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研究課題名（和文）Designing of novel bone-inducing molecules by an experimental-computational approach

研究課題名（英文）Designing of novel bone-inducing molecules by an experimental-computational approach

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研究成果の概要（和文）：リン脂質分子の石灰化機構を解明するために第一段階として考えられる加水分解反応を調べた．疎水性部である炭素鎖が６個の炭素原子を含み，親水性部としてcholineおよびserineを含むphosphatidylcholine (PC6)およびphosphatidylserine (PS6)の２種類のリン脂質分子を取りあげた．さらに，Caイオンを囲む２個のリン脂質分子からなる系を計算の対象とした．加水分解反応の機構は複数の素反応からなり，活性化エネルギーを基準に，素反応が生じる順番を特定した．その結果，2PC6Caと2PS6Caにおける加水分解反応の機構は多くの点において異なることがわかった．

研究成果の学術的意義や社会的意義
Recovery from bone fracture is slow and is a growing global social problem as the percentage of elderly people increases. Accelerating bone fracture repair would reduce treatment costs. The final aim of our research is to develop biomaterials that mineralize quickly and lead to fast bone formation.

研究成果の概要（英文）：To elucidate the mechanism of mineralization of phospholipids, we first assumed that the hydrolysis reactions are the first step in the mechanism, releasing the phosphate group from the molecule to the aqueous solution and facilitating the formation of hydroxyapatite, for example. The dynamics of relevant elementary reactions was analyzed via quantum chemical calculations. Phospholipids having 6 carbons in the hydrophobic tail, and choline (PC6) or serine (PS6) as the hydrophilic head were chosen as the reactants. We started analyzing reactions of single molecures and then complexes composed of two phospholipids surrounding a Ca ion (2PC6Ca or 2PS6Ca). The steps involved in the hydrolysis reactions were identified, and the energies of reactants, transition states and products were determined. From the values of activation energy and heat release, the sequences through which the steps proceed were determined, and differences between the hydrolysis of 2PC6Ca and 2PS6Ca were found.

研究分野：Analysis of chemical reactions

キーワード：mineralization　quantum chemistry　reaction dynamics　reaction mechanisms

１．研究開始当初の背景

Bone regeneration is an important step in various reconstruction processes such as orthopedic and dental treatment. In recent years, various biocompatible materials (examples: hydroxyapatite, beta-tricalcium phosphate, carbonate apatite), growth factors (examples: bone morphogenetic protein 2 [BMP-2], fibroblast growth factor 2 [FGF-2]) and cell transplantation technology have been developed and applied for tissue regeneration. However, the major problems are the long time (i.e., 3 months) and costs required for bone regeneration. Therefore, solving these problems is an important issue, especially in the current super-aging society.

The co-investigator Hara has been analyzing the very initial process of bone formation based on a Biology-Material Science integrative approach, and has made the following paradigm-shifting discoveries:

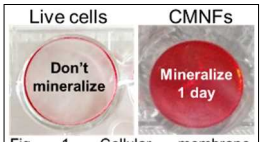

Fig. 1. Cellular membrane nanofragments (CMNFs) can mineralize in just 1 day, but live cells mineralize in 2-3 weeks.

1- Cell (plasma) membrane nanofragments (PMNFs) were the nucleation site for mineral formation *in vivo*. Also, the PMNFs collected from cultured cells were found to mineralize in just 1 day, whereas the conventional methods for *in vitro* mineralization using cells require at least 2 to 3 weeks (**Fig. 1**). These results indicated that PMNFs could be a strong material for bone regeneration. However, since cell membrane contains phospholipids and numerous proteins, there was a need to clarify which factor in the PMNFs was the main mineralizing factor.

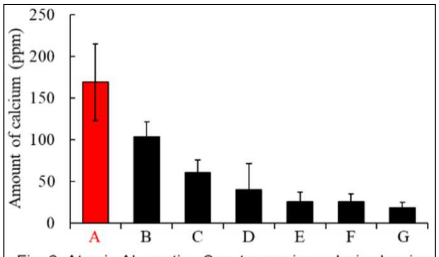

Fig. 2. Atomic Absorption Spectroscopic analysis showing the mineralization ability of different. Note that "phospholipid A" shows the highest mineralization ability.

