[Original report]

Physical properties of the central dark lines in biological apatite of vertebrate calcified tissues and synthetic octacalcium phosphate

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Abstract

Vertebrate teeth and bones consist largely of biological apatite crystals. Octacalcium phosphate (OCP) has been speculated to be a potential candidate for a constituent of the central dark lines in biological apatite crystals. However, if OCP and the central dark line can be shown not to be identical, then some pathway other than the OCP one might operate to produce the apatite crystals. In this study we used transmission electron microscopy and X-ray diffraction to look for possible differences between the central dark line and OCP. X-ray diffraction analysis revealed that the sharp reflection peak of 1.855 nm in d-value, characteristic of OCP, disappeared after the synthetic OCP had been heated up to 150°C for 1 hour. However, electron micrographs provided clear evidence that the central dark lines persisted in the examined biological apatite crystals even after heating at 600°C. These findings indicate that OCP cannot be a candidate for a constituent of the central dark lines in the biological apatite crystals in vertebrate teeth and bones.

Key words: apatite crystal; central dark line; electron microscopy; octacalcium phosphate; X-ray diffraction analysis.

Introduction

Teeth and bones in vertebrates are calcified tissues consisting largely of apatite crystals, also called "carbonated apatite crystals". Under the electron microscopy, biological apatite crystals show dark linear pattern, known as the central dark line, which is recognized as the platy nuclei of carbonated apatite crystals in several calcified hard tissues (Marshall and Lawless, 1981; Nakahara, 1982; Nakahara and Kakei, 1983; Cuisinier et al., 1992; Kakei et al., 2000, 2001). This line is also called the central electron-opaque line, dark contrast line (Rönnholm, 1902; Nylen, 1964; Frazier, 1968, Bris et al., 1986), or central planar defect (Nelson and Mclean, 1984; Nelson et al., 1986, 1989).

Octacalcium phosphate (OCP) has been widely accepted to act as a precursor for the apatite crystal formation in all calcified vertebrate tissues including pathologic mineralization (Brown et al., 1962, 1981, 1987; Wiess et al., 1981; Nelson and Mclean, 1984; Nelson et al., 1986, 1989; Chickerur et al., 1987; Iijima et al., 1991, 1992; Maike et al., 1993). Also, some investigators have considered that the central dark line might consist of a single unit cell thickness of OCP embedded in an apatite matrix in mature crystals (Brown et al., 1962, 1981, 1987; Wiess et al., 1981; Chickerur et al., 1987; Nelson et al., 1989; Iijima et al., 1991, 1992; Maike et al., 1993). In other words, a remnant of OCP might be responsible for the formation of the central dark line (Brown et al., 1962, 1981, 1987; Chickerur et al., 1987; Wiess et al., 1981; Nelson and Mclean, 1984; Nelson et al., 1986.)

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1989; LeGeros et al., 1989; Iijima et al., 1991, 1992; Miale et al., 1993). In addition, a preferential dissolution is said to take place at the central region of apatite crystal containing the central dark line due to the higher dissolution rate of OCP compared to that of apatite (Nelson et al., 1986, 1989). Furthermore, OCP shows sensitivity to electron-beam bombardment, as does the central dark line (Nelson and McLean, 1984). As a consequence, synthetic apatite crystals with an OCP lattice in the center of the crystal might serve as a reference for crystals in vertebrate hard parts due to the morphological similarity (Nelson et al., 1989; Iijima et al., 1991, 1992; Miale et al., 1993). However, the nature of the central dark lines has still remained unresolved, although many researchers have regarded the appearance of this line to be an important step for the apatite formation in vertebrate teeth and bones.

Because OCP is structurally unstable, the X-ray diffraction pattern of OCP was reported to disappear at approximately 180°C (Brown et al., 1962, Bigi et al., 1990). Based on infrared spectra analysis, it was suggested that the existence of OCP was difficult after heating at approximately 400°C (Fowler et al., 1966; Bigi et al., 1990). On the other hand, the disappearance of the central dark lines in apatite crystals seems to occur only at relatively high temperatures (unpublished data). Whereas it is almost impossible to analyze the constituents of the central dark line directly, this condition led us to believe that a thermal treatment would be an effective means to decisively distinguish OCP from the central dark line. Therefore, to determine whether OCP is a candidate for a constituent of central dark lines in biological apatite crystals, we sought to demonstrate a difference in the physical properties between OCP and the central dark line.

