Control Over Aragonite Crystal Nucleation and Growth: An In Vitro Study of Biomineralization

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Abstract: Calcite and aragonite are the two most stable polymorphs of calcium carbonate. Even though they have very similar structures, many organisms are able to selectively deposit one polymorph and not the other. Recent in vitro studies have shown that one or more mollusk shell matrix macromolecules are capable of specific polymorph nucleation, provided they are in an appropriate microenvironment.[1] In this study we examine aspects of the structure and function of some of the components of this β-chitin–silk fibroin in vitro system. We also show, by scanning electron microscopy, that the chitin framework is very porous, thus facilitating the diffusion of ions and macromolecules into the structure. Fluorescent light microscopy and scanning electron microscopy (SEM) demonstrate that the silk fibroin is intimately associated with the chitin framework. One particular fraction purified from the assemblage of mollusk-shell macromolecules extracted from an aragonitic shell layer is able to specifically induce aragonite crystal formation in vitro. These crystals are needle-shaped, whereas the aragonite crystals induced by the total assemblage of macromolecules are shortened into ellipsoids. This implies that other components in the assemblage modulate crystal growth. Finally, testing of a series of Asp and Leu or Glu and Leu containing synthetic peptides in the in vitro assay system shows that only $(Asp\text{-}Leu)_n$ is capable of specifically inducing aragonite formation. All these observations demonstrate that polymorph specificity is dependent upon the amino acid sequence, the conformation of specific protein(s) in the mollusk shell and the microenvironment in which crystal nucleation and growth takes place.

Keywords: aragonite · biomineralization · calcite · crystal growth · peptides

Introduction

Crystal formation processes in biology are often very well controlled. Characteristically, the resulting crystals are oriented in specific directions, and have shapes and sizes that are quite different from their inorganic counterparts. In the case of $CaCO_3$-containing skeletons, there is also usually complete control over which of the various polymorphs are formed.[2,3] The most common biologically formed polymorphs are calcite and aragonite, which are structurally very similar. The major difference between these two polymorphs is that the carbonate groups in calcite are all in a plane, whereas in aragonite they are slightly staggered and rotated.[4] The manner in which organisms control polymorph formation is not well understood, and is particularly perplexing bearing in mind the small differences in structure and stability at ambient temperature between polymorphs. One frequently suggested mechanism involves the introduction of a specific inhibitor of calcite (such as magnesium), which allows the aragonitic polymorph to form.[5–7] Magnesium is known to induce aragonite formation from sea water, and in vitro at ratios of $Mg/Ca > 4$,[8] but has never been shown to be operative in vivo.

Falini et al.[1] recently addressed the question of calcite/aragonite polymorphism using mollusk shells as a starting model (where calcite and aragonite often compose two different coexisting shell layers). They assembled in vitro a complex containing the major matrix components present in a mollusk shell, namely β-chitin,[9] silk-fibroin-like protein,[10,11] and water-soluble acidic macromolecules.[12–14] They obtained β-chitin from the normally unmineralized pen of the squid, *Loligo* (which belongs to the Cephalopoda, a class within the mollusk phylum). Silk fibroin was extracted and purified from the cocoon of the silk moth, *Bombyx mori*. They then diffused into the matrix aspartic-acid-rich glycoproteins extracted from either an aragonitic mollusk shell layer or a calcitic

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  0947-6539/98/0403-0389 $ 17.50+.25/0

layer. When this assemblage was placed in a saturated solution of calcium carbonate, multicrystalline spherulites formed within the complex. These were aragonitic if the added macromolecules were from an aragonitic shell layer, or calcitic if they were derived from a calcitic shell layer. In the absence of acidic glycoproteins no mineral formed within the complex. However, crystals of vaterite, another (relatively unstable) polymorph of $CaCO_3$, did form on the complex surfaces. Falini et al.[1] also observed an intimate relation between the assembled organic complex and the crystals that formed. The morphologies of the latter varied according to the source of the added macromolecules.

Belcher et al.[15] have produced an analogous in vitro system that is also capable of specifically inducing the formation of aragonite or calcite crystals. They used the so-called "green layer" from the abalone shell and added macromolecules from either a calcitic layer or an aragonitic layer of the abalone. As yet, little is known about the composition and structure of the green layer.

In an artificial system (not including any biogenic constituents), Litvin et al.[16] have recently shown that aragonite formation can be induced under a Langmuir monolayer composed of 5-hexadecyloxyisophthalic acid (C_{16}ISA).

These studies clearly showed that considerable control can be exerted in vitro over the crystal formation process. This includes control over nucleation and the type of polymorph formed, as well as over crystal growth. The present study was aimed at elucidating some of the factors involved in specific aragonite nucleation, as well as characterizing the microenvironment in which crystal growth takes place in the system devised by Falini et al.[1]

The strategy adopted was first to examine aspects of the ultrastructure of the β-chitin from squid pen following the earlier investigation of Hunt and El Sherief.[17] We then addressed the question of the spatial relations between the chitin framework and the added silk fibroin proteins. In the third stage, we purified the soluble macromolecules from an aragonitic shell layer of the mollusk *Atrina*, in order to better understand their individual functions in the in vitro system. Finally, in order to further clarify the possible roles of specific amino acid sequences, we used the in vitro systems to test a series of synthetic peptides.

