Bone diagenesis: New data from infrared spectroscopy and X-ray diffraction

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Abstract

This paper combines non-destructive high-resolution Fourier transform infrared spectroscopic techniques (attenuated total reflectance in the mid-infrared — ATR, and diffuse reflectance in the near-infrared — NIR) with X-ray diffraction and Rietveld analysis, in the study of bone diagenesis. Sixty fossil bones from two Upper Miocene sites in Greece (Pikermi and Chalkoutsi) and one Upper Pleistocene site in Cyprus (Aghia Napa) are investigated in comparison to various mineral and biological apatites. Diagenetic trends, common to all these sites include a subtle but systematic decrease of the unit cell volume and α-axis of carbonate hydroxylapatite, as well as a parallel increase of the coherence length along the c-axis. Chemometric modelling reveals that the changes in the unit cell and the coherence length are highly correlated to (and can be predicted on the basis of) the ATR spectra. Besides using chemometrics as a convenient predictive tool, we have been able to identify that the correlation with the XRD data is primarily based on the intensity of infrared bands at 577, 865 and 1092 cm⁻¹, as well as on the position of the ν₃ phosphate mode at ca. 960 cm⁻¹. These structural changes constitute the vibrational signature of diagenesis throughout our set of bone samples and can be accounted for by the stabilization of a distorted CO₃²⁻ species in the B-sites of apatite, and to a lesser extent by the substitution of OH⁻ by F⁻. NIR spectroscopy allowed for the identification of a well-defined H₂O species, absorbing at 5318 and 7240 cm⁻¹. This species is labile, appears to characterize mostly biogenic apatite, and is therefore considered to be chemisorbed on the surface of the crystallites.

1. Introduction

Bone diagenesis leading to the preservation of skeletal material over geological time is a highly complex phenomenon involving the physical, chemical, histological and mechanical alterations that occur at different time scales from the time of death and depend on the local geochemical conditions (Clarke and Barker, 1993; Hedges and Millard, 1995; Hedges, 2002; Reiche et al., 2003; Trueman et al., 2004). The geochemical aspects of bone diagenesis have been studied by a variety of physicochemical techniques including optical and electronic microscopy (Jans et al., 2004), X-ray diffraction (Chipera and Bish, 1991; Person et al., 1995), vibrational spectroscopy (Surovell and Stiner, 2001; Lee-Thorp and Sponheimer, 2003) and chemical analysis (Trueman and Turross, 2002). Bone itself is a heterogeneous, composite material, constructed from an intimate association of collagen fibres and plate-like inorganic crystallites (Weiner and Price, 1986; Weiner and Traub, 1992). The latter are analogous to poorly crystallized carbonate hydroxylapatite, and similar to the mineral earlier known as “dahelite” (Ca₁₀(PO₄, CO₃)₆(OH)₂)(Posner, 1985; Person et al., 1995). In turn, apatite is a very stable, yet versatile, hexagonal crystal (PO₄/m, a=9.4 Å, c=6.9 Å, Vcell=530 Å³) with many non-biogenic, mineral or synthetic, members (White et al., 2005).

In chemical terms, bone diagenesis has been associated with a variety of mostly anionic substitutions (e.g. halide for OH⁻; CO₃²⁻ for PO₄³⁻ or OH⁻) as well as with changes in crystallinity (Shemesh, 1990; Surovell and Stiner, 2001; Trueman et al., 2004). Despite serious research efforts, a detailed scenario for bone diagenesis remains elusive. Often, the proposed models are based on limited numbers of specimens from very specific localities, or lack a broader interdisciplinary perspective.

This work contributes towards understanding bone diagenesis over a large collection of fossilized specimens from three locations of different geological age in Greece and Cyprus. All samples reported here are taken from long bones, while a broader study including dental tissues from the same sites is in preparation. The fossilized specimens as well as a set of mineral and biogenic reference compounds are studied by new crystallographic and vibrational techniques. Emphasis is given in the identification of common structural features underlying bone diagenesis in the three sites...
investigated, and the establishment of correlations between chemical bonding and unit cell parameters.

