Analysis and Molecular Modeling of the Formation, Structure, and Activity of the Phosphatidylserine-Calcium-Phosphate Complex Associated with Biominalization*

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The nucleational core of matrix vesicles contains a complex (CPLX) of phosphatidylserine (PS), Ca\(^{2+}\), and inorganic phosphate (P\(_i\)) that is important to both normal and pathological calcification. Factors required for PS-CPLX formation and nucleational activity were studied using in vitro model systems and molecular dynamic simulations. Ca\(^{2+}\) levels required for and rates of PS-CPLX formation were monitored by light scattering at 340 nm, assessing changes in amount and particle size. Fourier transform infrared spectroscopy was used to explore changes in chemical structure and composition. Washing with pH 5 buffer was used to examine the role of amorphous calcium phosphate in CPLX nucleational activity, which was assessed by incubation in synthetic cartilage lymph with varied pH values. Addition of 4 Ca\(^{2+}\)/PS was minimally required to form viable complexes. During the critical first 10-min reaction period, rapid reduction in particle size signaled changes in PS-CPLX structure. Fourier transform infrared spectroscopy revealed increasing mineral phosphate that became progressively deprotonated to PO\(_4^{3-}\). This Ca\(^{2+}\)-mediated effect was mimicked in part by increasing the Ca\(^{2+}\)/PS reaction ratio. Molecular dynamic simulations provided key insight into initial interactions between Ca\(^{2+}\) and P\(_i\), and the carboxylic, amino, and phosphodiester groups of PS. Deduced interatomic distances agreed closely with previous radial distribution function x-ray-absorption fine structure measurements, except for an elongated Ca\(^{2+}\)–N distance, suggesting additional changes in atomic structure during the critical 10-min ripening period. These findings clarify the process of PS-CPLX formation, reveal details of its structure, and provide insight into its role as a nucleator of crystalline calcium phosphate mineral formation.

One of the essential features of mineral-induced matrix vesicles (MV)\(^2\) is the presence of a nucleation core (1, 2) that contains lipid-calcium-phosphate complexes (CPLX) capable of inducing mineral formation when incubated in a synthetic cartilage lymph (SCL) (3–6). A critical component of CPLX is the acidic phospholipid, phosphatidylserine (PS) (3, 7, 8), which is known to have a high affinity for Ca\(^{2+}\) (9, 10). PS is largely confined to the inner leaflet of the MV membrane (11), and electron micrographs of calcifying MV show the electron-dense calcium phosphate precipitate juxtaposed along the inner leaflet of the MV membrane (12).

Discovery of the nucleation core stems from the early finding that acidic phospholipids, especially PS, are complexed with Ca\(^{2+}\) at sites of early mineralization (13, 14). PS-Ca\(^{2+}\)/P complexes (PS-CPLX) are present at the early stages of almost all calcifying tissues as follows: growth plate cartilage (14), tumors (15), bone (16), and especially MV (8). Synthetic PS-CPLX formed in the absence of Mg\(^{2+}\) has been found to be a powerful nucleator that rapidly induces hydroxyapatite (HA) formation when incubated in SCL (3, 4); however, when formed from Mg\(^{2+}\) and HCO\(_3^−\)-containing buffers, PS-CPLXs are weak nucleators unless certain lipopholic proteins (17, 18), such as the annexins (5, 6), are also included. Annexin-A5, a major lipid-dependent Ca\(^{2+}\)-binding protein of MV (19–22), potently activates the nucleational activity of Mg\(^{2+}\)-containing PS-CPLX (5).

A key requirement in the construction of all PS-CPLXs is that P\(_i\) must be present with the lipid before introduction of Ca\(^{2+}\) (4, 7, 23). This feature appears to occur in cells that form MV where high levels of P\(_i\) are known to be present (24), and intracellular (cytoplasmic) levels of Ca\(^{2+}\) are low until MV formation is in progress (25–29). Interaction of Ca\(^{2+}\) with PS in the absence of P\(_i\) leads to the formation of 2:1 PS-Ca\(^{2+}\) complexes that chelate Ca\(^{2+}\) and have no nucleational activity (7, 30).

Addition of high concentrations of Ca\(^{2+}\) to P\(_i\)-rich solutions causes the formation of amorphous calcium phosphate (ACP), an ephemeral mineral phase (31, 32) that rapidly and spontaneously converts to HA unless stabilized by various agents, such as Mg\(^{2+}\) (33, 34), certain proteins (35–37), and acidic lipids such as PS (7). Interaction with PS during formation of ACP leads to production of PS-CPLX (38).

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2 The abbreviations used are: MV, matrix vesicles; ACP, amorphous calcium phosphate; CHARMM, Chemistry at HARvard Molecular Mechanics; CPLX, complexes; FTIR, Fourier transform infrared spectroscopy; HA, hydroxyapatite; MD, molecular dynamics; PS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; PS-CPLX, PS-Ca\(^{2+}\)/P complexes; RDF-EXAFS, radial distribution function x-ray-absorption fine structure; SCL, synthetic cartilage lymph; VMD, visual molecular dynamics; TES, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethy]l]amine)ethanesulfonic acid.
Although P$_2$-containing CPLXs can induce HA formation, in vivo many factors appear to be involved in regulating its formation and activity. To elucidate these factors, we have begun a series of systematic studies, both physical and theoretical, to more fully understand and visualize how they contribute to the formation and nucleational activity of PS-CPLXs. Because elevated P$_2$ appears to be an essential feature during in vivo CPLX formation, in our physical studies we used a P$_2$-rich KCl-KP$_2$ buffer to construct the complexes. We varied the ratio of Ca$^{2+}$ to PS, as well as their "ripening" time before introduction to SCL, examining their physical properties, as well as their ability to trigger mineral formation when incubated in SCL. In our theoretical studies, we explored how PS interacts with P$_2$ and Ca$^{2+}$, using molecular dynamic (MD) simulations to elucidate the mechanism of their formation, as well as the packing arrangement of Ca$^{2+}$ and PO$_4^{3-}$ after they interact with specific functional groups of PS to form PS-CPLX.