Abstract in Hebrew

קלציט וארגוניט הם שני הפולימורפים היציבים ביותר מבין גבישי סידן פחמתי. למרות היותם בעלי מבנה דומה, בעלי חיים רבים מסוגלים לגרום לשיקוע מועדף של אחד הפולימורפים הללו על פני האחר. נסיונות in vitro הראו שמקרומולקולות מהמטריצה האורגנית של קונכית רכיכה, מעודדות נוקלאציה יחודית של פולימורף מסוים בהיותן בסביבה הביולוגית המתאימה. בעבודה זו אנו בוחנים, in vitro, היבטים שונים של מבנה ופעילות של מרכיבים מסוימים, במערכת הכוללת β-כיטין ומשי. באמצעות מיקרוסקופ אלקטרוני סורק אנו מראים שמבנה הכיטין הינו נקבובי, דבר המאפשר דיפוסיה של יונים ומקרומולקולות לתוך המבנה. תצפיות שנערכו במיקרוסקופ אור פלואורסנטי ובמיקרוסקופ אלקטרונים סורק, מצביעות על כך שהמשי משולב בתוך מבנה הכיטין. בניסיונות שבוצעו נמצא כי פרקציה מסויימת, שבודדה מתוך אוסף המקרומולקולות הממוצות משכבת הארגוניט בקונכיה, מעודדת באופן יחודי יצירה של גבישי ארגוניט in vitro. גבישים אלו הם בצורת מחטים, בעוד שגבישי הארגוניט הנוצרים בנוכחות אוסף המקרומולקולות כולו הם בעלי צורה אליפטית. דבר זה מרמז על כך שמרכיבים אחרים באוסף משפיעים על גדילת הגביש. בחינה, במערכת ה-in vitro, של סידרת פפטידים סינטטיים המכילים חומצה אספרטית ולאוצין או חומצה גלוטמית ולאוצין, מראה שהפולימר המורכב לסרוגין מחומצה אספרטית ולאוצין הינו בעל יכולת לעודד יצירה יחודית של ארגוניט. תצפיות אלו מראות, כי יצירת פולימורף מסויים באופן יחודי תלויה הן ברצף חומצות האמינו והקונפורמציה של חלבונים יחודיים, והן בסביבה הביולוגית בה מתרחשות נוקלאציה הגביש וגדילתו.

Results

Structural characterization of the chitin–silk-matrix macromolecules complex: After removal of the originally associated proteinaceous material, the chitin from the squid pen seen at low magnification (Figure 1A) has a lamellar structure with parallel fibers organized to form sheets. The fibers that lie along the surface appear smooth at these magnifications, as do the surfaces of the different sheets. At higher magnifications, however, the surface of each lamella is visibly porous (Figure 1B). The presence of pores might conceivably be due to the critical point drying prior to observation in the scanning electron microscope, but we note that a porous structure was also observed after freeze fracture in a transmission electron microscope (data not shown). No difference

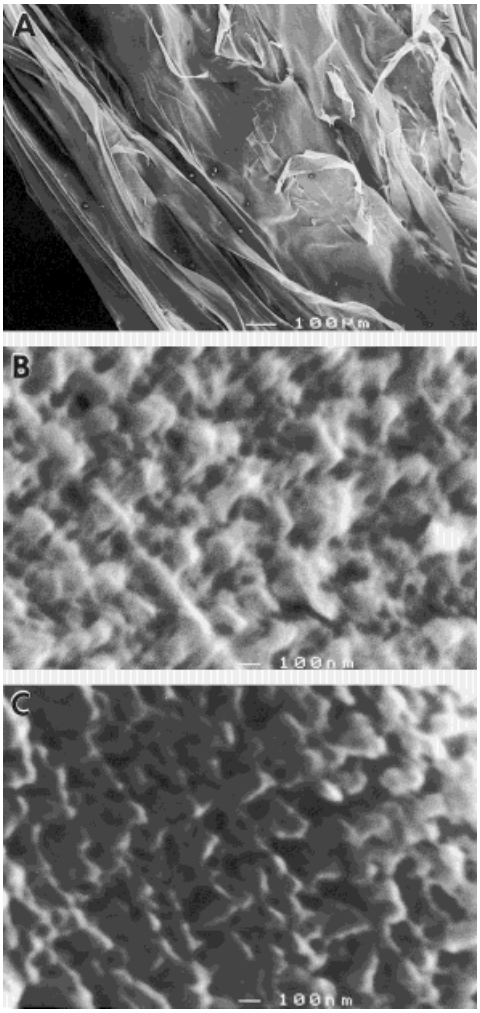

Figure 1. Scanning electron micrographs of chitin (A,B) and of chitin/silk assembly (C) after critical-point drying. A) View of the chitin surface and edge showing the lamellar structure. B, C) High magnification of the chitin surface before (B) and after (C) deposition of silk.

0947-6539/98/0403-0390 $ 17.50+.50/0 *Chem. Eur. J.* **1998**, *4*, No. 3

was observed in the appearance of the chitin in the SEM before or after deposition of silk fibroin (Figure 1C). Thus, silk does not appear to fill and/or block the pores, and we infer that it is spread on the chitin lamellae as a thin layer undetectable at a resolution of tens of nanometers (at least in the dried state).