2- Different phospholipids showed different mineralization efficiencies in *in vitro* experiments. Phospholipid "A" was able to mineralize more than the other phospholipids (**Fig. 2**). These results suggested that phospholipid "A" could be the major phospholipid involved in PMNF mineralization. However, although phospholipid "A" could show rapid mineralization *in vitro*, it did not induce bone regeneration *in vivo* when transplanted to mouse calvarial defects (**Fig. 3**). These results indicated that the mineralization of phospholipids is too weak to promote bone regeneration *in vivo*. Therefore, novel and more robust materials that can mineralize more strongly than phospholipid "A" are extremely on demand.

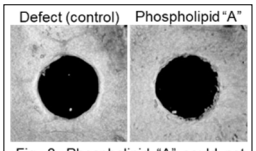

Fig. 3. Phospholipid "A" could not promote bone regeneration.

To elucidate these experimental findings, we analyze the reactions involved in phospholipid mineralization at the atomic/molecular level through Computational Chemistry. We first aim to understand the mechanisms of phospholipid mineralization, and then to design and develop novel phospholipids that can mineralize faster than phospholipid "A", and subsequently, be applied to bone regeneration.

２．研究の目的

The main purpose of this research project was to elucidate the mineralization mechanism of different types of phospholipids (e.g., phosphatidylserine, phosphatidylcholine) at the atomic level using quantum chemistry-based analysis (*in silico*). The results of this study will be important for designing novel phospholipids that can mineralize faster than the other commercially available phospholipids, and for future development of novel materials for bone repair.

３．研究の方法

1- We first assumed that hydrolysis is the first step in the mineralization mechanism because hydroxyapatites, for example, are composed of only phosphate, calcium and water. The phosphate group should probably, therefore, be released from the phospholipid molecules at an early stage of the

process. Detailed analysis of the reaction dynamics of relevant elementary steps in the hydrolysis of phospholipids allows the determination of the activation energy and the amount of energy drop. Detailed knowledge of the dynamics of various chemical events is expected to enable the identification of specific events that occur preferentially, and the sequence in which these events occur.

2- By comparing the dynamics of reactions that different types of phospholipids undergo, differences among various phospholipids can be determined, and the key factors (e.g., amine group, carboxyl group) that lead to these differences can be identified. Identification of the key structural elements is then expected to allow the design and synthesis of novel phospholipid molecules.

3- The quantum chemistry-based analysis of the dynamics of phospholipid reactions is conducted using Gaussian16 quantum chemistry software and a high-performance computer (HPC5000-XCL216TS). The dynamics of reactions as well as the vibrational frequencies of the stationary structures are determined under the B3LYP/6-31G(d,p) level of theory and the accurate activation energies are determined under the APFD/6-311+G(d,p) level of theory. To simulate the reactions in anaqueous solution, the Polarized Continuum Model (PCM) is utilized.

The way different hydrophilic head groups (e.g., serine, choline) and the hydrophobic tail are hydrolyzed is analyzed in detail. Through these comparative analyses, we can determine which head group leads to the easiest phosphate release (lowest activation energies). In this way, structural elements that are advantageous for phospholipid mineralization can be identified.

４．研究成果

The preliminary results shown in Fig. 2 were confirmed by subsequent experiments employing different amounts of phospholipids attached to the wall of the glass-bottom dishes. The amounts of phospholipids dissolved in the chloroform solution were reduced before taking the solution to the dish, so that fewer layers of molecules were expected to approach the glass wall. After evaporation of the chloroform, an aqueous solution containing $CaCl_2$ was supplied to the dish. Because the head part of phospholipids is hydrophilic and the tail is hydrophobic, the head part tends to dissolve in water, while the hydrophobic tail tends to be attached to the glass surface. After incubation of the dishes for 2 days, the amounts of mineralized calcium were measured and it was found that phospholipid “A” in Fig. 2 was phosphatidylserine (PS), “B” was phosphatidic acid (PA), and “C” was phosphatidylcholine (PC).

The phospholipids actually used in the experiments had long carbon chains as hydrophobic tails, but considering that the carbon chains do not participate directly in the reactions, in the simulations the length of carbon chains was restricted to 6 carbon atoms, for simplicity. We adopted molecules of phosphatidylserine (hereafter, PS6) and phosphatidylcholine (PC6) as the reactants because these molecules led to quite different mineralization efficiencies.

