Journal of Inorganic Biochemistry 103 (2009) 948–962

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Differential effects of zinc and magnesium ions on mineralization activity of phosphatidylserine calcium phosphate complexes

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ARTICLE INFO

Article history:
Received 29 October 2008
Received in revised form 6 April 2009
Accepted 8 April 2009
Available online 19 April 2009

Keywords:
Matrix vesicles
Biomineralization
Calcium
Phosphate
Magnesium
Zinc
Phosphatidylserine
Phospholipid–calcium–phosphate complexes
Hydroxyapatite
Annexin
Nucleation
Fourier-transform infrared spectroscopy

ABSTRACT

Mg^{2+} and Zn^{2+} are present in the mineral of matrix vesicles (MVs) and biological apatites, and are known to influence the onset and progression of mineral formation by amorphous calcium phosphate (ACP) and hydroxyapatite (HAP). However, neither has been studied systematically for its effect on mineral formation by phosphatidylserine–Ca^{2+}–Pi complexes (PS–CPLX), an important constituent of the MV nucleation core. Presented here are studies on the effects of increasing levels of Mg^{2+} and Zn^{2+} on the process of mineral formation, either when present in synthetic cartilage lymph (SCL), or when incorporated during the formation of PS–CPLX. Pure HAP and PS–CPLX proved to be powerful nucleators, but ACP took much longer to induce mineral formation. In SCL, Mg^{2+} and Zn^{2+} had significantly different inhibitory effects on the onset and amount of mineral formation; HAP and PS–CPLX were less affected than ACP. Mg^{2+} and Zn^{2+} caused similar reductions in the rate and length of rapid mineral formation, but Zn^{2+} was a more potent inhibitor on a molar basis. When incorporated into PS–CPLX, Mg^{2+} and Zn^{2+} caused significantly different effects than when present in SCL. Even low, subphysiological levels of Mg^{2+} altered the inherent structure of PS–CPLX and markedly reduced its ability to induce and propagate mineral formation. Incorporated Zn^{2+} caused significantly less effect, low (<20 μM) levels causing almost no inhibition. Levels of Zn^{2+} present in MVs do not appear to inhibit their nucleational activity.

1. Introduction

The *de novo* initiation and propagation of calcium phosphate mineral formation during vertebrate bone formation is a complex process. In growth plate cartilage, matrix vesicles (MVs) derived from the chondrocyte plasma membrane play a primary role in initiating the process of mineralization [1,2]. One of the essential features of active MVs is the presence of a nucleation core [3,4] that contains phospholipid–Ca^{2+}–Pi complexes (CPLX) capable of inducing mineral formation when incubated in synthetic cartilage lymph (SCL) [5–8]. A key component of CPLX is phosphatidylserine (PS) [5,9,10], a lipid with high affinity for Ca^{2+} [11,12]. Discovery of the nucleation core is rooted in the early finding that acidic phospholipids, especially PS, are complexed with Ca^{2+} at sites of initial mineral formation [13,14]. PS–Ca^{2+}–Pi complexes (PS–CPLX) are present at early stages of development of almost all calcifying tissues: growth plate cartilage [14], tumors [15], bone [16], dentin [17], and in particular MV [10]. Pure synthetic PS–CPLX is a powerful nucleator capable of rapidly inducing hydroxyapatite (HAP) formation when incubated in SCL [5,8]; however, when formed from Mg^{2+}-containing Pi-rich buffers, PS–CPLXs are weak nucleators unless certain phospholipid-dependent Ca^{2+}-binding proteins, e.g. the annexins [6,7] are also included. Annexin A5, a major protein of MV [20–23], potently activates the nucleational activity of Mg^{2+}-containing PS–CPLX [7].

The mechanism by which PS–CPLXs are formed has been recently investigated [24], as well as how annexin A5 enhances CPLX activity in the presence of certain MV lipids and collagen [25]. However, little is known as to why Mg^{2+}-containing PS–CPLXs have such poor nucleating activity. While Mg^{2+} is known to affect the ability of ACP to convert to HAP [26–28], the nucleational activity of PS–CPLX is known not to depend on ACP [24,29].

Further, MVs are known to contain substantial amounts of Zn^{2+} [30], which paradoxically is known to powerfully block mineral formation by MVs when present in SCL [30,31]. The inhibitory effects of Zn^{2+} in MV mineralization have been linked to its influence on the Ca^{2+}-channel activity of annexin A5 [32]; and Zn^{2+} is known to influence the *in vitro* formation and stability of ACP [33,34]. However, little is known of the effect of Zn^{2+} on the nucleational activity of PS–CPLX, although there is preliminary evidence indicating an inhibitory effect [8].

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doi:10.1016/j.jinorgbio.2009.04.004

Thus, the studies reported here were aimed at determining the effects of Mg^{2+} and Zn^{2+} on the ability of PS–CPLX to induce mineral formation. As positive controls in these studies, the effects of these ions in SCL on mineral formation by both HAP and ACP were also studied. To clarify their regulatory effects, these ions were also studied when incorporated at graded levels into PS–CPLX before incubation in SCL to induce mineral formation.

2. Experimental procedures

The studies performed on these biologically relevant nucleators encompassed several approaches. We first established the effect of varying the seeding volumes on the rate and amount of mineral formation by each nucleator. Next, we examined the effects on mineral formation of varying the levels of Mg^{2+} and Zn^{2+} present in the SCL in which these nucleators were seeded. Finally, we examined the effects of incorporating various levels of Mg^{2+} and Zn^{2+} into the PS–CPLX before seeding into normal SCL. These studies were performed to gain insight into the biological roles these metal ions play in regulating the rate of formation and growth of calcium phosphate minerals under *in vivo* conditions.