For the study of the relative distributions of the different components (chitin, silk and matrix proteins), the fluorescent dye rhodamine isothiocyanate was first covalently bound to soluble matrix proteins extracted from the nacreous layer of the shell of *Atrina serrata.* The labeled proteins were adsorbed on polystyrene tissue-culture dishes, on the β-chitin framework, and on the assembly of β-chitin and silk fibroin. Fluorescence was then monitored with a fluorescence microscope and compared in the different systems. No fluorescence was observed when the labeled proteins were adsorbed on polystyrene. Labeled protein adsorbed on chitin showed weak fluorescence (Figure 2A), whereas labeled protein adsorbed on the assembly of chitin and silk showed more intense fluorescence (Figure 2B). In the latter, characteristic features of the chitin framework, such as lamellae and fibers, were also highlighted by the fluorescent dye. When the same procedure was applied using gelatin instead of silk, the intensity of the fluorescence was as weak as on chitin in the absence of silk. This indicates that the silk fibroin has a more specific role than just forming a protective coat that traps the protein within the chitin pores, as is presumably the case with gelatin.

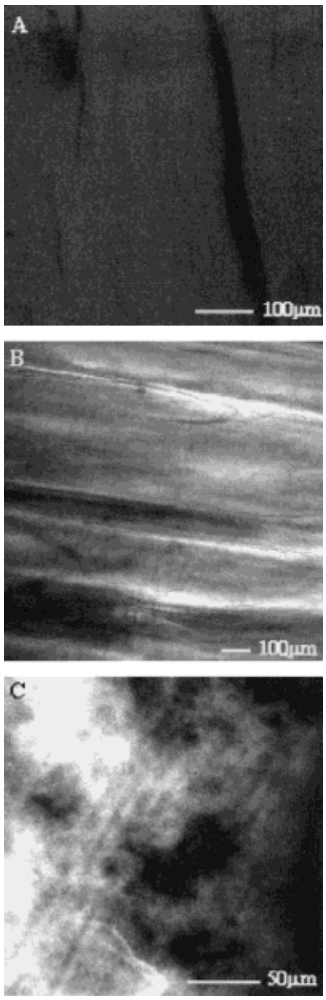

Figure 2. Fluorescence micrographs of A,B) rhodamine-labeled matrix proteins adsorbed on chitin (A) and on chitin/silk assembly (B); C) rhodamine-labeled silk fibroin adsorbed on chitin.

Rhodamine isothiocyanate labeled silk fibroin was also adsorbed on polystyrene and on chitin. The former has a very weak fluorescence relative to that on chitin. The labeled silk adsorbed on chitin fluoresced strongly (Figure 2C) and highlighted the lamellae as well as the macroscopic fibers of the substrate, implying a more specific spatial adsorption of the protein on the chitin polysaccharide fibers. In order to verify that the dye itself does not have a preference for one of the substrates, the free dye was allowed to adsorb on chitin with or without silk. No fluorescence was observed in either case (data not shown). The fluorescence experiments show that the soluble acidic macromolecules are adsorbed directly on silk fibroin, which is itself deposited on chitin.

Isolation of aragonite-nucleating macromolecular fractions: These experiments were aimed at isolating and characterizing components of the assemblage of macromolecules associated with the aragonitic layer that are capable of inducing in vitro aragonite nucleation. The choice of monitoring aragonite rather than calcite induction was motivated by the more stringent requirements for aragonite nucleation, as aragonite is the less stable of the two polymorphs.

The macromolecules associated with aragonite were extracted from the nacreous layer of the shell of the mollusk *Atrina serrata* after complete dissolution of the mineral. A recently developed ion-exchange method for dissolution of the mineral in the absence of any chelating agent or acidic denaturing conditions was used.[18]

The yield of protein was, on average, 0.01 mole % amino acids per unit weight of mineral, with the following typical composition of major amino acids (mole % of total protein): Asx (25), Gly (18), Ser (14), Ala (9), and Glx (8). The assemblage of macromolecules was fractionated by FPLC on an ion-exchange column. Figure 3 shows a typical chromatogram of the eluting fractions. Each fraction was adsorbed separately onto the assembled substrate composed of β-chitin and silk fibroin. The residual protein in solution was removed by extensive washing, and calcium carbonate crystallization was induced. The products of crystallization were characterized by infrared spectroscopy, light microscopy, and SEM. Three different macromolecular preparations were separated and assayed in this way, all of which gave similar separation profiles and the same activity of the corresponding fractions.

Fractions A–C induced the formation of vaterite and calcite on the surface of the substrate together with very few spherulites within the complex (<10 per 25 mm^2). The few spherulites that did form were a mixture of vaterite, calcite, and aragonite. Fractions D and E produced mostly aragonite within the complex with as little as 2 μg mL^{-1} of protein introduced in solution at the adsorption step. The activity of fraction E was especially remarkable, as massive crystallization, exclusively of aragonite, occurred inside the chitin complex (>200 per 25 mm^2), while very few crystals formed on the substrate and around it.