Finally, to further elucidate the processes by which PS-CPLX induces mineral formation when exposed to SCL, the ability of both native and acid-washed complexes to induce and support mineral formation was studied by varying the pH of the SCL. Because acid washing is known to solubilize and remove ACP (23), the ability of acid-washed versus unwashed PS-CPLX to induce mineralization would reveal whether ACP, whose conversion to crystalline mineral is highly sensitive to pH (39), is directly involved.

**EXPERIMENTAL PROCEDURES**

**Synthesis of PS-Ca-P$_2$ Complexes**—The methods used were modeled after conditions known to be present in calcifying growth plate cells at the time of MV formation as follows: high levels (20–25 mM) of P$_2$ in a K$^+$-rich (>100 mM) intracellular environment (24). For studying the effect of CPLX reaction time, a stock solution of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (PS) emulsion (4X) was prepared by drying 5 mg of PS in chloroform under N$_2$ to form a thin film in a test tube. Then 2 ml of KCl (150 mM)-KP$_2$ buffer (10 mM, pH 7.5) was added; and the tube then sonicated for 2–4 min in a water bath at 25 °C to disperse the lipid and form small unilamellar liposomes. For making 200 μl of CPLX, 50 μl of the 4X PS stock (0.16 μmol) was diluted with 150 μl of the KCl-KP$_2$ buffer. Then 9.6 μl of 100 mM CaCl$_2$ (Ca$^{2+}$/PS molar ratio, 6:1) was added dropwise with constant stirring to form the complex. The stirring (ripening) times studied varied from 1.5 to 10, 30, and 60 min, although some were allowed to ripen for 18 h. The complexes were sedimented by centrifugation at 13,000 × g for 5 min; and after decanting the supernatant, the pellets were resuspended in 200 μl synthetic cartilage lysine (SCL) and chilled to 0 °C to prevent further change. Prior to measuring mineral formation, 50 μl of the suspension was diluted to 1 ml with SCL. Samples (140 μl per microplate well, n = 4) of each complex preparation were used for measurement of mineral formation.

For studying the effect of the Ca$^{2+}$/PS ratio used in making CPLX, the volume of CaCl$_2$ was varied. The PS concentration in the KP$_2$ buffer used for making PS-CPLX was fixed at 0.8 mM; to produce the 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 Ca$^{2+}$/PS mixing ratios, the final CaCl$_2$ concentrations required were 1.6, 3.2, 4.8, 6.4, 8, and 9.6 mM. 200 μl of CPLX reaction mixture was prepared using a reaction time of 10 min before harvest by centrifugation. The CPLX pellet was resuspended in 200 μl with SCL; 50 μl of the suspension was diluted to 1 ml with SCL; 140 μl of the diluted suspension was transferred in quadruplicate to the microplate wells, incubated at 37 °C, and assayed for mineral formation as follows.

**Microplate Mineralization Assay**—Mineral formation was monitored by a previously described 96-multwell microplate assay system (1, 40) based on the light-scattering method of Brecicevic and Furedi-Milhofer (41). Kinetic analysis of mineral formation was made using both first-derivative and 5-parameter curve-fitting analysis, as recently described (5). In brief, 140-μl samples (see above) of CPLX resuspended in SCL were distributed into 4 wells of a 96-half-area Costar microplate, measurement being made automatically at 15-min intervals using a Labsystems iEMS Reader MF microplate reader (Needham Heights, MA). Mineral formation (light scattering at 340 nm) was recorded over a 0–16-h period. Background absorbance at 340 nm at time 0 was used to evaluate initial conditions and was subtracted from each successive measurement to reveal the progress in mineral formation. SCL contained 2 mM Ca$^{2+}$ and 1.42 mM P$_2$ in addition to 104.5 mM Na$^+$, 133.5 mM Cl$^-$, 63.5 mM sucrose, 16.5 mM TES, 12.7 mM K$^+$, 5.55 mM glucose, 1.83 mM HCO$_3^-$, 0.57 mM Mg$^{2+}$ (42, 43). For most of the studies, the pH of SCL was adjusted to 7.5, the pH observed in the extracellular fluid of growth plate cartilage (24, 44). However, for the study of the role of ACP in unwashed and mild acid-washed PS-CPLX, the pH of the SCL was adjusted to various pH levels from 7.0 to 8.6 (see below).

**Fourier Transform IR Analysis of CPLX**—For FT-IR analyses, the CPLX pellets harvested after the various ripening times were chilled to 0 °C; the supernatant fluid was decanted; and the tubes were allowed to drain, wiped to remove residual fluid, and then lyophilized after freezing at −80 °C. A sample of the dried pellet (1 mg) was incorporated into a KBr (300 mg) pellet formed under vacuum at 12,000 p.s.i. pressure and then examined over a range from 4000 to 400 cm$^{-1}$ using a model 1600 series spectrometer from PerkinElmer Life Sciences (43).

**Effect of SCL pH on CPLX Mineralization**—For studies on the role of ACP in PS-CPLX mineral formation, both unwashed and acid-washed PS-CPLX (Ca$^{2+}$/PS ratio = 6, ripening time = 10 min) were investigated. After harvest by centrifugation, the PS-CPLX was either used directly or after washing with pH 5 (10$^{-5}$ M HCl) at 0 °C for 10 min, followed by sedimentation, brief washing in distilled deionized water, and resedimentation. The unwashed and acid-washed PS-CPLX samples were then incubated in a series of 12.7 mM TES-buffered SCL solutions and adjusted to pH values of 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, and 8.6 to study mineral formation as described above.