2. Materials and methods

The material used in this study, consists of sixty fossil bones (mainly metapodials) from older and recent excavations in two Upper Miocene (Pikermi and Chalkoutsi, Greece) and one Upper Pleistocene sites (Aghia Napa, Cyprus). The material coming from the fluvial deposits of Pikermi and the (currently coastal) area of Chalkoutsi represent the fossil genus Hipparion (Koumantakis, 1971; Marinos and Symeonidis, 1973) and belongs to the collections of the Palaeontology & Geology Museum of Athens University. The Aghia Napa material, coming from a semi-collapsed karstic shelter, is from the collections of the Geological Survey of Cyprus and consists of long bones of Phanourios minor, the dwarf hippopotamus of Cyprus, which lived on the island ca. 11,000–13,500 years ago (Theodorou et al., 2004; Stathopoulou, 2005). Comparison with other types of apatite, both mineral and biological is based on samples of relatively fresh bovine bone, bone meal (NIST Standard Reference material 1486), mineral carbonated fluorapatite (earlier known as “francolite”) from the phosphate ore deposit of Epirus, Greece (Perdikatis, 1991), two samples of the mineral fluorapatite (Durango – Mexico and Baikal – Siberia), a phosphate rock (NIST Standard Reference material 120C), a biological fluorapatite, as well as various calcites isolated from the bone cavities. All samples were measured as powders with no pre-treatment.

X-ray diffraction measurements were performed on a SIEMENS D500 Diffractometer with a secondary graphite monochromator and CuKa radiation. Slits of 1°/1°/1° at aperture diaphragm positions and 0.15°, 0.15° at detector and diffracted beam monochromator positions respectively (step 0.03°, time 5 s/step, 2 theta range 18°–90°) were employed. The Rietveld method (FULLPROF program) was used in order to calculate the crystal structure parameters and analyze the anisotropic broadening observed in the Bragg peaks of hydroxylapatite, which was the main phase observed (Rodriguez-Carvajal et al., 1991). Instrumental broadening effects were calibrated via a LaB6 standard (The Gem Dugout).

The mid-infrared spectra of the powders over the range 525–4000 cm⁻¹ have been measured on a Fourier transform instrument (Equinox 55 by Bruker Optics) equipped with a single reflection diamond ATR accessory (Duramap S3R by SensIR). Each spectrum represents the average of 256 scans at a resolution of 1 cm⁻¹ and a zero-filling factor of 2. ATR is characterized by a (frequency dependent) penetration depth of several μm. It is therefore ideal for the acquisition of infrared data of fine powders without distortions due to optical saturation and dispersion, and free of chemical alterations that can possibly occur in contact with a KBr matrix (hydration, ion-exchange etc). A critical comparison of ATR to the more commonly used transmission techniques in the study of minerals can be found in Madejová and Komadel, 2001.

The NIR spectra of the same samples have been measured via a fiber optic bundle probe operating in the diffuse reflectance mode over the frequency range 4000–8000 cm⁻¹ on a Fourier transform instrument (Vector 22 N by Bruker Optics). For the study of time dependent phenomena, an integrating sphere accessory has been used, equipped with a chamber for controlled atmosphere. The NIR spectra represent averages of 100 scans at a resolution of 8 cm⁻¹. Savitzky–Golay 2nd derivative analysis of the ATR and NIR spectra (instead of background subtraction and peak fitting) has been tuned in order to enhance the resolution of sharp features overlapping with broad bands (Leung et al., 1990). Typically, at a resolution of 1 cm⁻¹ and a zero-filling factor of 2, smoothing over 9 or 13 points is optimal for observing the sharpest features of the apatite spectra ($\nu_1$ of PO₄³⁻ or $\nu_2$ of CO₃²⁻). Broader features ($\nu_2$ PO₄³⁻, CO₃²⁻) require lower resolution and derivation over broader regions.