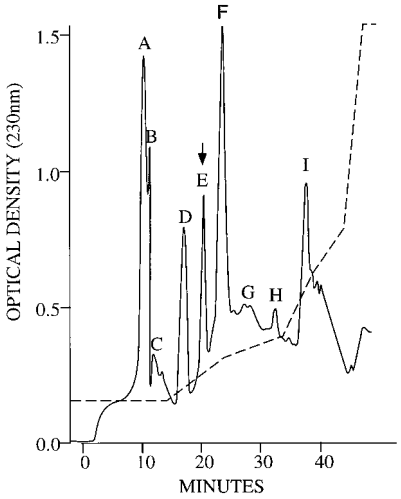

Figure 3. DEAE ion-exchange chromatographic separation of the total assemblage of soluble proteins extracted from the nacreous layer of *Atrina*. The dashed line represents the salt gradient applied during the elution. The arrow indicates the aragonite-nucleating fraction (fraction E).

SEM observations of the mineral phase produced by fraction E inside the substrate showed that the chitin–silk complex and the mineral are intimately associated, with each crystallite making contact with the organic matrix. The crystallites produced by fraction E are needle-shaped and often reach 1–2 μm in length (Figure 4B). This is in contrast to the aragonite single crystallites produced by the total assemblage of macromolecules; these are elliptical in shape, with sizes in the range 100–300 nm (Figure 4A). Preferential orientation of the needle-shaped crystallites was often observed locally. Massive aragonite crystallization was never observed in the presence of the total assemblage of macromolecules. Moreover, in order to induce aragonite nucleation with the total protein assemblage, at least 10 μg mL^{-1} of protein had to be used in the adsorption step (five times the concentration used with fraction E).[19] Lower protein concentrations produced mixtures of calcite and aragonite. In the absence of adsorbed protein, no crystallization occurs inside the substrate, indicating that aragonite formation is due to selective nucleation of this phase, rather than to calcite inhibition.

Fraction E is a small fraction within the entire macromolecular ensemble, consisting of only 6 mole % of the total amino acids. Its major amino acid components are (mole %): Asx (21), Gly (15), Glx (12), Ser (11) and Ala (7). Fraction E has a PI (isoelectric point) of less than 3 (determined by isoelectric focusing). Separation by gel electrophoresis shows that fraction E runs as a fairly wide band with an apparent molecular weight of 10–14 kDa (Figure 5). This band is absent from the assemblage of proteins extracted from the calcitic prismatic layer, strengthening the suggestion that this is a unique aragonite nucleating protein.

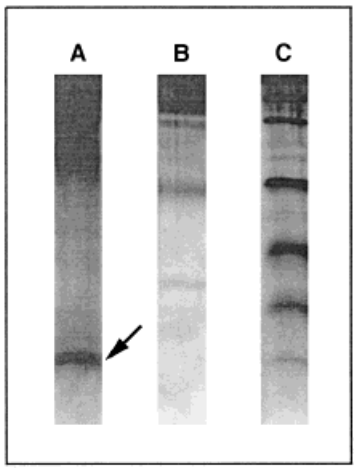

Figure 5. Silver-stained SDS-PAGE separation of A) the total assemblage of proteins extracted from the nacreous layer of *Atrina serrata* (the arrow denotes the position of fraction E); B) the total assemblage of proteins extracted from the prismatic layer of *Atrina serrata*. C) MW markers: 66, 48.5, 29, 18.4 and 14.2 kDa.

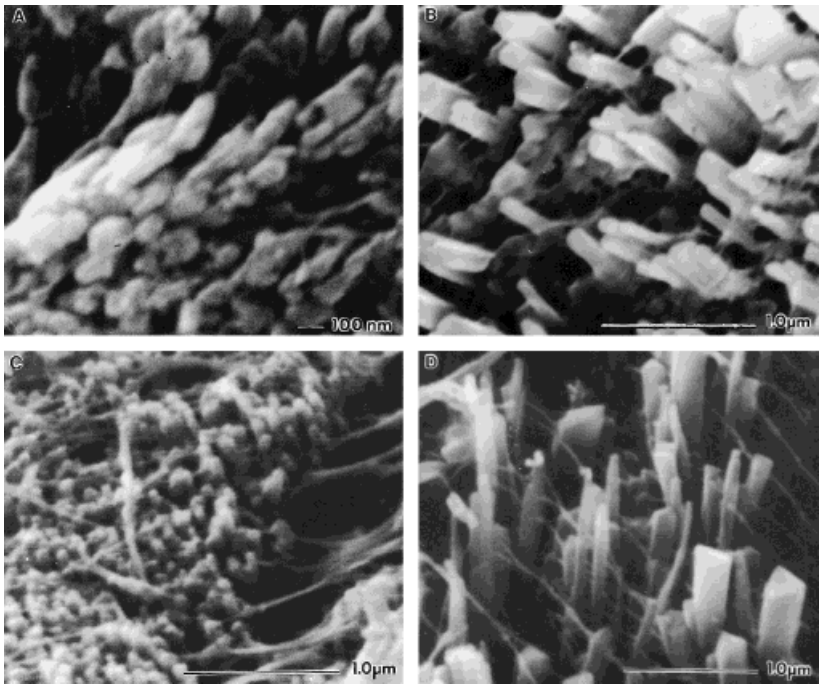

Figure 4. SEM micrographs of fractured sections of the spherulites of aragonite grown inside the β-chitin–silk fibroin assembly. A) Induced by the total assembly of macromolecules; B) induced by fraction E; C) induced by poly(Asp); D) induced by poly(Asp-Leu).

