromatographic conditions appropriate for separating almost all the commonly found amino acids (Weiner & Tishbee, 1981). Initially a comparison was made of the hydrolysate products of all the soluble components in one layer with those of the other, in order to identify sequence differences between the layers and

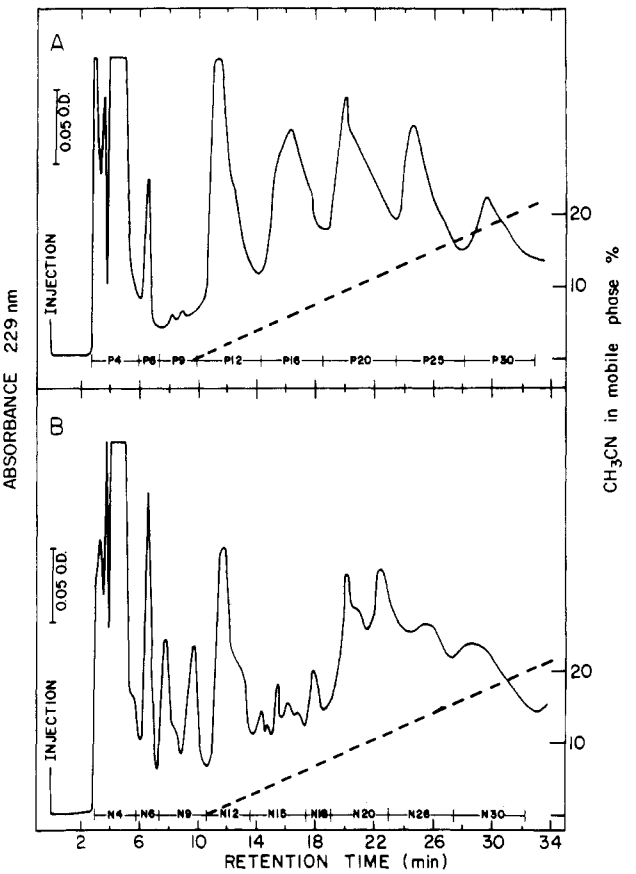

FIGURE 3: HPLC elution profiles obtained from the fraction 3 samples of the prismatic-calcite layer (A) and nacreous-aragonite layer (B). About one-third of the sample was injected. No major protein-containing peaks were eluted after about 20% acetonitrile in the mobile phase passed through the column. The mean gradient shown (dashed line) corrects for the time lag between the onset of the gradient program, which is concurrent with sample injection and the appearance of the first elevated concentrations of acetonitrile in the detector. The steplike form of the gradient (see Experimental Procedures) is not shown. The peaks were collected as designated.

to recognize those that are quantitatively abundant and hence present as multiple copies. The results are shown in Figure 4. The two chromatograms are very similar and are dominated by the presence of large peaks of free amino acids other

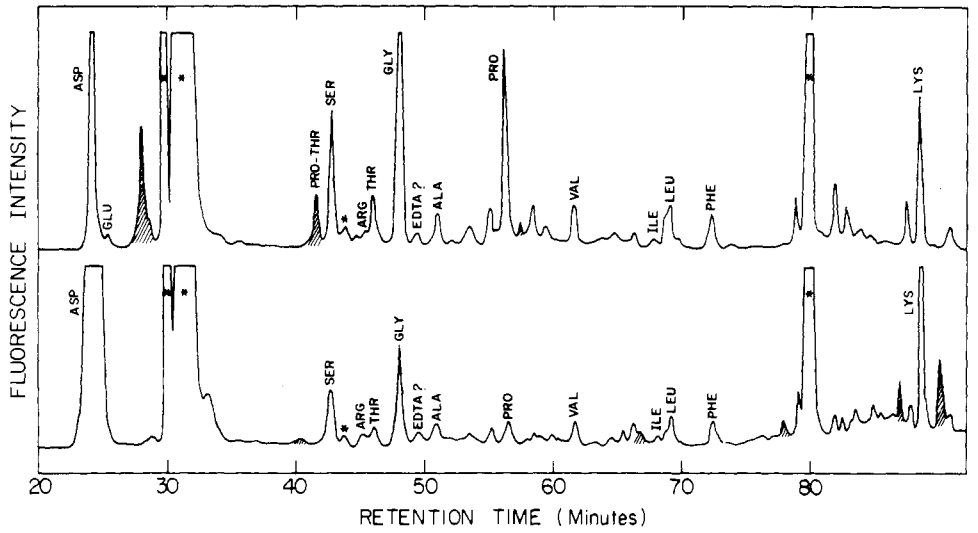

FIGURE 4: HPLC separation of the Dns products after partial acid hydrolysis of the total soluble matrix fractions from the prismatic-calcite layer (top) and nacreous-aragonite layer (bottom). The major differences between the two chromatograms are shaded. (*) Dns hydrolysis products. (EDTA?) EDTA hydrolyzed and reacted with Dns-Cl under the same conditions produces a peak with this retention time and a similar ultraviolet (254 nm)/fluorescence ratio. For the relative fluorescent intensities of equimolar amounts of Dns-amino acids see Weiner & Tishbee (1981). The first 20 min after injection is not shown, as no significant peaks elute during this period.