2.1. Chemicals

Synthetic phosphatidylserine (PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine, sodium salt, was obtained from Avanti Polar Lipids, Inc., Alabaster, AL. All other chemicals were of reagent grade and were purchased from either Fisher Scientific (Norcross, GA) or Sigma Chemical Co. (St. Louis, MO).

2.2. Preparation of synthetic cartilage lymph (SCL)

Standard SCL (pH 7.5) was prepared from stock solutions and stored at 4 °C until used; it contained 2.0 mM Ca^{2+} and 1.42 mM Pi, in addition to 104.8 mM Na^+, 134.7 mM Cl^-, 63.5 mM sucrose, 16.5 mM TES, 12.7 mM K^+, 5.55 mM glucose, 1.83 mM HCO_3^-, and 0.57 mM Mg^{2+} [35–37]. The effects of varying the concentrations of Mg^{2+} (0.57–2.57 mM) and Zn^{2+} (0–100 μM) in the SCL into which the following nucleators were incubated was studied. The levels of these ions were in the range of those present in cartilage extracellular and intracellular fluids [30,37,38].

2.3. Synthesis of amorphous calcium phosphate (ACP) and hydroxyapatite (HAP)

Synthesis of ACP and HAP were as previously described [39]. For ACP, 0.20 mL of 50 mM K_2HPO_4 was mixed with 0.55 mL of 150 mM Tris buffer (pH 8.0); to this mixture was added 0.25 mL of 50 mM $CaCl_2$ stock solution, with continuous stirring. After 12 min reaction, the cloudy precipitate was sedimented by centrifugation at ~13,000*g* for 5 min; the supernatant was decanted and the ACP pellet resuspended in 1 mL of chilled (0 °C) SCL to arrest further development [8]. In the initial studies, various volumes of this cold suspension ranging from 10 to 60 μL were added to 1 mL of SCL and used for measurement of mineral formation at 37 °C to establish convenient seeding levels. In subsequent studies, volumes of 40 μL/mL of ACP were used to study the effect of varying the level of Mg^{2+} or Zn^{2+} in the SCL on mineral formation.

For synthesis of HAP, 20 mL of 50 mM K_2HPO_4 was mixed with 55 mL of 150 mM Tris buffer (pH 7.5) containing 20 mg of NaN_3; to this mixture was added 25 mL of 50 mM $CaCl_2$ stock solution, with continuous stirring as above. The reaction was continued for 5 days at 37 °C to ensure complete conversion to stable HAP. In initial studies, various volumes of this suspension (2–12 μL) after centrifugation were added to 1.0 mL SCL and used to study further mineral formation; in subsequent studies 10 μL of suspension after centrifugation was added per mL of SCL and used for study of the effects of Mg^{2+} and Zn^{2+} levels in SCL.

2.4. PS–Ca–Pi complexes (PS–CPLX)

Synthesis of standard PS–CPLX was recently described in detail [24]; here, the effect of adding various levels of Mg^{2+} (0.0–1.5 mM) or Zn^{2+} (0–50 μM) during synthesis of the CPLX was also investigated. For preparing the standard CPLX, a 4×-stock solution of PS emulsion was prepared by drying 5 mg 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 2–3 min in a water bath at 25 °C to disperse the lipid and form small unilamellar liposomes. For making 200 μL of the Mg^{2+}-containing CPLXs, 50 μL of the 4× PS stock (0.8 μmol) was diluted with 150 μL of the 10 mM KCl–KPi buffer containing levels of $MgCl_2$ ranging from 0 to 1.5 mM. Then, 7.0 μL of 100 mM $CaCl_2$ was added drop-wise with constant stirring for 10 min to form the complex. 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 of SCL chilled to 0 °C, followed by brief sonication to ensure full dispersal of the complex [8]. Either 50 or 60 μL of this suspension was diluted to 1 mL with chilled SCL. Samples (140 μL per microplate well, $n = 4$) of the diluted suspension of each complex preparation were used for measurement of mineral formation. An essentially identical method was used for making the Zn^{2+}-containg CPLXs, except that $ZnCl_2$ was included in the KCL–KPi buffer at levels ranging from 0 to 50 μM. As above, the Zn^{2+}-containing CPLX pellets were resuspended in 200 μL of SCL; 50 or 60 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.

2.5. Microplate mineralization assay

Mineral formation was monitored by a previously described 96-multiwell microplate assay system [3,40] based on the light-scattering method of Brecevic and Furedi-Milhofer [41]. Kinetic analysis of mineral formation was made using both first-derivative and 5-parameter curve fitting analysis, as recently described [6]. In brief, quadruplicate 140 μL samples (see above) of CPLX resuspended in SCL were distributed into four 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-zero was used to evaluate initial conditions, and was subtracted from each successive measurement to reveal the progress in mineral formation.

2.6. Fourier-transform IR analysis of CPLX

For FT-IR analyses, the CPLX pellets harvested after the 10 min ripening time were chilled to 0 °C, the supernatant fluid was decanted; 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 psi pressure, and then examined over a range from 4000 to 400 cm^{-1} using a Perkin Elmer model 1600 series spectrometer [36].