The fractions which eluted at higher ionic strength produce more calcite inside the substrate than aragonite, and in some experiments only massive calcite crystallization occurred. Observation of the calcite spherulites by SEM showed that the individual crystallites retain their typical rhombohedral morphology. Thus the assemblage of macromolecules from the aragonitic layer contains components that after partial or complete purification have different activities in the in vitro assay system. Fraction E in particular has a remarkable ability to nucleate aragonite.

0947-6539/98/0403-0392 $ 17.50+.50/0 *Chem. Eur. J.* **1998**, *4*, No. 3

Polypeptide analogues of nucleating macromolecules: In order to further clarify the possible roles of specific amino acid sequences in aragonite nucleation, we performed a series of crystallization assays in which various polypeptides were used in place of the acidic proteins. The choice of peptides was based on the following information about soluble macromolecules present in mineralized tissues, and in particular those from mollusk shells. The high concentration of aspartic acid characteristic of many of this group of proteins[13] suggests that sequences of polyaspartic acid may be common in these proteins. Partial sequencing has confirmed that such stretches most probably do exist.[20] Weiner and Hood[21] used partial acid hydrolysis of mollusk-shell soluble proteins to infer that stretches of (Asp-Y) n-type sequences may also be present. Peptides with a repeating Asp-Ala sequence as well as $(Asp)_n$ have been shown to possess inhibitory activity in the crystallization of $CaCO_3$ from solution.[22] Mollusk shells, as well as other mineralized tissues, are also known to have a minor assemblage of proteins that are rich in glutamic acid. In certain sponge and ascidian spicules they are associated with amorphous calcium carbonate.[23] We therefore also used polyglutamic acid and Glu-containing repeating motifs. We chose to use leucine to separate Asp or Glu residues, as its hydrophobic nature encourages interaction with the relatively hydrophobic silk fibroin. The following polypeptides were therefore used: 1) poly(Asp-Leu), 2) poly(Leu-Asp-Asp-Leu), 3) poly(Glu-Leu), 4) poly(Leu-Glu-Glu-Leu), 5) poly-(Asp) and 6) poly(Glu).

Circular dichroism measurements of polypeptide 1 indicate that in a 1:2 Ca:carboxylate solution, this polypeptide assumes a β-sheet conformation.[24] The carboxylate groups of the alternating Asp residues are thus exposed on one side of the sheet, while the hydrocarbons of the leucine residues are exposed on the other side. The resulting sheet has a hydrophobic face that can bind to a hydrophobic substrate, and a hydrophilic face that can be in contact with the solution. Polypeptide 2, in contrast, assumes a predominantly α-helical conformation in the same solution. Polypeptides 3 and 4 assume conformations similar to 1 and 2, respectively, but differ from them owing to the higher conformational freedom of the Glu side-chain carboxylates. In addition, the pK_a of Glu (4.25) is higher than that of Asp (3.65). In solution, polypeptide 5 assumes mainly a β-sheet conformation, and 6 is disordered. Note, however, that the conformation of a polypeptide when adsorbed on the framework is not necessarily the same as in solution.[25] The results of the crystallization experiments are summarized in Table 1.

Table 1. Calcium carbonate polymorphs formed inside the chitin or chitin–silk fibroin assembly containing different polypeptides. The mineral phase shown in parentheses indicates the quantitatively minor component formed.

Polypeptide	$CaCO_3$ polymorphs formed inside substrate of: β-chitin + silk fibroin + polypeptide	β-chitin + polypeptide
poly(Asp)	aragonite,[a] calcite (vaterite)	calcite (vaterite)
poly(Glu)	calcite,[a] aragonite (vaterite)	calcite (vaterite)
poly(Asp-Leu)	aragonite/–	–
poly(Leu-Asp-Asp-Leu)	–	–
poly(Glu-Leu)	–	–
poly(Leu-Glu-Glu-Leu)	–	–

[a] Usually the most abundant polymorph.

Poly(Asp) and poly(Glu) always induced massive deposition of spherulites (100–400 per 25 mm^2) inside the chitin, with little or no crystallization on the surface or around the substrate assembly. Significantly, both aragonite and calcite and usually a few vaterite spherulites were formed. With poly(Asp), aragonite spherulites were more abundant in 9 cases out of 15, while with poly(Glu) mainly calcite spherulites were formed in 6 cases out of 9. SEM examination of the aragonitic spherulites showed that the single crystallites have the shape of elongated rods (100–200 nm) (Figure 4C). The calcite crystallites maintain the typical rhombohedral morphology of synthetic calcite, with dimensions of about 400 nm.

When poly(Asp-Leu) was used, spherulites were formed inside the chitin in only 4 out of 12 experiments. Significantly, and in contrast to all the other experiments, these were exclusively composed of aragonite. Furthermore, the aragonitic crystallites were in the shape of elongated needles (Figure 4D), similar to those obtained from protein fraction E.