1- Hydrolysis of a single phospholipid

The reactions leading to hydrolysis of a single PS6 molecule in an aqueous solution follow four steps, as shown in Fig. 4. Two routes were found: in R1, the P–O bond linked to the serine group is hydrolyzed first, and in R2 the P–O bond linked to the glycerol group is hydrolyzed first. Because the carbon chains linked to the glycerol group are hydrophobic and are therefore expected to be attached to the glass surface, if R1 proceeds first, only the serine group is released to the aqueous solution, while if R2 proceeds first, the whole moiety composed of phosphate and serine is released to the solution.

The first step in each route is the approach of two water molecules to PS6 resulting in the association of an OH group to the central P atom, which becomes pentavalent, and an H atom links to the O atom of a P–O bond. It was found that the hydrolysis of the P–O bond linked to serine/glycerol was easiest when the approaching water molecules came from the side opposite to the serine/glycerol group. Therefore, in the first steps of R1 and R2 in Fig. 4 refer to approach of water molecules from the side opposite to serine and from the side opposite to glycerol, respectively. The product of the first step in R1 and R2 is a pentavalent (PO5) phosphate, in which the leaving groups serine and glycerol, respectively, are at equinox position, and the remaining three O atoms are at equatorial position. This reaction dynamics agrees with the SN2 mechanism of phosphate hydrolysis reported in the literature. The first step has the largest activation energy in each route, indicating that a PS6 molecule would not be hydrolyzed so easily.

In the second step of R1 the hydrolysis of serine proceeds through the migration of an H atom from the NH_3 group of serine to the O atom linked to P (the transition state is shown in Fig. 5, where the dashed bond is the P–O being broken). The H atoms in the NH_3 moiety of serine are loosely bonded to N, and can migrate quite easily towards the P–O bond, leading to the release of serine from the phospholipid.

After serine is released, the third step is a water molecule association step (similar to the first step) to the P atom, from the side opposite to the glycerol group, which occurs following a dynamics similar to that of the first step described above but now the activation energy is slightly smaller and eventually the P–O bond linked to glycerol is hydrolyzed, leading to the formation of H_3PO_4 (phosphoric acid).

In R2, both the first (OH association) and the second (water molecule attack) have activation energies larger than those of the first and second steps of R1, indicating that the hydrolysis of the serine group proceeds first and then the hydrolysis of the glycerol group can follow. In addition, before the fourth step takes place, one of the two OH groups in the PO5 intermediate between TS3 and TS4 of R2 of Fig. 4 needs to turn 180 degrees so that it becomes parallel to the P–O bond linked to glycerol. Because the intermediate is quite unstable, the need for an extra step makes the cleavage of the P–O bond linked to glycerol difficult to proceed. Eventually, phosphoric acid will be formed in the solution, and the moiety composed of glycerol and the hydrophobic carbon chains remains attached to the glass surface.

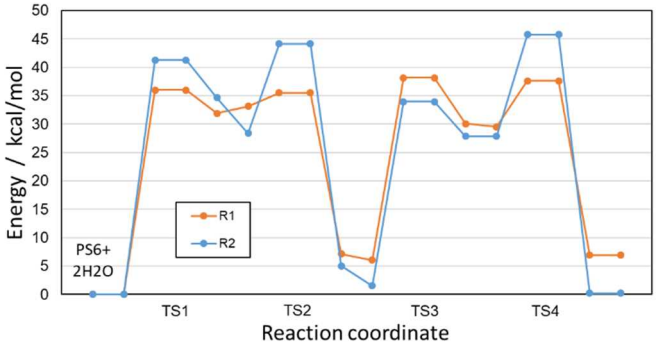

Fig. 4 Energy variation in the hydrolysis of a single PS6

The hydrolysis of a single PC6 molecule follows a reaction dynamic similar to that shown in Fig. 4, with the difference that the activation energy of the first step (OH association to the central P atom) is larger in the case of PC6. The second step of choline or glycerol hydrolysis seems to occur easily, but if choline is released to the aqueous solution first, then the hydrolysis of glycerol becomes very difficult. Therefore, if the bond to glycerol is broken first, releasing the whole choline–phosphate group to the aqueous solution, then the bond to choline can be hydrolyzed afterwards.