Materials and methods

The experimental use of animals was approved by the Institutional Animal Care and Use Committee at Meikai University. Bone and enamel samples were dissected from anesthetized adult Sprague-Dawley rats, and the surrounding soft tissues were removed. After having been dried at room temperature, samples were kept in a plasma reactor (Yamato) at 270 W, 90 cm² of oxygen current per minute, for 20 hours in order to eliminate organic substances. Each sample for electron microscopy was heated at 400°C, 500°C, 600°C or 620°C for 1 hour in a muffle furnace (MF-02, YUNIKA) controlled within ± 2°C. Synthetic OCP was prepared using the method described by LeGeros (1988), and then heated at 150°C, 200°C, 300°C, 400°C, 500°C or 600°C for 1 hour in the muffle furnace. These samples were then subjected to X-ray diffraction analysis for assessing the effects of heat on the crystals.

X-ray powder diffraction analysis was carried out by using a RINT-2000 Rotoflex (Rigaku) under the following conditions: copper (Cu) target; accelerating voltage of 40 kV; 200 mA beam current; “auto” for graphite monochromator (slit system); scanning speed of 2 degrees (2θ) per minute; and scanning range from 4.0 to 60.0 degrees 2θ.

Samples without fixation were directly dehydrated by the passage through a series of ascending concentrations of ethanol, and then embedded in Araldite 502 according to the method described by Kakei et al. (2001). Blocks were cut with a diamond knife on a Porter Blum MT2 ultramicrotome (SORVALL). Thin sections having a dark gray to silver interference color (approximate thickness of 50 to 60 nm) were mounted on uncoated fine grids (400 mesh) and examined under a JEM 100CX electron microscope (JEOL) operating at an accelerating voltage of 80 KV.

Results

X-ray diffraction patterns of synthetic OCP, bone, and enamel are shown in Figure 1. The synthetic OCP before thermal treatment (Fig. 1) showed a sharp reflection at approximately 1.855 nm in d-value, which was assumed to indicate the existence of OCP in this sample. This sharp reflection completely disappeared by heating at 150°C for 1 hour, and substitutively appeared a new reflection of 1.705 nm (Fig. 1b). This reflection then shifted to 1.566 nm after heating at 200°C (Fig. 1c), and the peak intensity was small compared with that of 1.855 nm before heating (Fig. 1a-c). This reflection then disappeared upon heating up to 400°C, suggesting that the synthetic OCP had decomposed completely (Fig. 1d). On the other hand, although the reflection patterns ranging from 20.0 to 40.0 degrees in 2θ were broad at 150°C (Fig. 1b), the samples heated at over 200°C revealed a decrease in OCP crystallinity.
Fig. 1. X-ray diffraction analysis of the effect of heat treatment on the different materials (synthetic OCP, bone apatite, enamel apatite). A sharp reflection peak of 1.855 nm in d-value, characteristic of OCP, is observed in synthetic OCP without heat treatment. Heating of OCP over 150°C results in progressive alteration of OCP. a, OCP without heat; b, OCP heated at 150°C; c, at 200°C; d, at 400°C; e, at 600°C; f, bone apatite after plasma ashing; g, enamel apatite after plasma ashing.

from the unheated state, suggesting that a shift from OCP to crystalline products had taken place (Fig. 1c, d). The X-ray diffraction pattern of synthetic OCP heated from 200 to 400°C (Fig. 1c, d) indicated a poor crystallinity resembling that of bone more than that of enamel (Fig. 1f, g). However, the degree of crystallinity was increased by the heating treatment. Also, a reflection peak of approximately 0.301 nm in d-value became distinct when the sample was heated at 500°C (data not shown), and this peak became more apparent after heating at 600°C (Fig. 1e).

Electron micrographs (Fig. 2a-c) clearly showed that the central dark lines remained in both enamel and bone apatite crystals heated at 400°C, 500°C or 600°C, although the enamel apatite crystals seemed to be more sensitive than those of bone to heating. In addition, in the case of bone samples (Fig. 2d), the presence of central dark lines was recognized in apatite crystals heated at 620°C. However, it was difficult to observe the central dark lines in the apatite crystals heated over 625°C, suggesting the thermal vanishing point for the central dark lines in biological apatite crystals to be approximately 625°C.