All the crystallization experiments included, as controls, a system of chitin alone and of chitin and silk without polypeptides. No crystallization was observed inside either of these substrates (in 15 cases out of 15).

The importance of the silk fibroin layer for the adsorption of the acidic macromolecules and the polypeptides is reflected in the results of the crystallization experiments (Table 1). In the absence of silk, no aragonite was formed with any of the polypeptides, analogous to the experiments performed with adsorbed proteins.[1] Poly(Asp) and poly(Glu) induced formation of calcite and small amounts of vaterite inside the chitin. The density of the spherulites (20–50 per 25 mm^2) was, however, lower than that formed with the whole assembly including silk. Changing the order of assembly such that the polypeptides were first adsorbed on the chitin and the silk was subsequently introduced gave the same results as the assembly of chitin and polypeptides in the absence of silk, namely no aragonite formed.

Discussion

Crystal nucleation studies using acidic macromolecules extracted from the shells of different mollusks, and employing two different in vitro assays, showed that these specialized macromolecules are involved in the control of calcite–aragonite polymorphism in vitro.[1,15] It appears, however, that the nucleation of the desired polymorph also requires an appropriate microenvironment, which at least in part reproduces the biological environment of crystal growth. In the case of the system devised by Falini et al.,[1] we used three separate but convergent approaches to better understand the microenvironment of crystal growth. From the ultrastructure of the artificial assay system (comprising chitin and silk) we elucidated structural and biophysical parameters important in the process, such as diffusion of ions and macromolecules, adsorption of proteins and their relative juxtaposition.

Purification of the acidic matrix macromolecules and testing of the function of each purified fraction in the system provided information about the components of the macromolecular mixture, their nucleating potential, and other possible functions. From the use of ordered synthetic polypeptide analogous, we gained more insight into the control of polymorph formation at the protein sequence level.

Examination of the squid pen β-chitin framework with SEM showed that its fibers form a highly porous layered structure. This would enable the diffusion of both ions and large macromolecules into the structure, and could provide potential sites for crystal nucleation and growth. Addition of silk to the chitin framework does not alter the appearance of the structure, nor does it form a recognizable separate layer. However, it does penetrate the chitin and associate to its fibers. The presence of the silk is essential for the adsorption of the acidic macromolecules. We observed a significant enhancement of fluorescence upon adsorption of labeled macromolecules to chitin after silk was added, and we noted that the order in which silk and polypeptides are introduced into the system affected the results. As polymorph control occurs only in the inner layers of the chitin – silk substrate and never on the outer surface, we infer that the chitin – silk assembly serves not only as a substrate onto which macromolecules adsorb, but also as a three-dimensional framework contributing to the proper microenvironmental conditions for controlled nucleation.

In the absence of acidic macromolecules, no crystals are formed inside the framework. The total assemblage of macromolecules extracted from the aragonitic layer selectively induces aragonite nucleation with very high fidelity.[1] After separation of the constituents of this assemblage by liquid chromatography, one fraction was isolated which produces aragonite in very high yields, while other fractions nucleate calcite and some hardly nucleate any crystals at all. Interestingly, the calcite nucleating fractions are those which elute from the anion-exchange column with high salt concentrations, indicating that they are strongly polyanionic. Calcite also forms when macromolecules extracted from the calcitic layer are introduced into the system.[1] Consistent with these two observations, the calcite-inducing macromolecules are known to be much more strongly acidic than the aragonite-inducing ones.[26,27] The polyanionic polypeptides, poly(Asp) and poly(Glu), induced massive nonspecific nucleation of both calcite and aragonite. It may be envisaged that the polyanions create a cation sink, to which calcium is strongly attracted. The resulting local, supersaturated, condition is probably the reason for the absence of specificity in polymorph nucleation, as the activation energy required for the nucleation of both polymorphs is exceeded. (We note that poly(Asp), with a predominantly β-sheet conformation, produces more aragonite than poly(Glu), which in solution assumes a predominantly disordered conformation.)

Unlike the strongly anionic proteins that induce calcite in vitro, the fraction which induces only aragonite is unique to the aragonitic layer. Its amino acid composition does not differ substantially from the average composition of the total assemblage, indicating that the characteristics that lead to its special nucleation activity may be identified only at the level of sequence and structure. We note that recently the first total sequence of a protein from mollusk nacre was determined.[28] It has domains with repeating Gly – Xaa – Asn (where Xaa = Asp, Glu or Asn) sequences. In the present experiments, poly(Asp-Leu), which adopts the β-sheet conformation, also induces aragonite formation exclusively or else does not nucleate at all. In thermodynamic terms, this may be interpreted as the polypeptide that provides the correct structural organization of the nucleation site not producing a sufficient ion concentration to overcome the required energy of activation for nucleation.[29] Concomitantly, the other polypeptides containing the same relative amounts of negative charge never induce any crystallization. Poly(Leu-Asp-Asp-Leu) has the same average composition as poly(Asp-Leu), but assumes a different conformation, namely predominantly α-helical. The other two ordered synthetic polypeptides used here, poly(Glu-Leu) (β sheet) and poly(Leu-Glu-Glu-Leu) (α helix), contain Glu rather than Asp. All these results seem to indicate that nucleation of aragonite is not exclusively the result of the Ostwald rule of stages. The macromolecules involved in calcium carbonate nucleation in vivo are also preferentially Asp-rich,[13] however, the reason for this is not known. Interestingly, Glu-rich macromolecules have been observed in two different natural biological systems, both of which are associated with the stabilization of amorphous calcium carbonate, and thus they presumably function as inhibitors rather than inducers of nucleation.[23]