### Table 1

Unit cell parameters and fluorine content of the reference materials used in this work

<table>
<thead>
<tr>
<th>Sample type</th>
<th>a-axis (Å)</th>
<th>c-axis (Å)</th>
<th>Vₚₑₑ (Å³)</th>
<th>F (wt.%)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA-1 (FAp)</td>
<td>9.3851</td>
<td>6.8688</td>
<td>525.32</td>
<td>1.92</td>
<td>This study</td>
</tr>
<tr>
<td>FA-2 (FAp)</td>
<td>9.3916</td>
<td>6.8831</td>
<td>525.76</td>
<td>2.02</td>
<td>This study</td>
</tr>
<tr>
<td>&quot;Francolite&quot; (CO₃–FAp)</td>
<td>9.3235</td>
<td>6.8971</td>
<td>519.22</td>
<td>3.71</td>
<td>This study</td>
</tr>
<tr>
<td>NIST 1486 (bone meal–Hap)</td>
<td>9.4264</td>
<td>6.8961</td>
<td>530.64</td>
<td>800 µg/S⁺</td>
<td>This study</td>
</tr>
<tr>
<td>NIST 120C (phosphate rock–FAp)</td>
<td>9.3429</td>
<td>6.8961</td>
<td>521.32</td>
<td>1.82</td>
<td>This study</td>
</tr>
<tr>
<td>LP-1 (biological CO₃–FAp)</td>
<td>9.354</td>
<td>6.9067</td>
<td>523.35</td>
<td>3.15</td>
<td>This study</td>
</tr>
<tr>
<td>Hap</td>
<td>9.422</td>
<td>6.8839</td>
<td>529.17</td>
<td></td>
<td>Sudarsanan and Young (1978)</td>
</tr>
<tr>
<td>Hap</td>
<td>9.432</td>
<td>6.881</td>
<td>530.14</td>
<td></td>
<td>Kay and Young (1964)</td>
</tr>
<tr>
<td>&quot;Francolite&quot; (B-CO₃–FAp)</td>
<td>9.3248</td>
<td>6.9002</td>
<td>519.59</td>
<td></td>
<td>Mackie and Young (1974)</td>
</tr>
<tr>
<td>ClOHAp</td>
<td>9.541</td>
<td>6.838</td>
<td>539.07</td>
<td></td>
<td>Sudarsanan and Young (1978)</td>
</tr>
</tbody>
</table>

* Data from the NIST standard reference material reports.
Chemometric modeling by a mean-centered partial least squares algorithm (PLS1) has been performed with routines available in the OPUS QUANT software by Bruker Optics, and validated by the leave-one-out technique (Geladi and Kowalski, 1986).

Supplementary determinations of fluorine content on selected powdered samples subjected to pyrohydrolysis have been performed at the Natural History Museum in London by ion chromatography (Dionex DX300).

3. Results

The unit cell parameters of the reference samples, derived from the Rietveld analysis of the X-ray diffraction data are shown in Table 1. Plotting these data according to Handschin and Stern (1992) and including the unit cell parameters of the fossilized samples (Fig. 1), allows for the following comments:

a) Structural variations are manifested in the linear dependence of the $a$-axis length on the unit cell volume ($V_{cell}$). The $c$-axis shows no systematic variation.

b) All fossilized samples fall between the NIST 1486 bone meal standard, which is essentially a hydroxyapatite on the high $V_{cell}$ side, and the fluorinated and/or B-type carbonate apatites on the low $V_{cell}$ side. Chlorinated as well as A-type carbonate apatites exhibit very large values of $V_{cell}$ (Table 1). Note that the distribution of fossilized samples is much narrower than that mapped by the reference apatites, indicating that the effect of diagenesis on the unit cell dimensions is subtle.

c) The three fossiliferous sites are mapped as overlapping distributions of $(a, c, V_{cell})$ varying in the range (9.394, 6.895, 527.3)–(9.411, 6.899, 528.4) for Pikermi, (9.403, 6.897, 528.3)–(9.416, 6.90, 529.6) for Chalkoutsi and (9.41, 6.892, 529.2)–(9.425, 6.904, 530.1) for Aghia Napa.