than that of the expected aspartic acid. This observation confirms earlier studies showing that an $(Asp\text{-}Y)_n$-type sequence, where Y is a single amino acid, is quantitatively abundant in mollusk shell proteins (Weiner & Hood, 1975; Weiner, 1981). Major differences between the two chromatograms are shaded in Figure 4. Note that the peak eluting after 28 min in the prismatic-calcite layer is not a peptide. Its identity is unknown. One of the most prominent differences between the two chromatograms in Figure 4 is the presence of a peak with a retention time of 41.5 min (elutes just before Ser) in the prismatic-calcite layer (Figure 3A). This peak was identified as a dipeptide, Pro-Thr, by N-terminus and amino acid composition analysis. (It should be noted, however, that as Dns-Pro is not stable during 6 N HCl hydrolysis, quantitation is difficult, and the possibility does exist that the peptide contains more than one Thr residue.) Thus, it appears that the amino acid sequence -(Asp-Pro-Thr-Asp)- is unique to the prismatic-calcite layer, and as it is quantitatively abundant, it is presumably present in multiple copies.

A search for the protein(s) which contain the -(Asp-Pro-Thr-Asp)- amino acid sequence was undertaken by examining the partial acid hydrolysates of the six ion-exchange fractions of the prismatic-calcite layer. Only fraction 3 contained the Pro-Thr peptide. Each of the HPLC peaks in this fraction (Figure 3A) was analyzed and as a control, fraction 3 nacreous layer peaks (Figure 3B) were also studied. The appropriate portions of the partial acid hydrolysate chromatograms from the major protein-containing fractions are shown in Figure 5. Only peaks P12 and P16 of the prismatic-calcite layer contain this peptide and hence the -(Asp-Pro-Thr-Asp)- amino acid sequence. Note that fraction N20 also has a peak with a very similar retention time, but the ultraviolet/fluorescence ratio is completely different. The proportions of Ser, Thr, Gly, and Ala released from the $(Asp\text{-}Y)_n$-type sequence, where Y is one of these amino acids, are very similar in all the peaks, except for N12 (Figure 5).

Peaks P12 and P16 constitute 26.5% and 14.3%, respectively, of all the protein eluted from the HPLC separation shown in Figure 3A (see Table I). Fraction 3 contains 39% of the total amount of protein eluted from the ion-exchange column. Thus, these peaks together constitute about 16% of the total soluble protein in the prismatic-calcite shell layer.

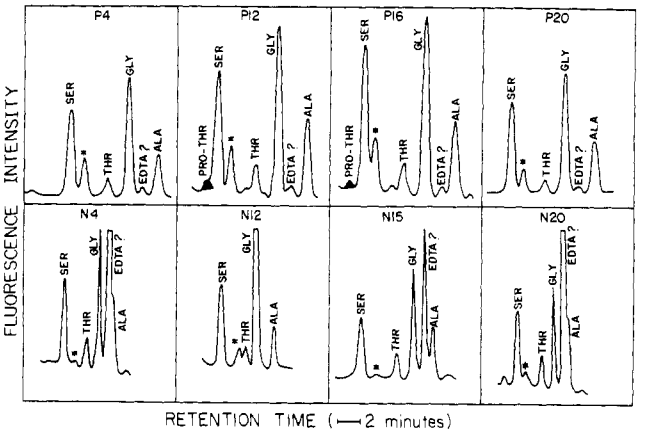

FIGURE 5: Portions of the HPLC chromatograms obtained from the partial acid hydrolysates of the major protein-containing peaks shown in Figure 3. Shaded peaks are identified as the Pro-Thr dipeptide. (*) Dns hydrolysis product. (EDTA?) EDTA hydrolyzed and reacted with Dns-Cl under the same conditions produces a peak with this retention time and a similar ultraviolet (254 nm)/fluorescence ratio.