The presence of both aragonite and calcite nucleating proteins together in the aragonitic nacreous layer raises some questions regarding the roles of these proteins in the in vivo mineralization process. As calcite is the most stable polymorph, the presence of calcite-nucleating proteins in the assemblage of matrix macromolecules should lead to at least partial calcite precipitation. As this does not occur, we infer that either the proteins are not reconstituted in their correct conformations in the artificial system, or they influence each other such that in the assemblage the separate components do not behave as individuals.

SEM shows that the calcite precipitated in the presence of the total assemblage of macromolecules derived from a calcitic shell layer was composed of individual unmodified rhombohedral crystallites.[1] On the other hand, aragonite spherulites that formed in the case of adsorption of the total assemblage of proteins from the aragonitic layer were composed of elliptically-shaped crystallites. This morphology is very different from the thin needles typical of nonbiogenic aragonite. The elliptical shape may be indicative of the presence of proteins that inhibit or modify the crystal morphology during growth. Since the proteins are adsorbed onto the framework, their ability to participate in modulation of growth and morphology implies that the framework is in intimate contact with the growing crystallites on a nanometer scale. In agreement with this observation, use of the purified aragonite-nucleating protein resulted in the formation of needles, as did the use of the polypeptide poly(Asp-Leu). Thus, needles form in the absence of other growth-modulating factors.

This study has taken us one step further in the understanding of the importance of the microenvironment for the

0947-6539/98/0403-0394 $ 17.50+.50/0 *Chem. Eur. J.* **1998**, *4*, No. 3

specific nucleation of aragonite. Structural characterizations of the different components of the organic matrix, as well as study of the relations between them and the crystals, are necessary to better understand the nature of the nucleation site in vitro. This in turn will shed light on the control of crystal nucleation and growth in vivo.

Experimental Section

Skeletal element preparation: Shells of the bivalve mollusk *Atrina serrata* were collected alive off the coast of Florida (USA). The soft parts were removed and the shells stored dry. The calcitic prismatic layer was mechanically separated from the inner nacreous layer, and each layer was washed with 10% ammonium hydroxide solution followed by extensive washes with double-distilled water (DDW). The shell layers were then ground in a mortar and decalcified by an ion-exchange resin as described elsewhere.[18] After complete decalcification and extensive dialysis against DDW, the soluble and insoluble materials were separated by centrifugation at 3000 *g* for 10 min. The soluble material was concentrated by lyophilization and stored at 4 °C for further analysis. The protein concentrations of the soluble macromolecules were determined by amino acid analysis.

Amino acid analysis: Aliquots of the soluble protein solution were lyophilized and hydrolyzed under vacuum in HCl (6N, 0.3 mL) at 112 °C for 24 h after flushing twice with nitrogen. Following evaporation of the HCl, the hydrolysates were analyzed on an automatic amino acid analyzer (HP amino quant).

FPLC separation of the macromolecular ensemble: High-performance liquid chromatography was carried out with an FPLC system (Pharmacia) equipped with an anion-exchange Mono Q column (Pharmacia, HR 5/5). The column was equilibrated with a buffer solution of 0.02 M Tris base, pH 8.0. The buffer solution and a 2 M NaCl solution in buffer were degassed under reduced pressure in an ultrasonic bath for 10 min. A 40 min, two-step gradient was initiated following a 10 min isocratic run at 0.2 M (0.5 M, 1.6 M) NaCl. The proteins were eluted at room temperature at a flow rate of 0.7 mL min^{-1} and monitored both at 230 nm and 280 nm. The pooled fractions were extensively dialyzed and their protein concentrations determined by amino acid analysis. The fractions were stored at 4 °C prior to analysis.

Polyacrylamide gel electrophoresis (PAGE) of proteins: Minigels (0.75 mm thick) of 10% polyacrylamide were used with the discontinuous buffer system of Laemmli.[30] Usually 1–10 μg protein (determined by amino acid analysis) was applied per gel lane. The gels ran at 35 mA for 1 h on a Hauffer minigel apparatus and were silver-stained according to ref. [31].

Crystal growth experiments: Pieces of pure β-chitin (≈ 0.5 cm^2) were placed in Nunc multidishes (well diameters of 1.5 cm) and immersed in calcium chloride solution (10 mM, 0.75 mL) for 2 h. Following the removal of the calcium chloride solution, silk fibroin solution (≈ 2%, 0.1 mL) was added to the chitin and air-dried. The assembly was then treated for 1 h with methanol (80%, 0.75 mL), followed by three washes with calcium chloride solution (10 mM). A total volume of 0.75 mL calcium chloride solution (10 mM) containing the protein or polypeptide sample (10–100 nmole amino acid mL^{-1}) was introduced into the wells and incubated overnight at room temperature on a rocking table. Following the removal of the protein solution and extensive washes with $CaCl_2$ solution (10 mM), the substrate was overlaid with fresh $CaCl_2$ solution (10 mM, 0.75 mL). The wells were covered with aluminum foil, which was punctured by a needle. Crystals were grown for 2 d by slow diffusion of ammonium carbonate in a closed desiccator.[1] Each crystallization experiment included controls of pure calcium chloride solution, pure β-chitin, and β-chitin with silk without protein. The complete assembly of chitin, silk, and soluble macromolecules induced the formation of crystals of $CaCO_3$ inside the chitin–silk substrate. These crystals were referred to as spherulites despite that fact that they were not always spherical, since the many crystallites from which these spherulites are constructed appear to nucleate from discrete centers of nucleation.