Note that decreasing $V_{cell}$ parallels increasing F content in the reference materials (Table 1), while the corresponding analysis of the fossilized bones indicates that the Aghia Napa samples have very low fluorine contents (average value 0.16 wt.% F). Chalkoutsi and Pikermi samples contain 1.13±0.2 wt.% F and cannot be distinguished from each other.

In Fig. 2 (top) we show the absorption spectra of a representative fossilized bone over the NIR (left) and ATR (right) spectral ranges. Five frequency ranges are detailed in the lower part of Fig. 2, where all the
spectra are shown in the 2nd derivative mode, grouped per site. The spectra are not scaled on the y-axis but are shown offset for clarity. From right to left, these ranges correspond to a) the ν4 modes of the PO43− units, b) the ν2 modes of the carbonate radicals, c) the ν1 and ν3 modes of PO43−, d) the stretching–bending combination mode of H2O species, and e) the stretching overtone of the OH− intermediate frequency component at 577 cm−1. For the following observations.

Bands due to the PO43− units appear weaker and poorly defined in the Aghia Napa samples, presumably due to their higher bandwidth. However, the intensities of the bands attributed to OH− or CO32− appear similar in all sites. In addition to the well-known ν4 modes of PO43− units in hydroxylapatite that appear split at ca. 560 and 600 cm−1, an intermediate frequency component at 577 cm−1 is observed mostly in the Pikermi and Chalkoutsi samples. This feature seems to be accompanied by a weaker component at 613 cm−1. Similarly, the bands from Aghia Napa lack a ν2 (CO32−) mode at 865 cm−1, which is very characteristic of the Pikermi and Chalkoutsi samples. Calcite in the sample, identified as a distinct phase by XRD, produces a characteristic infrared peak at 712 cm−1 (not shown in Fig. 2) and can contribute to the intensity of the 871 cm−1 component. A weaker ν2 (CO32−) mode at 847 cm−1 is also attributed to calcite.

The 6982 cm−1 NIR band is clearly due to the overtone of the fundamental OH− mode, observed at 3574 cm−1 (not shown). A weak band at 7067 cm−1 is assigned to traces of dioctahedral AlAlOH moieties from clay secondary minerals, but the assignment of a stronger mode at 7240 cm−1 remains unclear. In order to elucidate its origin, we have subjected fossilized bone powders to exposure to ethanol vapour. Both bands at 5318 and 7240 cm−1 are shown to decrease in a synchronous manner, with a parallel increase of the 2ν(C–H) bands of ethanol (5600–6000 cm−1). The intensity of the 2ν(OH−) at 6982 cm−1 remains unchanged. This is a clear indication that ethanol adsorbs on apatite by substituting a very labile H2O species identified by its combination and overtone modes at 5318 and 7240 cm−1, respectively. This adsorption process is reversible and appears to be a property of biogenic apatites, since it has not been observed in any of the mineral reference apatites studied in this work. We assign this feature to a surface H2O species in agreement with Ishikawa et al. (1989) who studied a synthetic, carbonate-free, colloidal calcium hydroxylapatite and reported a sharp H2O combination mode at 5308 cm−1.

4. Discussion and conclusions

The above presentation demonstrates that the three bone populations from the different sites can be differentiated (albeit with overlap) by either XRD or vibrational spectroscopy. This implies that the structural alterations occurring at a microscopic level and sensed by vibrational spectroscopy are reflected in variations of the unit cell parameters. Beyond fingerprinting, the exact correlation between chemical and crystallographic structure is considerably more complicated.

