Colloids and Surfaces B: Biointerfaces 155 (2017) 466–476

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Protocols

Effect of the presence of cholesterol in the interfacial microenvironment on the modulation of the alkaline phosphatase activity during *in vitro* mineralization

B.F. Favarin[a], M.A.R. Andrade[a], M. Bolean[a], A.M.S. Simão[a], A.P. Ramos[a], M.F. Hoylaerts[b], J.L. Millán[c], P. Ciancaglini[a,*]

[a] Depto. Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto da Universidade de São Paulo (FFCLRP-USP), Ribeirão Preto, São Paulo, Brazil
[b] Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium
[c] Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA

ARTICLE INFO

Article history:
Received 30 January 2017
Received in revised form 20 April 2017
Accepted 25 April 2017
Available online 26 April 2017

Keywords:
Lipid interface
Biomineralization
Alkaline phosphatase
Monolayer
Proteoliposome
Sterol
Cholesterol

ABSTRACT

Mineralization of the skeleton starts within cell-derived matrix vesicles (MVs); then, minerals propagate to the extracellular collagenous matrix. Tissue-nonspecific alkaline phosphatase (TNAP) degrades inorganic pyrophosphate (PP_i), a potent inhibitor of mineralization, and contributes P_i (Phosphate) from ATP to initiate mineralization. Compared to the plasma membrane, MVs are rich in Cholesterol (Chol) (~32%) and TNAP, but how Chol influences TNAP activity remains unclear. We have reconstituted TNAP in liposomes of dipalmitoylphosphatidylcholine (DPPC) or dioleoylphosphatidylcholine (DOPC) combined with Chol or its derivatives Cholestenone (Achol) and Ergosterol (Ergo). DPPC plus 36% sterols in liposome increased the catalytic activity of TNAP toward ATP. The presence of Chol also increased the propagation of minerals by 3.4-fold. The catalytic efficiency of TNAP toward ATP was fourfold lower in DOPC proteoliposomes as compared to DPPC proteoliposomes. DOPC proteoliposomes also increased biomineralization by 2.8-fold as compared to DPPC proteoliposomes. TNAP catalyzed the hydrolysis of ATP more efficiently in the case of the proteoliposome consisting of DOPC with 36% Chol. The same behavior emerged with Achol and Ergo. The organization of the lipid and the structure of the sterol influenced the surface tension (γ), the TNAP phosphohydrolytic activity in the monolayer, and the TNAP catalytic efficiency in the bilayers. Membranes in the Lα phase (Achol) provided better kinetic parameters as compared to membranes in the Lo phase (Chol and Ergo). In conclusion, the physical properties and the lateral organization of lipids in proteoliposomes are crucial to control mineral propagation mediated by TNAP activity during mineralization.

1. Introduction

In vertebrates, biomineralization consists in the orderly deposition of minerals composed of phosphate and calcium ions, to form a calcium phosphate salt that resembles hydroxyapatite (HA) ($Ca_{10}(PO_4)_6(OH)_2$). Chondrocytes and osteoblasts initiate this process by releasing matrix vesicles (MVs) [1,2], which possess various hydrolytic enzymes including tissue-nonspecific alkaline phosphatase (TNAP). TNAP displays phosphomonohydrolytic activity, which generates inorganic phosphate (P_i) from inorganic pyrophosphate (PP_i) and adenosine-5-triphosphate (ATP) and has a crucial role in calcification. TNAP is attached to the cell and MV membranes *via* a glycosylphosphatidylinositol anchor (GPI-anchored), which provides the enzyme with lateral mobility relative to the membrane [3]. This mobility allows TNAP to accumulate in specific lipid microenvironments associated with ordered microdomains that are rich in sphingolipids, glycosphingolipids, and cholesterol (Chol) [4–8].

Ciancaglini et al. [9] have demonstrated that the catalytic properties of TNAP vary depending on the microenvironment around the enzyme. Distinct forms of the enzyme (associated with membranes, solubilized with detergent, or treated with phosphatidylinositol-specific phospholipase C) display different specificities for biological substrates, indicating that the GPI-anchor and the components of the membrane greatly affect enzyme kinetics.

* Corresponding author at: Depto Química, FFCLRP-USP, Av. Bandeirantes, 3900, 14040-901, Ribeirão Preto, SP, Brazil.
E-mail addresses: pietro@ffclrp.usp.br, pietro.ciancaglini@gmail.com (P. Ciancaglini).

http://dx.doi.org/10.1016/j.colsurfb.2017.04.051
0927-7765/

Liposomes, specifically large unilamellar vesicles (LUVs), mimic the structure of the lipid membrane [10]. On the other hand, proteoliposomes are vesicles that can mimic MVs in which one or more proteins have been incorporated or inserted [11]. Recent studies by our research group [10–16] have shown how different compositions of lipids affect the function of TNAP during mineralization and have explored the application of proteoliposomes as MV biomimics *in vitro*. MVs present high content of Chol, sphingomyelin, and phosphatidylserine, resembling the content of rafts in the plasma membrane [17,18]. According to Thouverey et al. [19], MVs consist of around 36% phosphatidylethanolamine (PE), 26.5% phosphatidylcholine (PC), 3.5% phosphatidic acid (PA), 7% phosphatidylinositol (PI), 16.5% phosphatidylserine (PS), and 11% sphingomyelin (SM).

Because MVs are rich in phospholipids and Chol, lipids in different states of organization can influence the functionality of the membrane and the properties of the proteins present in the membrane. Therefore, the effect of saturated (DPPC) and unsaturated (DOPC) lipids in MVs containing sterols deserves special attention.