FTIR Spectrometry: Individual spherulites were removed mechanically with a needle under an optical microscope and mounted in an infrared transparent diamond pressure cell (High Pressure Diamond Optics, Tucson (AZ, USA)). The spectra were collected (4 cm^{-1} resolution) by means of a computer-controlled spectrometer (MIDAC Corporation, Costa Mesa (CA, USA)). The absorption peaks characteristic of vaterite are at 877 and 744 cm^{-1}, of calcite at 876 and 713 cm^{-1}, and of aragonite at 860 and 713 cm^{-1}.[32]

Rhodamine isothiocyanate labeling: This was performed according to ref. [33]. Rhodamine B isothiocyanate (Sigma) was dissolved in dry dimethyl sulfoxide at 1 mg mL^{-1}. A solution of *Atrina* nacreous protein (2 mg mL^{-1}) in sodium carbonate solution (0.1 M) was prepared. The dye solution (30 μL) was added slowly in 5 μL aliquots to the protein solution (0.6 mL), which was gently but continuously stirred during the addition. The coupling reaction was left in the dark for 8 h at 4 °C, NH_4Cl was added to make up a 50 mM solution, and the reaction was incubated for a further 2 h in the dark at 4 °C. The separation of the unbound dye from the conjugate was achieved by dialysis of the solution in a dialysis tube (Spectrapor 3 tubing, MW cutoff 3500 daltons) against DDW for 12 h in a cold, dark room. The absorbance of a solution of labeled protein was measured at 575 nm (rhodamine λ_{max}) and 280 nm and the ratio OD_{575}/OD_{280} found to be 1.22. The concentration of the labeled protein was determined from amino acid analysis to be 3.27 nmol amino acid per microlitre. This corresponds to approximately one rhodamine per 200 amino acids.

Rhodamine isothiocyanate labeling of silk was performed following the same procedure. A 500 μL dye solution was added in 10 μL aliquots to a silk solution (10 mg mL^{-1} in 0.1 M sodium carbonate), incubated as above and dialyzed (MW cutoff 12000–14000). $OD_{575}/OD_{280} = 0.717$.

Fluorescence experiments: The substrates chitin, silk, and the chitin/silk assembly were prepared according to the procedure described above. The adsorption protocol for the labeled proteins was the same as that described for the nonlabeled proteins, except that the 24 h incubation was carried out in the dark. Gelatin was prepared by dissolving gelatin powder (2 g; Biochemical) in DDW (10 mL) at 40 °C, and adsorption was performed as for the silk sample. The intensity of the fluorescence was monitored by a laser scanning confocal imaging system (MRC-1024) operating at the wavelength of rhodamine B.

Preparation of the polypeptides: The following polypeptides were synthesized as described in ref. [24]: poly(Asp-Leu), MW = 9500 Da; poly(Leu-Asp-Asp-Leu), MW = 5000 Da; poly(Glu-Leu), MW = 15000 Da; and poly(Leu-Glu-Glu-Leu), MW = 15400 Da. The polypeptides were dissolved and dialyzed against DDW for 24 h at 4 °C. Poly(L-glutamic acid) (MW = 9700 Da; Sigma) and poly(L-aspartic acid) (MW = 9900; Sigma) were dissolved in DDW and kept frozen.

Preparation of samples for SEM: In order to preserve the structure of the organic material (chitin, chitin + silk), the assemblage was first fixed by immersion in a 2% glutaraldehyde fixative solution for 1 h. The sample was then washed 3 times with sodium cacodylate buffer (0.1 M) for 5 min each time, and finally 3 times with DDW. The sample was transferred to the appropriate holder and critical-point dried (PELCO CPD-2). The dehydrated sample was fixed to an aluminum stub using double-sided carbon tape, sputter-coated with gold, and examined with a JEOL 6400 scanning electron microscope at 15 kV. For characterization of the crystals, single spherulites were mechanically removed from the chitin with a needle, dried, transferred to a stub covered with double-sided carbon tape and sputter coated with gold.

Circular dichroism (CD) measurements: Different concentrations of the polypeptides in aqueous solutions (ranging from 10^{-3}–10^{-4} M) were prepared and their CD spectra were recorded at room temperature (λ = 190–250 nm) with a Jasco J-500 spectrometer. Measurements were also performed in $CaCl_2$ solutions with a ratio [Ca]:[polypeptide] = 1:2.

Acknowledgements: We thank Giuseppe Falini for providing us with squid pens and silkworm cocoons and Scott Brande for providing *Atrina* shells. This research was supported by a United States–Israel Binational Foundation grant.

Received: August 13, 1997 [F793]

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