One way of discussing the variations in Vcell would involve the presence of carbonates in the fossilized bone apatite and the equilibrium between substitutions in OH− (A-type, large Vcell) and PO43− (B-type, low Vcell) sites. There is a widespread assignment of the 880 and 871 cm−1 ν2 (CO32−) modes of carbonate apatite to A- and B-type substitutions, respectively (Rey et al., 1989; Shemesh, 1990; Sponheimer and Lee-Thorp, 1999), while A-type carbonates are usually connected to conditions of high temperatures. However, the intensity of the 880 cm−1 feature in the 2nd derivative spectra of Fig. 2
precludes a positive correlation with the observed trends in $V_{cell}$. Based on this criterion, the Aghia Napa samples would have the lowest $V_{cell}$, opposite to the results of the Rietveld analysis. Recent studies have indicated that the A-type CO$_3^{2-}$ substitution in synthetic apatite, prepared at high temperatures and pressures, occurs in two structural locations (Fleet and Liu, 2004). The A1-type substitution (1540 and 1455 cm$^{-1}$) occurs in the apatite channel and is equivalent to the A-type substitution observed in biological apatites, while the A2-type carbonate (1565 and 1505 cm$^{-1}$) is situated in a stuffed channel position and is a high pressure equivalent. There are overlaps present between them as well as with the B-type carbonate bands, which appear at about 1455 and 1410 cm$^{-1}$. Conditions of high temperatures and pressures are expected to be uncommon in fossilization. Therefore, such A2-type carbonates should not be anticipated in our samples (Fleet and Liu, 2004, 2005; Wopenka and Pasteris, 2005). The 1540 cm$^{-1}$ band, characteristic of A1-type carbonates (corresponding to the A-type of biological apatites) has not been observed in our spectra (detail not shown). This is contrary to the results of Sponheimer and Lee-Thorp, 1999, whose work was realized on enamel. As a result, although we can observe the 880 cm$^{-1}$ band, we conclude that A-type CO$_3^{2-}$ species, should they exist in our samples, are far from being a majority species. Instead, the diagenetic profile investigated appears to involve various types of CO$_3^{2-}$ B-substitution.

An additional chemical factor affecting the dimensions of the unit cell, compatible with the XRD data, is the extent of the substitution of F$^-$ in the OH$^-$ positions of hydroxylapatite. The fluoride ion does not have a discrete vibrational signature, although the appearance of a band at ca. 1090 cm$^{-1}$ is claimed to be indirectly associated with its presence (Shemesh, 1990; Wright and Schwarcz, 1996; etc). This band is observed at 1092 cm$^{-1}$ in our spectra, and its intensity in the 2nd derivative (strongest in Pikermi, weakest in Aghia Napa) agrees qualitatively with the observed trends in both $V_{cell}$ and $a$-axis. However, we observe that chemical analysis of selected fossilized bones from the three sites indicates that the Pikermi and Chalkoutsi groups both have fluoride content of 1.13±0.2, while very little fluorine is detected in Aghia Napa. In addition, the reference carbonate fluorapatite mineral containing 3.71 wt.% F ("francolite", Table 1), exhibits a very weak 1092 cm$^{-1}$ band (data not shown), while the two reference low-carbonate fluorapatites FA1 and FA2 exhibit a very strong 1092 cm$^{-1}$ band despite their relatively low F content (ca. 2 wt.%). Therefore, we argue that the origin of the 1092 cm$^{-1}$ band and its sharpness or intensity in the spectra of fossilized bones is not simply related to the fluoride content, but instead reflects a more complex structural alteration scheme.