**Molecular Dynamic Simulation of PS-CPLX Formation**—To explore how PS interacts with Ca$^{2+}$ and P$_2$, we used molecular dynamic (MD) simulation to gain insight into the atomistic mechanism by which PS-CPLX may form. We focused on PS, because as noted above PS-based Ca$^{2+}$-P$_2$ complexes have long been associated with normal and pathological calcification in vivo. Accordingly, a nanoscale PS-CPLX domain consisting of PS, Ca$^{2+}$, PO$_4^{3-}$, K$^+$, and H$_2$O was constructed and visualized.
Synthesis and Modeling of Phosphatidylserine-Ca-Pi Complexes

To perform MD simulations, a force field for the molecules of interest is required. One commonly used force field, CHARMM27 (47), contains parameters for a growing list of lipid molecules; however, PS is not among them. Therefore, we created parameters for PS starting from basic fragments of similar molecules already present in CHARMM27 (48, 49). Specifically, using parameters for the palmitoyl and oleoyl chains, and the glycerol backbone of phosphatidylethanolamine, and similar molecular fragments of serine and palmitate, we compiled initial parameters for PS. The partial charges of the atoms of the PS head group were further refined using \textit{ab initio} quantum chemical calculations using Gaussian 03W (Gaussian, Inc., Wallingford, CT) (50) and Trident 1.0.0 (Wavefunction, Inc., Irvine, CA) software with O-methyl-L-serine phosphate anion as the model compound; its geometry was first optimized in water. Then using a stepping stone approach in which the geometries using the Hartree-Fock/3–21G and Hartree-Fock/6–31G* basis sets were sequentially optimized (51), the partial charges of the PS head group atoms were determined and added to the CHARMM27 topology library. Nondegenerate bond, angle, and dihedral parameters for PS geometric were from Pandit and Berkowitz (52) and added to the CHARMM27 parameter file. The nonbonded interaction parameter for Ca$^{2+}$ in the presence of PO$_4^{3-}$ was refined to be more representative of the properties of the system based on the methods of Marchand and Roux (53) and Zahn (54).

using Tool Command Language scripts run from within Visual Molecular Dynamics (VMD) software (45). An assembly of six PS molecules was first created, orienting the lipid tails approximately coincident with the z axis of the simulation cell, with the phosphodiester phosphate atoms of the head group ~8 Å apart in a loose hexagonal arrangement 4 Å below the x-y plane. To mimic the basic unit of ACP (31, 46), 9 Ca$^{2+}$ and 6 PO$_4^{3-}$ atoms were added in a random arrangement 4–7 Å above the PS head groups. Next, because CPLXs are prepared in KCl-containing solutions, to neutralize the negative charge of PS, 6 K$^+$ atoms were added to the ensemble. Finally, the system was hydrated by adding bulk H$_2$O above the hydrophobic region of PS. The complete system contained 1130 atoms.

For studying the effect of CPLX reaction (ripening) time, stock solution of PS emulsions (4×) were prepared by drying 5 mg of PS in chloroform under N$_2$ to form a thin film in a test tube. Then 2 ml of KCl (150 mM)-KPi buffer (10 mM, pH 7.5) was added; and the tube then sonicated for 1–2 min in a water bath at 25 °C to disperse the lipid and form small unilamellar liposomes. Of the 4 ml of the KCl-KPi buffer for making 200 µl of CPLX. Then 9.6 µl of CPLX. Then 9.6 µl of 100 mM CaCl$_2$, was added dropwise with constant stirring to form the 6:1 Ca$^{2+}$/PS (molar ratio) complexes. The reaction (ripening) times studied were 1.5, 3.0, 5.0, 6.5, 8.0, 10, 30, and 60 min. The complexes were then sedimented by centrifugation at 13,000 g for 5 min, and after decanting the supernatant; the pellets were resuspended in 200 µl, then 50 or 60 µl of SCL for 50 µl of PS-CPLX (A) and 0.048 µmol/ml of SCL for 60 µl of PS-CPLX (B). Note that there was no significant effect of ripening time on the rate or amount of mineral formation.

![FIGURE 1. Effect of reaction time for PS-CPLX synthesis on subsequent mineral formation when incubated in SCL. For studying the effect of CPLX reaction (ripening) time, stock solution of PS emulsions (4×) were prepared by drying 5 mg of PS in chloroform under N$_2$ to form a thin film in a test tube. Then 2 ml of KCl (150 mM)-KP buffer (10 mM, pH 7.5) was added; and the tube then sonicated for 1–2 min in a water bath at 25 °C to disperse the lipid and form small unilamellar liposomes. Of the 4 ml of the KCl-KPi buffer for making 200 µl of CPLX. Then 9.6 µl of 100 mM CaCl$_2$, was added dropwise with constant stirring to form the 6:1 Ca$^{2+}$/PS (molar ratio) complexes. The reaction (ripening) times studied were 1.5, 3.0, 5.0, 6.5, 8.0, 10, 30, and 60 min. The complexes were then sedimented by centrifugation at 13,000 g for 5 min, and after decanting the supernatant; the pellets were resuspended in 200 µl, then 50 or 60 µl of SCL for 60 µl of PS-CPLX (B). Note that there was no significant effect of ripening time on the rate or amount of mineral formation.](image-url)
Synthesis and Modeling of Phosphatidylserine-Ca-Pi Complexes

Molecular dynamic simulations were run using nanoscale MD version 2.6 (Theoretical Biophysics Group and the National Institutes of Health Resource for Macromolecular Modeling and Bioinformatics, Beckman Institute, University of Illinois, Urbana-Champaign) (55) on a dual-core dual-5150 Xeon processor machine. The system was minimized to remove unfavorable van der Waals contacts using the conjugate gradient algorithm of nanoscale MD (56) for 100 steps, then warmed and equilibrated at 310 K using a 2.0-fs time step. The warmed structure was then subjected to MD simulations with at least 8 Å of solvent between the lipid head groups and the periodic boundary. A Langevin thermostat with a damping coefficient of 2.0 ps$^{-1}$ was used. Bond lengths were fixed for all TIP3-explicit water molecules (57). Short range nonbonded interactions were cut off smoothly between 12 and 15 Å, and simulations were run for 2.0 ns.