2- Hydrolysis of phospholipids in the presence of Ca^{2+} ions

When Ca^{2+} ions are present in large amounts in the aqueous solution, they may link to the O atom in the P=O group of the phospholipids due to electrostatic attraction. In addition, water molecules are expected to surround the Ca^{2+} ion. The hydrolysis reactions of PS6 and PC6 in the presence of Ca^{2+} ions were thus calculated assuming a structure in which a Ca^{2+} ion is linked to P=O and surrounded by two water molecules. Here again the reactions proceed through multiple steps and two different routes, but now either serine, choline, or glycerol can be broken with same easiness. The third step (second OH association) becomes more difficult, though.

3- Hydrolysis of complexes of two phospholipids surrounding a Ca^{2+} ion

In the experimental results, phospholipids and Ca^{2+} ions are expected to form a large network. In order to consider a system in the simulations that is closer to the real situation, we analyzed the hydrolysis reactions of complexes composed of two phospholipids surrounding a Ca^{2+} ion (2PS6Ca and 2PC6Ca). For 2PS6Ca, the reactions that were found to proceed most easily were the subsequent release of the two serine groups. For 2PC6Ca, one choline group was found to be released first, and then the P–O bond linked to glycerol is hydrolyzed at the same P from which choline was released; only after this reaction at the PC6 of the other side of Ca^{2+} take place. The activation energies related to reactions of 2PS6Ca were lower than those of 2PC6Ca, showing that 2PS6Ca react more easily than 2PC6Ca. This agrees with the experimental results which showed that PS had a better mineralization efficiency than PC. H atoms can migrate easily from the NH_3 group of serine, and this seems to be a key factor facilitating the hydrolysis reactions.

After the first hydrolysis reactions, many other chemical events should take place in the process leading to production of precursors of bone formation. We now plan to investigate how the serine or choline groups released to the aqueous solution participate in the chemical reactions that should occur subsequently to hydrolysis.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計9件（うち招待講演　1件／うち国際学会　3件）

１．発表者名 程 雲昊、ハラ エミリオサトシ、国吉 ニルソン
２．発表標題 複数種類のリン脂質分子の加水分解反応における量子化学計算
３．学会等名 日本化学会第１０４春季年会
４．発表年 2024年

１．発表者名 程 雲昊, ハラ エミリオサトシ, 国吉 ニルソン
２．発表標題 複数種類のリン脂質分子の加水分解反応における量子化学計算
３．学会等名 日本化学会第１０４回春季年会
４．発表年 2024年

１．発表者名 Nilson Kunioshi, Emilio Satoshi Hara
２．発表標題 Computational investigations of the formation mechanism of bone-like minerals from phospholipids
３．学会等名 20th International Conference on Crystal Growth and Epitaxy, ICGE20（国際学会）
４．発表年 2023年

１．発表者名 Emilio Satoshi Hara
２．発表標題 Cell membrane as a potential cell-free therapeutic for rapid bone tissue engineering
３．学会等名 XII Latin-American Congress of Artificial Organs and Biomaterials（国際学会）
４．発表年 2023年

１．発表者名
ハラエミリオサトシ

２．発表標題
細胞膜を材料として用いた組織工学アプローチ

３．学会等名
第45回日本バイオマテリアル学会大会

４．発表年
2023年

１．発表者名
程 雲昊、ハラ エミリオサトシ、国吉 ニルソン

２．発表標題
リン脂質分子の石灰化反応における量子化学計算

３．学会等名
第１０３春季年会

４．発表年
2023年

１．発表者名
Hara ES, Anada R, Hatano E, Okada M, Matsumoto T.

２．発表標題
生体内初期石灰化を模倣したセラミックス複合材料の開発とその応用

３．学会等名
第61回セラミックス基礎科学討論会（招待講演）

４．発表年
2023年

１．発表者名
Nilson Kunioshi, , Emilio Satoshi Hara, Reina Kurihara, Takafumi Shiotani, Katsunori Yamaguchi

２．発表標題
Computational analysis of chemical reactions in the mineralization of phospholipids

３．学会等名
15th International Conference on Materials Chemistry (MC15)（国際学会）

４．発表年
2021年

１．発表者名 塩谷 恭史，ハラ エミリオ，山口 勉功，国吉 ニルソン
２．発表標題 量子化学計算によるリン脂質の石灰化機構の解析
３．学会等名 日本化学会第101回春季大会
４．発表年 2022年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

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７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関