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BIOLOGICAL CALCIFICATION: INVESTIGATION BY X-RAY ABSORPTION SPECTROSCOPY

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Résumé Les composés modèles, qui rassemblent aux phosphates de calcium déposés dans les systèmes biologiques, ont été synthétisés caractérisés par une variété de techniques physiques, et leurs spectres EXAFS enregistrés au dessus de la limite d'absorption K du calcium. Nous avons analysé le spectre de l'un de ces composés, l'hydroxyapatite, et à l'aide de ces résultats nous avons analysé les spectres de composés apparentés. Les spectres enregistrés au dessus de la limite d'absorption K du phosphorus fournissent de l'information complémentaire au sujet des structures des phosphates de calcium biologiques.

Abstract Model compounds which resemble the calcium phosphates deposited in biological systems have been synthesised, characterised by a variety of physical techniques and their EXAFS spectra recorded above the calcium K edge. The spectrum of one of the compounds, hydroxyapatite, has been analysed and the results used to aid the analysis of spectra from related compounds. Spectra recorded above the phosphorus K edge provide complementary information on the structures of biological calcium phosphates.

1. Introduction

X-Ray absorption spectra have proved especially useful for characterising biological calcium phosphates for two interdependent reasons. Firstly, calcified deposits and their model compounds may be poorly crystalline or amorphous and consequently difficult to characterise by X-ray powder diffraction. Secondly, calcium ions have a highly variable geometry [1] so that they have different coordination distances in the various phases of calcium phosphates. Spectra in the EXAFS region (20–400 eV) above the calcium K edge (4.038 keV; λ = 0.307 nm) have proved especially useful for characterising these structures because they are sensitive to short-range order around the calcium ions [2,3].

Three recent advances will be described here: (i) synthesis and characterisation of model compounds, (ii) detailed analysis of the spectra using spherical wave theory and (iii) preliminary experiments involving X-ray absorption spectra of biological calcium phosphates recorded above the phosphorus K edge (2.144 keV; λ = 0.578 nm). There have also been new applications of X-ray absorption spectroscopy to biological calcification, described elsewhere [4,5].

2. Model compounds

Initially the application of EXAFS spectroscopy to biological calcium phosphates involved comparison of their spectra with those from model compounds; more recently the spectra from these model compounds have been analysed in order to identify the structural differences encountered in biological systems [2,3]. Whichever approach is adopted, its success ultimately depends on the availability of well characterised model compounds.

Several compounds resembling hydroxyapatite, Ca₅(PO₄)₃OH (abbreviated to HAP), have been prepared and characterised by chemical analyses (for Ca, P and carbonate), X-ray powder diffraction and IR spectroscopy. Pure crystalline HAP was prepared by an established method [6].
A variation of this technique was used to prepare an amorphous calcium phosphate (ACP) which gradually matured into a poorly crystalline form of HAP when maintained in a moist condition for 20 h at pH 10 and 20°C. Freeze drying stabilises the product at any stage of the maturation process. The preparative procedure was designed to yield pure compounds rather than to mimic any biological process. Carbonate ions were also incorporated into the products by the addition of specific concentrations of ammonium carbonate to the ammonium phosphate solution used in the preparation.

EXAFS spectra of these model compounds, recorded above the calcium K edge, are shown in Fig. 1 together with the moduli of their Fourier transforms. Maturation of ACP into HAP leads to the appearance of fine structure in the EXAFS spectrum as a result of the development of long-range order in the structure. The spectrum of poorly crystalline HAP closely resembles that

![EXAFS spectra](image-url)
of bone mineral. Incorporation of carbonate ions (4% by weight, as in bone mineral) delays the maturation of ACP into HAP.

3. Analysis of spectra

The spectrum of pure crystalline HAP has recently been analysed using the coordinates of the known crystal structure and the exact spherical wave theory appropriate for a polycrystalline sample [7]. HAP has a complicated crystal structure in which the unit cell contains two formula units of Ca₅(PO₄)₂OH with two structurally distinct calcium sites. A weighted linear combination of the two calcium environments was constructed and the results grouped into composite shells when atoms of the same type were less than 0.01 nm apart; the validity of this grouping was tested by trial calculations [7]. The theoretical EXAFS spectrum, calculated using *ab initio* phase shifts, is compared with that obtained experimentally in Fig. 1(a). To achieve this fit, the radii of the shells surrounding calcium and their Debye–Waller factors were systematically varied. Several shells were omitted when calculating the spectra for reasons which are well understood [7,8]. Thus, for example, EXAFS spectra are insensitive to the positions of the phosphorus atoms at 0.37 nm from calcium in model compounds, and presumably biological calcium phosphates, because the photoelectrons backscattered from these atoms interfere with those scattered forwards by oxygen atoms situated around 0.24 nm from calcium [7,8].
Fig. 1(b) shows that, with slight modification, this model can explain the features of the EXAFS spectrum of ACP. This fit was achieved by considering only the four inner coordination shells appropriate for HAP; coordination distances for the oxygen and phosphorus shells had to be varied by no more than 0.007 nm, although their Debye—Waller factors had to be increased (e.g. from 0.013 to 0.023 x 10^-2 nm^2) presumably because of the greater static disorder in ACP. It is anticipated that now a model for the EXAFS spectrum of HAP has been developed, it can be used as the basis for analysing spectra from other model compounds and bone mineral — in the same way as it has been applied to the analysis of the spectrum from ACP.

4. Phosphorus spectra

Fig. 2 shows the X-ray absorption spectra recorded above the phosphorus K edge from some of our synthetic calcium phosphates and bone mineral. Specimens were powdered in an agate mortar with acetone and spread on to a copper block; when the acetone evaporated, a layer of specimen (1 cm x 10 cm) adhered to the block — spectra were recorded on the soft X-ray beamline developed at the Synchrotron Radiation Source of the SERC Daresbury Laboratory [9].

Our preliminary experiments indicated that the region 0—60 eV above the phosphorus K edge was most sensitive to the environment of the phosphorus atom in calcium phosphates. The spectrum of HAP, Fig. 2(a), is clearly different from that of brushite (CaHPO_4.2H_2O), Fig. 2(b), in this region. Furthermore, the spectrum of bone mineral, Fig. 2(c), is distinct from that of the fully crystalline HAP but clearly resembles that of poorly crystalline HAP, Fig. 2(d). We believe that X-ray absorption spectra recorded in this region above the phosphorus K edge will complement spectra recorded above the calcium edge for characterising biological calcium phosphates.

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