RESULTS

Effect of Reaction (Ripening) Time—The effect of varying the reaction time before harvesting the PS-CPLX was investigated. For these studies a Ca$^{2+}$/PS mixing ratio of 6.0 was used, and the 0.80 mM PS in the 10 mM KPi, pH 7.5, buffer would yield an initial Ca$^{2+}$ level of 4.8 mM. Thus, the Ca$^{2+}$/P$_i$ ion product would be 48 m$^2$, a value sufficient to induce rapid formation of ACP (37). The reaction times were varied as follows: from 1.5, 3.0, 5.0 to 10 min for short ripening periods; 30 min, 1 h, and overnight (18 h) for longer ripening times. The effects of varying the reaction time were assessed in three different ways as follows: (a) the effect on light scattering at 340 nm at $t_{ri}$; (b) the effect on the ratios of the intensity of FTIR spectral absorbance bands; and (c) the effects on the rates of mineral formation when either the 50 or 60 μL of diluted PS-CPLX was incubated per ml of SCL.

All of the PS-CPLX samples formed were powerful nucleators and began mineral formation as soon as they were introduced into the SCL, but there was no obvious systematic difference between ripening times in the rate or extent of mineral formation (Fig. 1, A and B). By using 60 μL of CPLX per ml of SCL (Fig. 1B), there was less variance in the overall pattern than with 50 μL (Fig. 1A). Detailed analysis of the initial and final rates of mineral formation confirmed that there was no correlation between ripening time and rate of mineral formation (data not shown).

However, upon further examination, significant differences did emerge. The initial background absorbance (light scattering) at 340 nm of the nucleators (Fig. 2) for both the 60 μL (squares) and the 50 μL (diamonds) PS-CPLX samples, showed significant and rapid decreases during the first 10 min of ripening, after which there was little further change. This decrease in light scattering is indicative of changes in the structure of the CPLX during this early ripening period rather than aggregation, which would have caused an increase in light scattering.

This was confirmed by examining changes in the FTIR spectrum of PS as it was reacting with Ca$^{2+}$ and $P_i$ to form CPLX. Shown in Fig. 3A are the spectra of pure PS (0 min) and that of the CPLX after a 3-, 5-, and 10-min reaction with Ca$^{2+}$ and $P_i$. Evident is the increasing intensity of the P–O stretch bands at 1032 and 1110 cm$^{-1}$ relative to the intensity of the C–H stretch bands of the lipid acyl chains at 2921 and 2852 cm$^{-1}$, as well as the changing pattern in the P–O stretch region (1032–1110 cm$^{-1}$) during this incubation period. To quantitate these changes, we measured the ratios of the intensity of key bands in the FTIR spectrum (Fig. 3B). For example, the ratio of the intensity of the principal P–O stretch band of tribasic phosphate PO$_4^{3-}$ (1032 cm$^{-1}$) to the 1063 cm$^{-1}$ (Fig. 3B, triangles) and the 1110 cm$^{-1}$ (diamonds) bands typical of immature HPO$_4^{2-}$-containing calcium phosphates increased significantly during the first 10 min of ripening, indicating some deprotonation of the inorganic phosphate. Furthermore, in Fig. 3C the ratio of the P–O stretch band (1032 cm$^{-1}$) of the calcium phosphate to the C-H scissoring and bending band (1467 cm$^{-1}$) (triangles) and the C-H stretch band (2921 cm$^{-1}$) (diamonds) of the PS acyl side chains increased significantly during the first 10 min of CPLX ripening, with minimal changes thereafter with increasing ripening time. The same pattern was seen in the ratio of the P–O stretch band (1110 cm$^{-1}$) of immature calcium phosphate to the C–O stretch band of the acyl ester of PS (1234 cm$^{-1}$) (Fig. 3C, squares). These increases reveal an accretion of nascent mineral with the lipid during this phase of CPLX ripening.

Thus, during the first 10 min of CPLX ripening, physicochemical changes were occurring that affected their structure.

Effect of Variation of Ca$^{2+}$/PS Mixing Ratios—We next explored the effects of varying the ratios of Ca$^{2+}$ to PS during formation of PS-CPLX, which were examined both with respect to their effect on the structure of the CPLX and the effect on subsequent mineral formation when incubated in SCL. The ripening time was set at 10 min, because as seen above, little change in CPLX structure occurred with longer reaction times. The Ca$^{2+}$/PS mixing ratios studied were 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0. Because the level of PS in the 10 mM KPi, pH 7.5, buffers was 0.80 mM, the levels of added Ca$^{2+}$ were 1.6, 3.2, 4.8, 6.4, 8.0, and 9.6 mM. Thus, with the 10 mM P$_i$ buffer these led to

![FIGURE 2: Effect of reaction time for PS-CPLX synthesis on light-scattering background (as at 340 nm). PS-CPLX samples were reacted for 1.5, 3.0, 5.0, 6.5, 8.0, 10, 30, and 60 min and processed as described in Fig. 1. Shown is the initial background absorbance (light scattering) before incubation at 37°C in SCL, which was measured at 340 nm. The concentration of the PS in the CPLX samples was 0.80 mM. Diamonds, 50 μL of PS-CPLX; squares, 60 μL of PS-CPLX. Note the parallel patterns for both levels of PS-CPLX showing a rapid decline in light scattering during the initial 10-min reaction period. Minimal further change was seen with longer reaction times.](image-url)
Ca^{2+}/P_{1} mixing ratios ranging from 0.16 to 0.96; and Ca^{2+} × P_{1} ion products varying from 16 to 96 mM\(^2\).

