NEXAFS study of carbonate substituted bioapatite

O V Petrova¹, K A Bakina¹,² and H Ehrlich³

¹Komi Science Centre Ural Division of the Russian Academy of Sciences, 167982, Syktyvkar, Russia
²Pitirim Sorokin Syktyvkar State University, 167001, Syktyvkar, Russia
³Institute of Electronic and Sensor Materials, TU Bergakademie Freiberg, 09599, Freiberg, Germany

E-mail: teiou@mail.ru

Abstract. The investigation of unique carbonate substituted bioapatite of Champsocephalus gunnari icefish jaw and skull bones was carried out using NEXAFS spectroscopy. It has been established that these bones contain the B-type carbonate substituted hydroxyapatit with a content [CO₃]²⁻ anion of about 0.79-3.07 wt.%.

1. Introduction

The bone is a multi-level hierarchical structured biomineral composite which includes organic, mainly collagen (more than 90%) and inorganic components (bioapatite) together with lipids and water. The bone is characterized by a high content of the mineralized phase, for vertebrates it is about 66% of the weight and 50% of the volume, while the percentage of the mineralized component in fish bones compared to mammals remains to be significantly less, thus fish bone have less density. The ratio of the organic and inorganic components of mineralized tissues determines their physical properties (hardness, strength and elasticity) [1-3]. The base of vertebrates hard tissue is poorly crystallized bioapatite, with the individual crystallites size in on the order of 50*25*5 nm and low concentration of the hydroxyl group [OH] compared to the abiotic crystalline apatite [1].

In hard tissues (bone, dentin and enamel) of mammals and prevailing number of fish, bioapatite is represented as hydroxyapatite Ca₁₀(PO₄)₆(OH)₂ and its carbonate substituted forms. According to 3D-super-lattise model of bone the hydroxyapatite nanocrystals are separated one from other by hydrate nanolayers with dissolved OH⁻, [PO₄]³⁻ and [CO₃]²⁻ anions and Ca²⁺ dications. Bioapatite nanocrystals separated by the aqueous solution, form conglomerates of coplanar assembly which grow around collagen fibers [4]. Another basic component of bioapatite is amorphous calcium phosphate [5].

It is generally accepted that carbonate substituted forms of hydroxyapatite classified as either A-type where the [CO₃]²⁻ replace the [OH] group, or B-type with replacement of [PO₄]³⁻. Previous reports demonstrated the prevailing presence of B-type substitution in bioapatites [6-8]. Depending on the type of mineralized tissue and its functions in the living organism, the [CO₃]²⁻ content in bioapatite varies from 1 to 9 wt.%. Despite the fact that interaction between the protein and mineral component in bone tissue is carried out by substitution of the carbonate group [CO₃]²⁻ on the phosphate anion [PO₄]³⁻ and the formation of B-type carbonate-substituted hydroxyapatit, the role of [CO₃]²⁻ ion implantation during bioapatite mineralization remains unclear up to now. The presence of carbonate groups in the bioapatite increases its reactivity and solubility, which directly affect the processes of re- and...
demineralization of solid tissues [2,9]. Thus the carbonate concentration and the ratio of carbonate 
[CO$_3^{2-}$] to phosphate [PO$_4^{3-}$] anion concentration are important parameters that are characterized 
bioapatite’s with functional differences [2]. Moreover these parameters are differ with the age 
of biomaterial and play an important role in the study of fossil bone remains [10-12]. 

Due to the complexity of the [CO$_3^{2-}$] anions quantitative determination, the B-type carbonate 
substituted hydroxyapatite is characterized by calcium to phosphorus atomic proportion (apatite 
module) [1,11]. However, the NEXAFS spectroscopy allow reliably to determine the presence of 
anion [CO$_3^{2-}$] in the hard tissue bioapatite by the characteristic high-intensity $\pi^*$-resonance in the 
absorption C1s-spectrum and determine the [CO$_3^{2-}$] and [PO$_4^{3-}$] anions concentration and its ratio. 
Moreover NEXAFS sensitive to atomic and chemical composition and allow to indicate the 
correlation between bioapatite hierarchical organization and local electronic structure of its mineralized 
phase [4,12,13]. Our preliminary studies provide strong evidence that NEXAFS spectroscopy is the 
most suitable method to examine structural changes and differences of organic and inorganic 
functional groups within biomaterials [14-16]. 