Their amino acid compositions (Table I) indicate that they are acidic, being rich in both Asx and Glx. Titration of the carboxyl groups using the method of Hoare & Koshland (1967) indicates that 55% are indeed Asp and Glu, as opposed to Asn and Gln. The P12 and P16 peaks were rechromatographed through a gel filtration column under denaturing conditions in order to assess their homogeneity and estimate their apparent molecular weights. As only microgram quantities of protein were available from the HPLC (Tables I and II), the carboxyl groups were radioactively labeled with [^{14}C]glycine ethyl ester (Hoare & Koshland, 1967). As the negatively charged groups of the molecules are neutralized, this modification has the added advantage of reducing the possibility of aggregation. Both P12 and P16 peaks eluted from the gel filtration column as single entities, and their apparent molecular weights are 6500 and 16 000. As the carboxyl groups have been derivatized with glycine methyl (and ethyl) ester, the apparent molecular weights of P12 and P16 are in excess by about 1000 and 1500, respectively. Both proteins have glycine as their N terminus.

Discussion

A comparison of the soluble matrix constituents of the outer prismatic (calcite) layer with the nacreous (aragonite) layer shows that approximately half are common to both layers and half are specific to one of the layers. Two of the proteins specific to the prismatic-calcite layer contain an amino acid sequence, -(Asp-Pro-Thr-Asp)-, which is not present in any of the aragonitic matrix constituents.

The outer prismatic and nacreous layers from the shell of the same organism differ primarily with respect to mineralogy and ultrastructure. The cells responsible for the formation of each layer are all part of the mantle epithelium but are spatially separated and have different structures (Crenshaw, 1980). About half the soluble matrix constituents are common to both layers, and one can assume that they perform the same function in each. On the other hand, those components specific to one or the other of the layers may be assumed to relate to the different properties of the layers, including the mineralogical difference.

The selection of the constituents responsible for mineralogical difference requires additional assumptions. The basic assumption is that the protein amino acid sequences are such that the calcium ions are bound at regular intervals which match some specific Ca–Ca spacing in the mineral lattice. Aspartic acid is usually the predominant acidic residue of these proteins (Table I and II) and is presumed to be involved in calcium binding. A gross comparison of the cleavage products of all the combined soluble proteins in each layer showed that many of the Asp-containing sequences, e.g., $(Asp\text{-}Gly)_n$ or $(Asp\text{-}Ser)_n$, are quantitatively abundant and common to both shell layers, as has been reported previously (Weiner & Hood, 1975; Weiner, 1981). These types of sequence are presumably not responsible for the mineralogical difference. One possibility is that this type of sequence inhibits the growth of calcium carbonate (Weiner, 1981) or is simply an integral part of every calcium binding site. The most conspicuous amino acid sequence difference between the shell layers is the presence of an -(Asp-Pro-Thr-Asp)- sequence in the prismatic-calcite layer, which is not present in the nacreous-aragonite layer. Significantly, similar amino acid sequences in which Asp residues are separated by a dipeptide were previously found to be confined to the prismatic-calcite layer of another bivalve, *Pinctada margaratifera* (Weiner, 1981). In this case the dipeptides are Gly-Ser and Ser-Gly. The presence of a Pro residue in the unique Asp-containing sequence of *M. californianus* may, however, imply a difference in secondary conformation between the two polypeptide chains.

Just two proteins (P12 and P16) contain the specific -(Asp-Pro-Thr-Asp)- sequence. They have been purified to homogeneity by gel filtration and proven to be individual chain species by N-terminal analysis. Their relative apparent molecular weights are 5500 and 14 500, respectively. It may be presumptive to claim that these two proteins are responsible for the deposition of calcite and not aragonite in the outer prismatic layer. However, these two proteins can be considered as "serious candidates" for inducing the formation of calcite crystals.

The next step in analyzing the role of these proteins in mineralization is to determine their in vivo locations. Amino acid sequence information is also required. Even with the relatively small quantities of material available from an analytical HPLC column (tens of micrograms), major portions of their amino acid sequences could be determined by using new automated microsequencing techniques (Hewick et al., 1981), or antibodies could be raised against these proteins to aid in the determination of their in vivo locations. Information of this type, together with definitive information on the precise spatial relationship between the matrix proteins and mineral crystallographic axes, is essential for improving our understanding of the functions of these and other matrix proteins in biomineralization.