The first observed effect upon increasing the Ca\(^{2+}\)/PS mixing ratios was a progressive increase in the light-scattering background of the CPLX when introduced into SCL (Fig. 4A). At a Ca\(^{2+}\)/PS ratio of 2:1, the background absorbance at 340 nm was 0.329 ± 0.001; this increased progressively to 0.383 ± 0.001 at a Ca\(^{2+}\)/PS ratio of 4:1 and leveled off at 0.416 ± 0.002 at a Ca\(^{2+}\)/PS ratio of 10:1. This increase appears to have resulted from the larger mass of CPLX formed when higher amounts of Ca\(^{2+}\) were added. However, when structural features of the PS-CPLXs were examined by FTIR (Fig. 4B), significant differences were also seen in the ratio of the intensity of the principal P–O stretch band of trisaccharide phosphate PO\(_4^{3-}\) (1033 cm\(^{-1}\)) to those typical of immature HPO\(_4^{2-}\)–containing calcium phosphates: 1064 cm\(^{-1}\) (squares), 1074 cm\(^{-1}\) (diamonds), 1110 cm\(^{-1}\) (triangles), and 991 cm\(^{-1}\) (circles). Note the distinct decline in the ratio of the intensities of the P–O stretch bands typical of immature or HPO\(_4^{2-}\)–containing calcium phosphates as the Ca\(^{2+}\)/PS mixing ratios increased. This indicates that the higher
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**A – Background As @ 340 nm**

**B – Mineral P–O Frequency Intensity Ratios**

- 1110/1033
- 1064/1033
- 1074/1033
- 991/1033

**C – Mineral P–O to Lipid C–H & C–O Frequency Intensity Ratios**

- 1110/1467
- 1032/1234
- 1110/1740
- 1032/2851

**FIGURE 4. Effect Ca$^{2+}$/PS mixing ratios on background absorbance and the ratios of the intensity of FTIR frequencies of PS-CPLX.** PS-CPLXs were produced as described under “Experimental Procedures.” For studying the level of added Ca$^{2+}$, the lower the proportion of HPO$_4^{2-}$ to PO$_4^{3-}$ in the resulting CPLX.

Furthermore, in Fig. 4C, when the intensity ratios of the absorbance of the mineral-related P–O stretch bands at 1110 and 1032 cm$^{-1}$ were compared with (a) the C–H scissoring and bending band (~1467 cm$^{-1}$) of the PS acyl side chains (circles), (b) the C–O stretch band (~1234 cm$^{-1}$) of the acyl ester of PS (triangles), (c) the C–O stretch band (~1740 cm$^{-1}$) of the ester carbonyl of PS side-chain fatty acids (diamonds), or (d) the C–H stretch band (~2851 cm$^{-1}$) of the acyl side chains (squares), a progressive increase is seen in the ratio of the mineral associated P–O stretch bands to the phospholipid associated C–H and C–O bands, at Ca$^{2+}$/PS mixing ratios above 4.0. Thus, at progressively higher Ca$^{2+}$/PS mixing ratios the calcium phosphate content of the PS-CPLXs progressively increased. This increase was reflected in the ability of the CPLXs to induce mineral formation when incubated in SCL.

As shown in Fig. 5A, the CPLX formed from a 2:1 Ca$^{2+}$/PS mixing ratio did not induce significant mineral formation (open diamonds). However, as the Ca$^{2+}$/PS ratios were increased from 4:1 to 6:1, 8:1, 10:1, and 12:1, there were progressive increases in the rate and amount of mineral formation. Fig. 5B shows that the initial rate (0–1 h of incubation) of mineral formation (RMF-I, circles) was the most sensitive to the increasing Ca$^{2+}$/PS mixing ratios; the subsequent rates (2–4 h of incubation, RMF-2, triangles) and (10–16 h of incubation, RMF-3, squares) were only marginally affected, as was the overall rate (0–16 h of incubation, RMF-overall, diamonds).
was reflected in the amounts of mineral formed at different stages when the PS-CPLXs were incubated in SCL (Fig. 5C).

Molecular Dynamics Simulation of PS-CPLX Formation—As indicated under “Experimental Procedures,” the simulation of PS-CPLX formation was arbitrarily started as a loose monolayer of six PS molecules set below a random arrangement of Ca\(^{2+}\), PO\(_4^{3-}\), K\(^+\), and water molecules (Fig. 6A), roughly approximating conditions used in the in vitro studies. It was constructed using VMD software and is shown from a vertical (left) and a lateral view (right). After 2 ns of molecular dynamic simulation at 310 K, a snapshot taken from a lateral view of the system reveals an extensive network of Ca\(^{2+}\)–O bonds (Fig. 6B, dashed lines). For clarity, only a portion of the magnified array is shown consisting of five of the Ca\(^{2+}\) ions (Fig. 6B, small CPK spheres), of which two are shown in 6-fold coordination to oxygen atoms (licorice) of the carboxylate group of PS, the free PO\(_4^{3-}\) (licorice) and water (licorice). Further stabilization of the PS-CPLX results from inter- and intramolecular hydrogen bonds (Fig. 6B, dashed lines) that form between the PS polar head group serine ammonium hydrogens and the oxygen atoms of the free PO\(_4^{3-}\) as well as the oxygen atoms of the phosphodiester groups of PS. Some of the computed Ca\(^{2+}\)–O bond lengths after simulation are indicated. Table 1 presents several key interatomic distances and, where direct comparison was possible, are in good agreement with RDF-EXAFS measurements for the PS-CPLX complex (58) and associated ACP (59). The principal exception is the Ca\(^{2+}\)–N distance, which in the molecular simulation is substantially greater than in the RDF-EXAFS measurement (see “Discussion”).

pH Effect on CPLX Mineralization—Another key issue regarding the nature of the PS-CPLX was whether the Ca\(^{2+}\) and P\(^{2-}\) associated with the PS molecules were simply as loosely bound ACP, or whether they were integrated into a more discrete and stable structure. To answer this question two forms of PS-CPLX were constructed, one of which was washed briefly with 10\(^{-5}\) M HCl, pH 5, to remove any loosely associated ACP; the other was left unwashed and used as formed. The PS-CPLX was made using a Ca\(^{2+}\) /PS mixing ratio of 6; the PS concentration was 0.80 mM; Ca\(^{2+}\) was 4.8 mM. Because ACP has a well defined narrow pH-dependent conversion to HA when incubated in SCL (39), these two forms of PS-CPLX were then incubated in a series of TES-buffered SCLs with pH values ranging from 7.0 to 8.6.