The present work continuation of the study of Champsocephalus gunnari (C. gunnari) icefish hard 
tissues [3,12] and aimed at determining the [CO$_3^{2-}$] and [PO$_4^{3-}$] concentration, its ratio and content of 
[CO$_3^{2-}$] group in bioapatite of C. gunnari icefish jaw and skull bones.

2. Experimental details

2.1 Sample preparation

For the investigation of bioapatite structure and composition selected jaw and skull bones of C. 
gunnari icefish together with the calcite CaCO$_3$ as a control sample were taken. The detailed 
description of C. gunnari icefish bone formation and structure peculiarities was published earlier in 
[3,12]. The preliminary treatment of selected icefish bones were carried out into 6 % H$_2$O$_2$ solution for 
24 hours with subsequent mechanical separation from muscles. For finalize cleaning of the hard 
tissues from attached soft tissues the samples, the ultrasonic bath with 15 % aqueous solution of 
NaOH has been used for 2-4 hours at room temperature. Finally the samples were washed in distilled 
water and then dried at 50°C on air. The samples for NEXAFS measurements were prepared ex situ in 
air by pressing of powdered investigated matter into the clean copper plate surface.

2.2. NEXAFS spectroscopy study

NEXAFS of the powdered samples under study were carried out using monochromatic synchrotron 
radiation at the RGBL BESSY-II [17]. All absorption measurements were performed under vacuum 
conditions 10$^{-9}$ torr in the experimental chamber. NEXAFS-spectra were measured by the total 
electron yield mode (TEY) with an energy resolution about 0.01eV. NEXAFS spectra were collected 
by TEY-signal monitoring by the drain current detection with Keithley 617 microammetr. The spectra 
were normalized to the incident photon flux, which was defined as a ratio between TEY-signal from a 
clean Au-plate. The suppression and estimation of the nonmonochromatic background in TEY-signal 
was carried out by absorption Ti-filter method [18,19].

3. Results and discussion

The [CO$_3^{2-}$] anions concentration in C. gunnari icefish bones were examined by NEXAFS C1s-
spectrum analysis. It is known that some of fish species hard tissue contain mineralized phase in the 
form of fluoroapatite. However the neither of measured NEXAFS F1s spectra of investigated icefish 
bones indicates the presence of fluoride. This fact suggests that C. gunnari icefish include bioapatite in 
the form of hydroxyapatite. Moreover this fact was confirmed by NEXAFS P2p and Ca2p spectra 
analysis discussed in our previous studies [3,12]. 

NEXAFS C1s-spectra of selected C. gunnari icefish bones and CaCO$_3$ are presented in Figures 1 
and 2. All bone spectra demonstrate identical features. The peaks at 285.0 eV and 285.4 eV 
correspond with transitions from C1s-edge to $\pi^*$-unoccupied orbitals of benzol and phenol aromatic
cycle, respectively [20]. The structure between 287.3 eV and 288.6 eV is presumably connected with single, double and epoxy bonds of carbon and oxygen atoms [16]. The presence of mentioned spectral features assume the small amount of contamination in the samples.

Figure 1. NEXAFS C1s-absorption spectra of selected C. gunnari icefish jaw and skull bones and calcite CaCO3

The presence of relatively small resonance at 290.2 eV in bone spectra have the essential meaning and indicates the presence of substituted [CO3]2− in biological hydroxylapatite. The ratio α of the square of peaks associated with [CO3]2− anion in NEXAFS C1s-spectra of bones and pure CaCO3 corresponds to the ratio of [CO3]2− anion concentrations in the samples.