A more systematic investigation of the spectroscopic trends related to the observed changes in unit cell parameters is possible by chemometric modeling. The model was fed with the ATR spectra (2nd derivative, 25 pt smoothing) of all fossilized bones over the frequency range from 525 to 1200 cm$^{-1}$, and was found to require four ranks (vectors) in order to predict $V_{cell}$ or $a$-axis with RMS errors of cross validation equal to 0.24 Å$^2$ and 0.002 Å, and correlation coefficients $R^2$ of 90 and 92%, respectively. The prediction of the less variable c-axis affords an error of 0.001 Å, despite its lower correlation coefficient ($R^2=75$%). A comparison of the predicted vs. Rietveld $a$-axis is shown in Fig. 4a. Inspection of the calculated vectors (not shown) indicates that decreasing $V_{cell}$ or $a$-axis is linked mainly to increasing intensity of the bands at 577, 865 and 1092 cm$^{-1}$, as well as to the increasing frequency of the mode at ca. 960 cm$^{-1}$. Each one of these individual spectroscopic parameters can be extracted from the spectra and correlated to the $a$-axis or $V_{cell}$ (e.g. Fig. 4b), although the correlation is improved by the simultaneous consideration of all the above spectral changes. It appears that these changes reflect various aspects of a single diagenetic scenario, associated with the decrease of $a$-axis or $V_{cell}$. This scenario is common to all the three sites investigated and may be of broader relevance despite controversies in band assignments.

For example, the 865 cm$^{-1}$ band has been observed by many previous investigators (Rey et al., 1989; Michel et al., 1995) although its specific assignment is elusive. This band has been attributed to a "labile"
or “unstable” carbonate species. According to various authors, these terms may have originated from the fact that these species decreases with maturation of bone or/and the increase of crystallinity, while the relative intensities of the other carbonate species (A- and B-type) are stable. We must note that in our study, the appearance of this band is stronger in the more crystalline specimens. Similarly, the band at 1092 cm⁻¹ has been associated with the fluoride content of the bone based on its strong appearance in the spectra of fluorapatites. Our findings suggest that this assignment is not necessarily valid in our case. The 1092 cm⁻¹ band is linked to the aforementioned 866 cm⁻¹ band, and may be due to the (normally inactive) ν1 mode of CO₃²⁻ becoming infrared active as the CO₃²⁻ triangles assume a C₂ᵥ or C₃v configuration similar to that in aragonite. If this assignment is correct, the C₂ᵥ or C₃v configuration would be the diagnostically stable state and its appearance can be favored by the substitutions of F⁻ for O²⁻.

The ν₄ (PO₄³⁻) mode at 577 cm⁻¹ has been observed in the spectra of carbonate apatite (Shemesh, 1990; Michel et al., 1995) and has even been used to indicate the presence of F. According to Shemesh the intensity of the 605 cm⁻¹ increases relative to the 575 cm⁻¹ peak, with increasing degree of fluoridation. In the spectra of fossilized bones, this feature is often masked by the stronger components at ca. 560 and 600 cm⁻¹ and its presence is indirectly inferred from the shallower “valley” between them. An infrared splitting factor (SF) had been developed based on the complex bandshape of the ν₄ (PO₄³⁻) modes and is widely used as a crystallinity index for bone apatite (Weiner and Bar-Yosef, 1990; Lee-Thorp and Sponheimer, 2003; Trueman et al., 2004).

The coherence length of the apatite crystallites can be estimated from the Rietveld analysis of the XRD spectra (Chipera and Bish, 1991; Psychiats et al., 2001). Our analysis suggests that the coherent domains are rod-shaped with dimensions 350–1250 by 12–20 Å and the long dimension aligned with the c-axis. In Fig. 4c we show that the splitting factor (SF) determined from our ATR absorption spectra in the conventional manner of Weiner and Bar-Yosef (1990) correlates essentially with the coherence length along the c-axis. The splitting factor responds essentially to the relative intensity of the 577 cm⁻¹ component band observed only at high spectral resolution. Since the intensity of the 577 cm⁻¹ component parallels the intensity changes of the 865 and 1092 cm⁻¹ bands, any of the latter criteria would correlate with the coherence length (Fig. 4d).

Overall, the present study identified a subtle but systematic trend of the carbonate hydroxylapatite unit cell and coherence length parameters that appears to be common over all three sites investigated. Though this trend is highly correlated with the vibrational spectra of the fossil bones, it does not seem to involve any significant changes in the incorporation of CO₃²⁻ in A-type sites, nor can it be accounted fully by chemical or microscopic changes in the apatite structure. Experientia 25 (1), 5–8.

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