Fig. 7 shows the rates and extent of mineral formation produced by both the unwashed and acid-washed PS-CPLX, incubated in the SCLs of varying pH values. When incubated in SCL at pH 7.0 and 7.2 (Fig. 7, A and B), mineralization was relatively slow, with induction of mineral formation by the acid-washed CPLX being slightly delayed and progressing at a significantly slower rate than the unwashed complex. When incubated in SCLs at pH values between 7.4 and 8.0 (Fig. 7, C–F), both the unwashed and acid-washed CPLXs showed much more rapid mineral formation; however, again, the acid-washed CPLX showed a somewhat delayed slower rate of mineral formation. Incubated in the higher pH SCL buffers at pH 8.2 and 8.6 (Fig. 7, G and H), mineral formation was significantly reduced and
exhibited a biphasic character. However, with the acid-washed CPLX, there was no delay in induction of mineral formation, but there was still a distinctly slower rate of mineral formation. Generally speaking, although there were somewhat slower inductions and lower rates of mineral formation with acid-washed CPLXs, the general pattern observed for the unwashed and acid-washed CPLX was similar at all pH values.

The specific effects of acid washing can be seen more clearly when the different phases of mineral formation were measured (Fig. 8). The induction time (Fig. 8A), the initial rate (Fig. 8B), the final rate (Fig. 8C), and the final amount of mineral formation at 24 h (Fig. 8D) are shown. With the exception of the induction time (Fig. 8A) in which it can be clearly seen that the induction of mineral formation was significantly slowed, the patterns are quite similar. Thus, in contrast to ACP in which mineral formation is sharply confined to SCL pH values of 7.6–7.8, and in which virtually no mineral formed at pH 7.4 or ≥ 8.0, mineral formation by both the acid-washed and unwashed PS-CPLX occurred over a much broader range, much as was seen with isolated MVs (39). This indicates that the principal driving force for mineral formation by the PS-CPLX was because of the complex itself and not to associated ACP.

DISCUSSION

Many studies now indicate that PS-CPLX is a key component of the nucleational core that induces mineral formation in growth plate MVs (1, 2, 4). It has also been observed in a variety of pathological calcification ranging from tumors (15) to atherosclerotic plaques (60, 61). Here we aimed to elucidate more clearly how PS, Ca\(^{2+}\), and Pi interact during the formation of a nucleationally competent lipid-Ca-Pi complex. We explored this in a series of in vitro studies, as well as via molecular simulations in silico, comparing our findings, where possible, with previously published data. In this basically three-component system it was important to consider both the interaction between Ca\(^{2+}\) and Pi, between Ca\(^{2+}\) and the carboxylate and phosphodiester moieties of PS, and between Pi and the ammonium group of the polar head group serine of PS, as well as subsequent rearrangements that led to formation of the PS-CPLX.
Previous knowledge of the *in vivo* conditions under which PS-CPLX forms during the process of MV formation were of cardinal importance to these studies. For example, it is known that the Pi level of the cytoplasm of growth plate chondrocytes where MV formation occurs is unusually elevated at ~20 mM (24); on the other hand it is also known that for the formation of a nucleationally competent PS-CPLX that Pi must be present with PS before Ca$^{2+}$ is introduced to the system (4, 7, 23, 30). Because this situation occurs in growth plate chondrocytes when MV form (29), in these studies PS was prepared as small unilamellar vesicles in a Pi-rich KPi buffer into which Ca$^{2+}$ was introduced to initiate PS-CPLX formation. However, it was unclear what sequence of events occurred after introduction of Ca$^{2+}$ to the PS-KPi system. Therefore, we first examined the immediate products formed when Ca$^{2+}$ was introduced to PS in the Pi buffer. We found that all of the nascent complexes were nucleationally competent when incubated with SCL, regardless of the ripening time, and almost immediately induced nucleation of mineral formation. Detailed study, however, revealed that significant physicochemical changes occurred during the first 10 min after addition of Ca$^{2+}$.

The first apparent change was a rapid decrease in the extent of light scattering at 340 nm. Because it is well known that decreases in light scattering are closely correlated with a reduc-

### TABLE 1

| Atom pairs | MD simulation of PS-CPLX | RDF-EXAFS analysis of PS-CPLX$^a$ |
|------------|--------------------------|-----------------------------------|
| 1) Ca–O    |                          |                                   |
| Phosphate  | 2.27 ± 0.06              | 2.28 ± 0.10$^b$                   |
| PS carboxylate | 4.00 ± 0.10    | 2.44 ± 0.23$^a$                   |
| PS amine   | 2.38 ± 0.10              | 2.86 ± 0.09$^a$                   |
| PS amine   | 4.43 ± 0.10              | 3.38 ± 0.12$^a$                   |
| PS carboxylate | 4.45 ± 0.59$^a$         |                                   |
| 2) Ca–N    |                          |                                   |
| PS amine   | 4.36 ± 0.13              | 3.44 ± 0.35                       |
| 3) Ca–C    |                          |                                   |
| PS carboxylate | 2.85 ± 0.15    | 2.97 ± 0.07                       |
| PS amine   | 3.40 ± 0.22              | 3.13 ± 0.37                       |
| 4) Ca–Ca   | Ion pair with PO$_4^-$  |                                   |
| 3.71 ± 0.08 | 3.42 ± 0.29$^a$         |                                   |
| 3.70 ± 0.08 | 4.13 ± 0.36$^a$         |                                   |
| 3.70 ± 0.08 | 4.73 ± 0.23$^a$         |                                   |
| 5) Ca–P    | (Ca)$_3$(PO$_4$)$_2$ quintet |                             |
| 2.94 ± 0.05 | 3.18 ± 0.24$^a$         |                                   |
| 2.92 ± 0.06 | 3.22 ± 0.44$^a$         |                                   |
| 2.94 ± 0.06 | 3.39 ± 0.34$^a$         |                                   |
| 6) P–P     | PS phosphodiester       | 6.36 ± 0.34                       |

$^a$ Data are from Ref. 58.