Discussion

In the present study, the x-ray pattern of synthetic OCP showed a sharp reflection peak of 1.855 nm in d-value (Fig. 1a), which is consistent with a previously reported characteristic of OCP (LeGeros, 1974). The appearance of reflection peaks of 1.705 nm by heating at 150°C and 1.566 nm at 200°C suggests that a collapse of OCP might have taken place due to the emission of water molecules from the OCP structure caused by heating up to these temperatures. The sharp reflection peaks found in the range from 20.0 to 40.0 degrees in 2θ seemed to
show broader peaks when OCP was heated to 150°C (Fig. 1b), and these peaks resembled those of bone after OCP had been heated to over 200°C (Fig. 1c). This broadening of peaks caused by heating at 150°C is considered to reflect the transitional phase toward the apatitic form, which contains OCP-derived products such as defective apatite and pyrophosphate (Brown et al., 1962; Fowler et al., 1966). Furthermore, the reflection peak at approximately 0.301 nm might indicate the formation of pyrophosphate derived from OCP collapse by heating at over 400°C (Fig. 1e), based on the results of a preliminary experiment (data not shown). Judging from the disappearance of the peak at 1.855 nm, a large shift in the pattern of synthetic OCP was expected to take place at approximately 200°C (Fig. 1a-c). On the other hand, our electron microscopic observations revealed that the central dark lines in the biological apatite crystals remained visible even at 600°C for enamel and 620°C for bone (Fig. 2). From these findings, it is clear that the temperature for thermal alteration of OCP was relatively lower than the thermal vanishing point of the central dark lines in biological apatite crystals, suggesting that the physical properties of the central dark lines are different from those of OCP. Regarding the crystal structure, it has been thought that a single unit cell thickness of OCP shows somewhat wide and opaque lattice image and might contribute to a structure consisting of two alternating layers resembling the apatitic layer separated by water molecules (Brown et al., 1962; Bigi et al., 1990; Fowler et al., 1993). Thus, this structure produces a layer of two unit cells of apatite by hydrolysis (Nelson et al., 1986; 1989). However, we have never obtained any ultrastructural evidence for this kind of phenomenon (Nakahara and Kakei, 1983). Therefore, it is difficult to consider that the central dark line may consist of a single unit cell thickness of OCP embedded in an apatite matrix in mature crystals. In addition, although the electron-beam damage to and preferential dissolution of the central dark lines have been interpreted to result from the instability of OCP (Nelson et al., 1986), it may be difficult to consider that embodiment of OCP within apatite crystals would change its heat resistance and preferential dissolution. On the other hand, Raman analyses have afforded speculation that some kind of magnesium-carbonate compound, such as huntite or other minerals, would be formed during the nucleation stage (Casciani et al., 1979; Kakei et al., 1997), suggesting that the central dark line might consist of different mineral constituents.

We also note that the central dark lines in bone crystals showed a little more resistance against heating than those in enamel. This difference might be that the central region where the central dark line is sandwiched in the enamel crystallite is easily affected by electron-beam bombardment, resulting in the so-called “white spots” or “translucent spots” (Marshall and Lawless 1981; Bris et al., 1986). This, in turn, suggests that heat-induced damage to the central dark lines might occur at a lower temperature in enamel than in bone. In addition, the enamel samples used in this study were obtained from the immature region. Thus, these factors might contribute to the lower heat resistance of the central dark lines in the enamel crystallites.

Also, some researchers have pointed out the relationship between the central dark line and an electron-lucent line, the latter describing the lucent-zone as “lucent-line” which corresponds to the central dark line (Nakahara and Kakei, 1983; 1989). Although this lucent-zone appeared as an electron-lucent line in stained sections owing to its width of only 1 nm, it has been described as an unclear feature surrounded by organic substance in the initial mineralization (Nakahara and Kakei, 1983; 1989). This zone was described as showing no lattice image in unstained sections at the earliest stage of apatite formation, and the first sharp lattice line is thought to be created within this unclear feature as the first sign of platy nuclei, which would later become the central dark line (Nakahara and Kakei, 1983; 1989). Thus, we consider it unlikely that this electron-lucent zone would be the same substance as the central dark line. From the present findings, we conclude that the central dark line in biological apatite crystals may not contain OCP and suggest that a pathway other than the OCP one exists in vertebrate dental and skeletal systems (Kakei et al., 1997).

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References