Chol is an essential lipid component of the plasma membrane in animal cells, and Chol at low concentration occurs in certain intracellular membranes involved in vesicular communication with the plasma membrane [20]. Free cholesterol predominates in the membrane of MVs, accounting for 31.7% of the nonpolar lipids therein [21]. Although Chol has several functions, one of its main roles is to modulate the physical properties and lateral organization of the lipid bilayer of the plasma membrane [22,23]. Previous studies [13] have shown how increasing concentrations of Chol impact the thermodynamic parameters of liposomes: the variation in the enthalpy of the phase transition (ΔH) of liposomes decreases, and the mean diameter of liposomes composed of different DPPC/Chol molar ratios increases as compared to liposomes constituted by DPPC only. Incorporation of Chol into phospholipid monolayer and bilayer model membranes is clear from the broadening and the eventual elimination of the cooperative gel-to-liquid crystalline phase transition and its replacement with a phase displaying intermediate degree of organization [24,25]. Above the melting temperature of lipids, Chol organizes their acyl chains. Below the melting temperature, Chol prevents lipids from condensing into solid phases by intercalating between the phospholipids and reducing the flexibility of the surrounding chains [26]. Furthermore, the addition of Chol to mixtures of lipids generates two coexisting liquid phases, the liquid crystalline ($L\alpha$) and the liquid-ordered (L_o) phases [24,25,27,28]. Chol also decreases the area occupied per molecule in liposomes in the gel phase, thereby increasing lipid/lipid interactions and raising ΔH in binary and ternary systems [12,13].

Ergosterol (Ergo) differs from Chol (Fig. 1) by an additional double bond in ring B of the steroid nucleus at C7, C8 as well as a double bond between C22, C23 and an extra methyl group at C24 of the alkyl side-chain (Fig. 1). The additional double bond and the methyl group in the alkyl side-chain of Ergo provide this Chol analogue with a more rigid alkyl moiety, and the additional conjugated double bond of the steroid nucleus may produce a more rigid and planar ring B as well as rings B and C with flatter conformation [29]. Molecular dynamics simulations have suggested that Ergo occupies a considerably smaller effective molecular volume in DPPC bilayers [30] in the Lo state [31,32], probably because the off-axial mobility of the steroid nucleus and the alkyl side-chain in Ergo are smaller as compared to Chol, and because Ergo exerts greater condensing effect on the host phospholipid bilayer [33,34].

The third analogue in each molecular set, Cholestenone (Achol), differs from the Chol and Ergo by substitution of the —OH group for a keto group (Fig. 1). The existence of a double bond in ring A and of a keto group results in a conjugated arrangement in Achol [24]. These sterols (Chol, Ergo, and Achol) differ in both the nature and stereochemistry of the polar head group and in the position(s) of the double bond(s) [25].

Cholesterol analogues have frequently been used to evaluate how the functional groups in these molecules can interact with lipids at the interface of the membrane. In this context, this study focuses on the structural differences and on the interactions that sterols and lipids can establish and which consequently affect the activity of TNAP.

Our research group has standardized a methodology to obtain biomimetic vesicles containing TNAP incorporated into lipid microdomains, to enable biochemical and biophysical studies of these MV biomimics [12,13]. To understand the interaction between essential MV enzymes associated with the initiation and propagation of biomineralization, it is important to consider the microenvironment in which these enzymes work because this environment will determine the biological properties of the enzymes [35]. In this sense, the production of liposomes composed of different lipids (DPPC or DOPC) combined with different sterols may help to understand the modulation of the enzymatic activity during the propagation of minerals.

Diffuse reflectance ultraviolet-visible analysis (DRUV) and axisymmetric drop shape analysis (ADSA) can be used to study mimetic systems involving interfacial processes that cause spectroscopic changes. DRUV-ADSA allows the study of processes taking place within the drop and at the drop-air interface because this technique provides UV–vis spectrophotometric data and surface tension (γ) values simultaneously [36–38]. Enzymes, such as TNAP, attached to the lipid bilayer of the cell membrane *via* the GPI-anchor display surface activity [12,39]. The GPI moiety is hydrophobic, whereas the polypeptide chain is hydrophilic, so the anchored enzyme acts as a surfactant [40]. The DRUV-ADSA technique helps us to correlate the phosphohydrolytic activity of TNAP at the interface with the ability of TNAP-containing proteoliposomes to propagate biomineralization.

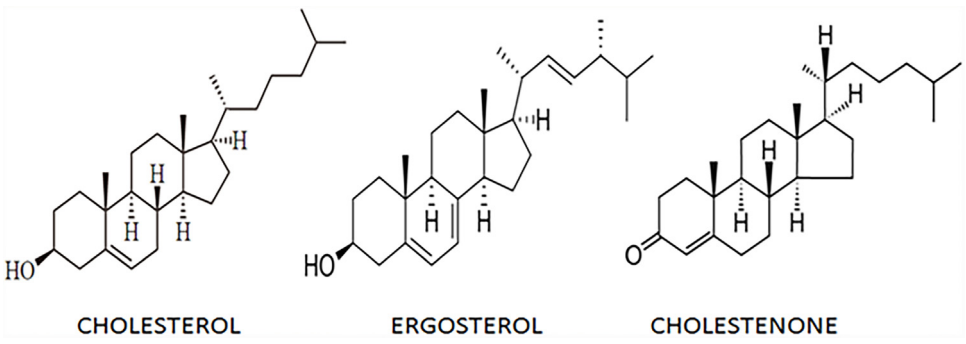

Fig. 1. Structural differences among the investigated sterols.