$^b$ Structural association is not specified.

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**FIGURE 7.** Effect of acid washing on mineral formation by PS-CPLX incubated in synthetic cartilage lymph with varied initial pH. To examine the contribution of ACP to the mineral-forming potential of PS-CPLX, mild acid (pH 5, 10 M HCl) treatment was used to remove unincorporated ACP from newly formed PS-CPLX. Comparison of the ability of the unwashed versus acid-washed complexes to form mineral was evaluated using a series of SCL solutions varying in pH from 7.0 to 8.6. Shown in A–H are the mineral formation patterns of the unwashed and acid-washed complexes incubated in SCL over the pH range of 7.0–8.6. Also shown are first derivative dA/dT curves that reveal the time of most rapid mineral formation. Note the small delay and reduction in mineral formation caused by the acid treatment. Also in G and H, note that there is a distinct change in the pattern of mineral formation. Instead of an initial rapid formation period, an initial slow rate is followed by a second impulse of more rapid mineral formation.
tion in particle size (62, 63), this suggested that some type of rearrangement must have occurred during this 10-min period that led to condensation of the PS-Ca\(^{2+}\)-Pi conglomerate. FTIR analysis revealed that there was a rapid increase in the ratio of PO\(_4^{3-}\) relative to HPO\(_4^{2-}\), which was accompanied by a significant increase in mineral phosphate relative to the lipid component. These findings indicate that interaction with Ca\(^{2+}\) induced significant deprotonation of the HPO\(_4^{2-}\) ion species, even as the calcium phosphate component was progressively combining with the phospholipid component to form the complex.

This was confirmed when we studied the effect of varying the Ca\(^{2+}\)/PS ratio used to induce formation of the PS-CPLX. We found that a minimal ratio of 4 Ca\(^{2+}\) per PS was required for formation of a viable complex, with greater additions of Ca\(^{2+}\) further potentiating the mineral-forming activities of the complex.

ACP had been a major component of the precipitate formed, there should have been major differences between the unwashed and acid-washed complexes in the amount of mineral formation. Our data show however that reduction in mineral formation in the acid-washed complex was only about 20%, indicating that ACP was a relatively minor factor. The finding that the patterns of mineral formation from the unwashed and acid-washed complexes were remarkably similar supports the concept that PS-CPLX initiates mineral formation and that auxiliary ACP only augments this process.

The small delay in the onset of mineral formation observed in the acid-washed complex probably resulted from protonation of surface phosphate ions, which would then need to be deprotonated before mineral formation could occur. This interpretation is supported by the finding that the delay in induction time was longest when incubated at pH 7.0–7.2 and
was progressively shortened when incubated at the higher pH values.

FTIR analyses revealed that Ca\(^{2+}\) caused significant deprotonation of HPO\(_4^{2-}\), even as the PS-complex was forming. Our molecular simulations data now provide important insight into this reaction process, elucidating the initial interactions that occur between Ca\(^{2+}\) with P\(_i\) and PS. The computed PS-CPLX structure reveals the initial packing arrangement of the polar head group of PS resulting from its interaction with Ca\(^{2+}\) and P\(_i\), when Ca\(^{2+}\) is introduced. To our knowledge, this is first depiction of the molecular assembly of the PS-Ca-P\(_i\) complex. Analysis of its atomic features provides clues to why it so efficiently induces mineral formation. The PS-CPLX structure shows hexa-coordinated bonding between Ca\(^{2+}\) and the oxygen atoms of the following: (a) free PO\(_4^{3-}\), (b) the carboxylate, (c) the phosphodiester of PS, and (d) water. It is evident that the NH\(_3^+\) group of PS contributes intermolecular hydrogen bonding with PO\(_4^{3-}\), as well as intramolecular bonding to oxygen atoms of the phosphodiester. From the 6.4 ± 0.3 Å intermolecular P–P distance between phosphodiester moieties of the PS head groups and their probable hexagonal packing arrangement in the lipid monolayer, this two-dimensional array would approximate the lattice parameters of HA.

Furthermore, there is good agreement between the simulated Ca\(^{2+}\)–O interatomic distances and those previously determined by RDF-EXAFS of PS-CPLX. This was also largely true of the Ca\(^{2+}\)–Ca\(^{2+}\) interionic distances and Ca\(^{2+}\)–C (carboxylate) distances (Table 1). The only significant difference was in the Ca\(^{2+}\)–N distance, which in the molecular simulation was 4.36 ± 0.13 Å, significantly longer than the Ca\(^{2+}\)–N distance (3.44 ± 0.35 Å) in PS-CPLX as analyzed by RDF-EXAFS. We simulated the PS-CPLX model for 10\(^7\) 2-fs time steps (2 ns); the system rapidly converged within the first 200 ps with the Ca\(^{2+}\)–N distance remaining stable thereafter. This suggests that transition events leading to the shortening of the Ca-N must take place at much greater time scales. Recall in our experiments that before addition of Ca\(^{2+}\), PS was present in the 10 mM pH 7.5 P\(_i\) buffer. The dominant ionic form of P\(_i\) would be HPO\(_4^{2-}\), which being in great excess would readily form an ion pair with the aliphatic amine of PS (64). Despite the fact that the p\(_K_a\) for deprotonation of HPO\(_4^{2-}\) is 12.3, interaction between Ca\(^{2+}\) and HPO\(_4^{2-}\) can lead to its deprotonation to PO\(_4^{3-}\) in aqueous solutions at much lower pH. From previous quantum molecular simulations (54), this involves sequential formation of an electrically neutral [Ca\(^{2+}\)→HPO\(_4^{2-}\)]\(^0\) ion pair, which then interacts with a second Ca\(^{2+}\) to form [(Ca1)\(^{2+}\)⋯(HPO\(_4^{2-}\))\(^0\)⋯(Ca2)\(^{2+}\)]\(^2+\). In this positively charged complex, deprotonation of HPO\(_4^{2-}\) to [Ca\(^{2+}\)⋯PO\(_4^{3-}\)⋯Ca\(^{2+}\)]\(^+\) is strongly favored thermodynamically.