The concentration nCO3 of [CO3]2− anion in CaCO3 and nPO4 of [PO4]3− anion in hydroxylapatite was calculated as: \( n_{\text{CO}_3} = \frac{\rho_{\text{CaCO}_3} N_A}{M_{\text{CaCO}_3}} \) and \( n_{\text{PO}_4} = \frac{\rho_{\text{HAP}} N_A}{M_{\text{HAP}}} \), where \( \rho_{\text{CaCO}_3} = 2.71 \text{ g/cm}^3 \) and \( \rho_{\text{HAP}} = 3.19 \text{ g/cm}^3 \) – average specific gravity of calcite and apatite taken from Mineralogy Database [20], \( M_{\text{CaCO}_3} \) and \( M_{\text{HAP}} \) – molecular weights of calcite and hydroxylapatite and \( N_A \) – Avogadro constant. Using this data the concentration \( n'_{\text{CO}_3} \) of [CO3]2− anion in carbonate substituted bioapatite of bone samples can be expressed as \( n'_{\text{CO}_3} = \alpha n_{\text{CO}_3} \) and the concentration \( n'_{\text{PO}_4} \) of [PO4]3− anion in carbonate substituted bioapatite as \( n'_{\text{PO}_4} = n_{\text{PO}_4} - \alpha n_{\text{CO}_3} \). In the next step the ratio \( \beta = \frac{n'_{\text{CO}_3}}{n'_{\text{PO}_4}} \) of [CO3]2− and [PO4]3− anion concentration and \( \omega_{\text{CO}_3} \) – content of [CO3]2− anion in wt.% in carbonate bioapatite of C. Gunnari icefish bones can be determined. The correlation between \( \beta \) and \( \omega_{\text{CO}_3} \) is:

\[
\frac{1}{\omega_{\text{CO}_3}} = \frac{M_{\text{HAP}}}{6M_{\text{CO}_3}} \cdot \frac{1 + \beta}{\beta} \cdot \frac{M_{\text{PO}_4}}{M_{\text{CO}_3}} + 1, \tag{1}
\]

where \( M_{\text{CO}_3} \) and \( M_{\text{PO}_4} \) are the molecular weights of [CO3]2− and [PO4]3− anion respectively.

The Table 1 contains determined for investigated C. gunnari icefish bones \( \alpha \), \( \beta \) and \( \omega_{\text{CO}_3} \) coefficients. In accordance with our approximations and measurements the \( \omega_{\text{CO}_3} \) content of [CO3]2− anion in carbonate substituted bioapatite of C. gunnari icefish jaw and skull bone is 0.79 and 1.85 wt.%, respectively. The obtained data are well correlated with literature. Some inconsistencies can be
associated with the use of average specific gravity of apatite $\rho_{\text{HAP}} = 3.19 \text{ g/cm}^3$ taken from Mineralogy Database [21], although it is known that the specific gravity of the carbonate substituted biapatite is less than one used in calculation. More other the $\alpha$ and $\beta$ ratio depend not only on the specific gravity but also on the content of amorphous calcium phosphate and aqueous solution of OH$^-$, [PO$_4$$^3-$] and [CO$_3$$^2-$] anions and Ca$^{2+}$ dications.

### Table 1. Characteristics of *C. gunnari* icefish bones

| Sample       | $\alpha$, % | $\beta$, % | $\omega_{\text{CO}_3}$, wt% |
|--------------|-------------|------------|-----------------------------|
| Jaw bone     | 1.54        | 2.26       | 0.79                        |
| Skull bone   | 3.59        | 5.41       | 1.85                        |

### 4. Conclusion

The comparative analysis of NEXAFS C1s-adsorption spectra of selected *C. gunnari* icefish jaw and skull bones shows that the bones contain the B-type carbonate substituted hydroxyapatite. The presence of anion [CO$_3$$^2-$] was clearly determined by the characteristic $\pi$-resonance in the absorption C1s-spectrum of bones. The quantitative assessment of ratio of peaks area associated with [CO$_3$$^2-$] in bones and CaCO$_3$ NEXAFS C1s-spectra allow to determine the relative concentration of [CO$_3$$^2-$] and [PO$_4$$^3-$] anions and the $\omega_{\text{CO}_3}$ content of [CO$_3$$^2-$] anion in carbonate substituted hydroxyapatite of *C. gunnari* icefish jaw and skull bones. The estimated $\omega_{\text{CO}_3}$ content is 0.79 and 1.85 wt.% for jaw and skull bone samples, respectively. The obtained data are well correlated with literature, but they can be slightly distorted due to the usage of the average specific gravity of apatite in the calculation.

### Acknowledgment

This work was supported by the Bilateral Program of the RGGI at BESSY II, RFBR and the Komi Republic No 20-42-110002 and grant of the President of Russian Federation No MK-3796.2021.1.2.

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