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Calcium-Dependent Inhibitory Region of Troponin: A Proton Nuclear Magnetic Resonance Study on the Interaction between Troponin C and the Synthetic Peptide N^{α}-Acetyl[FPhe^{106}]TnI-(104–115) Amide†

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ABSTRACT: To investigate the calcium-dependent regulation of muscle contraction, a synthetic analogue of the inhibitory region of troponin I, N^{α}-acetyl[FPhe^{106}]TnI-(104–115) amide, has been made by solid-phase peptide synthesis. This region represents the minimum sequence necessary for inhibition of actomyosin ATPase activity in the presence of tropomyosin [Talbot, J. A., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2798–2802]. Conformational changes induced by the formation of the synthetic peptide–troponin C complex are followed by proton nuclear magnetic resonance spectroscopy. Aliphatic (Leu and Val), aromatic (*p*-fluorophenylalanine), and charged (Arg) residues are perturbed by interaction with troponin C. In troponin C, peptide–protein interaction results in the redistribution of the Phe envelope of troponin C and perturbations in the aliphatic region. The observed effects on the protein resonances are in agreement with proposed interaction of the peptide with the N-terminal region of site III of troponin C. In the absence of calcium, this region of troponin I (104–115) is bound to actin–tropomyosin, inhibiting actomyosin ATPase activity [Talbot, J. A., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2798–2802]. Our results suggest that the binding site for this region of troponin I is induced in troponin C in the presence of calcium and the formation of this complex releases actomyosin ATPase inhibition.

Full calcium-sensitive control of actomyosin ATPase activity requires both the troponin complex and tropomyosin. Troponin I can inhibit the ATPase activity of actomyosin by itself, but this inhibition, at ionic strengths where tropomyosin neither binds nor inhibits actomyosin, is greatly enhanced by the addition of tropomyosin (Eisenberg & Kielley, 1974; Eaton et al., 1975; Talbot & Hodges, 1979). Release of this inhibition is mediated by troponin C in the presence of calcium. Troponin I fragment studies conducted by Syska et al. (1976) demonstrated that two fragments are of special interest with respect to the function of this protein. One fragment, consisting of residues 1–21, includes the site of phosphorylation catalyzed by phosphorylase kinase (Thr-11), while the second site, which includes residues 96–116, is adjacent to a second site of phosphorylation (Ser-117) (Moir et al., 1974). Phosphorylation at both these sites is completely inhibited by an equimolar amount of troponin C (Cole & Perry, 1975). Furthermore, affinity chromatography using troponin C linked to Sepharose and gel electrophoresis in the presence of troponin C both indicate that these fragments form complexes with troponin C (Syska et al., 1976). The fragment, residues 96–116, also inhibits actomyosin ATPase activity. As with troponin I, the inhibitory effect was potentiated in the presence of tropomyosin but was released by troponin C in the presence or absence of calcium. Talbot & Hodges (1979, 1981) have reported on the biological activity of several analogues of the region 96–116 of troponin I. Their results indicate that the sequence 105–114 is the minimum inhibitory sequence, and analogues of this region are excellent mimics of troponin I, a protein 17 times larger. The tropomyosin specificity of these peptides is demonstrated by comparing the results obtained with salmine, a basic protein also known to inhibit actomyosin ATPase activity. In the presence of tropomyosin, the inhibition by salmine is suppressed, while that of troponin I and the inhibitory peptides is enhanced.

In the present study, we have undertaken the investigation of the interaction of the inhibitory region of troponin I with troponin C, employing the synthetic troponin I analogue N^{α}-acetyl[FPhe^{106}]TnI-(104–115) amide.[1] The interaction is monitored by ^{1}H NMR spectroscopy to determine the nature of the residues in both species that are affected and thus the region of troponin C that binds the inhibitory region of troponin I during complex formation.

Materials and Methods

Materials. All chemicals and solvents were reagent grade. Poly(styrene-*co*-1% divinylbenzene)benzhydrylamine hydrochloride resin (0.49 mmol of NH_2/g of resin) was purchased

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[1] Abbreviations: FPhe, *p*-fluorophenylalanine; N^{α}-acetyl[FPhe^{106}]-TnI-(104–115) amide, synthetic N-terminal acetylated rabbit skeletal troponin I fragment, residues 104–115 with a C-terminal amide and fluorophenylalanine substituted at position 106 for phenylalanine; CB-9, cyanogen bromide fragment 9, residues 84–135 of rabbit skeletal troponin C; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ^{1}H NMR, proton nuclear magnetic resonance; TnI, troponin I; Tris, tris(hydroxymethyl)aminomethane; Boc, *tert*-butoxycarbonyl; DCC, dicyclohexylcarbodiimide; Tos, tosyl; Z(Cl), 2-chlorobenzoxycarbonyl; TLC, thin-layer chromatography.

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