Based on our current in vitro and in silico molecular modeling studies, we propose that within the 10-min period following addition of Ca\(^{2+}\) to the PS-containing P\(_i\) buffer, there is a series of critical, time-dependent interactions between Ca\(^{2+}\) and the [PS- NH\(_3^+\)⋯HPO\(_4^{2-}\)]\(^-\) ion pair that leads to formation first of a [Ca\(^{2+}\)⋯PS- NH\(_3^+\)⋯HPO\(_4^{2-}\)]\(^+\) adduct. Next, because of its net positive charge, deprotonation of the PS-NH\(_3^+\) (p\(_K_a\) = 9.8) rather than of HPO\(_4^{2-}\) (p\(_K_a\) = 12.3) would occur. In the result-

ing adduct, [Ca\(^{2+}\)⋯PS-NH\(_3^+\)⋯HPO\(_4^{2-}\)]\(^-\)], the electrically neutral amine, R-NH\(_2\), which possesses a pair of unshared electrons, would bond to Ca\(^{2+}\) producing the significantly shorter Ca–N distance indicated by the RDF-EXAFS data (59). Such Ca–N bonding in fact is a key feature in powerful chelating agents such as EDTA. However, the affinity of PS for Ca\(^{2+}\) is weaker, and the overall reaction does not occur instantly, taking in the order of 10 min, as indicated by the FTIR data. Thus, in our molecular simulation, this process would not yet have occurred, which would explain the longer simulated Ca–N distance. Our in vitro data, which show a progressive decrease in light scattering during this 10-min period, support the concept that deprotonation of the amine would lead to condensation of the PS-CPLX. In fact, in the discussion of the RDF-EXAFS paper, Taylor et al. (58) comment on the close Ca–N distance. Previous MD simulations of only Ca\(^{2+}\) in an aqueous solution containing NH\(_4\)OH show stable Ca-N radial distribution functions within 0.5 ns and yielded maxima positions of 2.7 and 4.6 Å for the first and second shell of hydration (65).

It is possible that future MD simulations of the PS-CPLX based on a combined quantum mechanical/molecular mechanics strategy will model the Ca-N distance more precisely. Furthermore, larger PS-Ca\(^{2+}\)-P\(_i\) complex systems need to be built and simulated to provide more precise statistical mechanics of this important amphipathic process. Nevertheless, the current findings unite quantum-mechanical considerations, spectroscopic data, and biomimetic in vitro modeling to elucidate and visualize the formation of the initial PS-CPLX structure. They provide insight into the atomic events that occur physiologically in the growth plate at a crucial stage of mineral formation required for normal bone development.

REFERENCES

1. Wu, L. N., Genge, B. R., Dunkelberger, D. G., LeGeros, R. Z., Concannon, B., and Wuthier, R. E. (1997) J. Biol. Chem. 272, 4404–4411
2. Wu, L. N., Yoshimori, T., Genge, B. R., Sauer, G. R., Kirsch, T., Ishikawa, Y., and Wuthier, R. E. (1993) J. Biol. Chem. 268, 25084–25094
3. Boskey, A. L., Goldberg, M. R., and Posner, A. S. (1978) Proc. Soc. Exp. Biol. Med. 157, 590–593
4. Wu, L. N., Genge, B. R., Sauer, G. R., and Wuthier, R. E. (1996) Connect. Tissue Res. 35, 309–315
5. Genge, B. R., Wu, L. N., and Wuthier, R. E. (2007) J. Biol. Chem. 282, 26035–26045
6. Genge, B. R., Wu, L. N., and Wuthier, R. E. (2007) Anal. Biochem. 367, 159–166
7. Cotmore, J. M., Nichols, G., Jr., and Wuthier, R. E. (1971) Science 172, 1329–1341
8. Wuthier, R. E., and Gore, S. T. (1977) Calcif. Tissue Res. 24, 163–171
9. Abramson, M. B., Katzman, R., and Gregor, H. P. (1964) J. Biol. Chem. 239, 70–76
10. Nash, H., and Tobias, J. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 51, 476–480
11. Majeska, R. J., Holwerda, D. L., and Wuthier, R. E. (1979) Calcif. Tissue Int. 27, 41–46
12. Anderson, H. C., Garinella, R., and Tague, S. E. (2005) Front. Biosci. 10, 822–837
13. Irving, J. T., and Wuthier, R. E. (1961) Arch. Oral Biol. 5, 323–324
14. Wuthier, R. E. (1968) J. Lipid Res. 9, 68–78
15. Anghileri, L. J. (1972) Expierientia (Basel) 28, 1086–1087
16. Boskey, A. L., and Posner, A. S. (1976) Calcif. Tissue Res. 19, 273–283
17. Boyan, B. D., and Boskey, A. L. (1984) Calcif. Tissue Int. 36, 214–218
18. Boyan-Salyers, B. D., and Boskey, A. L. (1980) Calcif. Tissue Int. 30, 167–174
