C. R. Chimie 19 (2016) 1665–1671

Contents lists available at ScienceDirect

Comptes Rendus Chimie

www.sciencedirect.com

Full paper/Mémoire

Solid state NMR of salivary calculi: Proline-rich salivary proteins, citrate, polysaccharides, lipids, and organic–mineral interactions

Yang Li [a], David G. Reid [a], Dominique Bazin [b, c], Michel Daudon [d], Melinda J. Duer [a, *]

[a] Department of Chemistry, University of Cambridge, Lensfield Rd., Cambridge CB2 1EW, UK
[b] Laboratoire de Physique des Solides, CNRS, Université Paris-Sud, 91405 Orsay, France
[c] Laboratoire de Chimie de la Matière Condensée de Paris Université Pierre et Marie Curie et Collège de France, 11 place Marcelin Berthelot, 75231 Paris cedex 05, France
[d] APHP, Hôpital Tenon, Service d'Explorations Fonctionnelles, 4 rue de la Chine, 75020 Paris, France

ARTICLE INFO

Article history:
Received 14 May 2015
Accepted 6 July 2015
Available online 3 January 2016

Keywords:
Apatite
Citrate
Polysaccharides
Proline-rich proteins
REDOR
Salivary proteins
Statherin

ABSTRACT

Solid state NMR (ssNMR) can characterize mineral (^{31}P) and organic (^{13}C) components of human salivary stones ($n = 8$). All show apatitic ^{31}P spectra. ^{13}C ssNMR indicates more protein, of more consistent composition, than apatitic uroliths, with prominent signals from Tyr, Phe, and His. Citrate and lipids, identified by dipolar dephasing (DD), and polysaccharides are also observable in varying amounts. ^{13}C{^{31}P} rotational echo double resonance (^{13}C{^{31}P} REDOR) identifies carbon atoms in close (<ca. 0.5 nm) proximity to phosphorus and therefore probably binding with mineral. Citrate, sugar, and carboxylate signals exhibit strong ^{13}C{^{31}P} REDOR effects, also observed for signals between 50 and 60 ppm, from protein α-carbons and, possibly, phosphoserines and phospholipids, and sometimes for a 35–40 ppm envelope containing Asp–Cβ and Glu–Cγ signals. Amino acid analysis indicates high proline and aromatic content. ^{13}C ssNMR and amino acid analyses are consistent with the preponderance of proline-rich salivary proteins such as statherin.

Pathological calcification can occur at multiple anatomical sites and under a variety of physiological conditions. The composition and structure of the resultant calcified materials may provide clues to the pathological causes and molecular mechanisms leading to calcification, and possibly suggest avenues for therapeutic intervention. Basic information on the composition of these materials can be obtained with simple chemical analyses such as determination of elemental composition. However, information which may be critical to identifying pathological causation, such as biomineral crystal structure, requires more sophisticated physicochemical methodologies which provide structural information to complement chemical analysis [1,2].

X-ray diffraction is a powerful tool for defining the spatial arrangement of atoms in crystalline or multicrystalline materials. It can be used to determine the crystal structure, and by comparison with published database "fingerprints" of chemically or synthetically well-defined materials, chemical composition in favourable cases. It is widely used in characterizing the crystalline components of biominerals including sialoliths [3–5]. Fourier transform infrared spectroscopy (FTIR) can also be used to identify the structure and physical phases of the material by probing the molecular vibrational modes which are functions of molecular and crystal structures [6]. In pure organic phases or mixed organic–inorganic composites,

* Corresponding author.
E-mail address: mjd13@cam.ac.uk (M.J. Duer).

http://dx.doi.org/10.1016/j.crci.2015.07.001
1631-0748/

the amide stretches due to the C–O and C–N backbone of a peptide, for instance, gives distinctive IR spectra which can be related to the backbone conformation. Common pathological biomineral phases, such as calcium oxalate solvates and polymorphs widespread in kidney stones, can be distinguished by zero-crossing-point first-derivative spectrophotometry [7], and different apatites and other calcium phosphate materials can be identified by assigning P–O stretches. Scanning electron microscopy (SEM) examines the surface of the calcified material, and is a useful method for characterizing the crystallite morphology [8]. These important physical methods, and others such as powder neutron diffraction (PND) and X-ray fluorescence (XRF), have long been widely used to study many kinds of pathological calcified materials to determine the molecular composition and establish possible links between structural and chemical properties and the etiopathogenesis of biomineralization [9].

Solid-state nuclear magnetic resonance spectroscopy (ssNMR) is widely used to study solid material structures by virtue of its sensitivity to the local environment of each NMR receptive atom [10]. Unlike X-ray scattering techniques, which rely on molecular crystallinity and long range order for interpretable data, ssNMR (like FTIR) can effectively study materials such as amorphous solids without long range order and provide information from which the local structure can be inferred. In addition NMR data are often easier to interpret in terms of molecular structure as each chemically unique atom gives rise to a single signal the frequency of which directly depends, often in an interpretable way, on its chemical environment. Considering the example of a protein, ^{13}C atoms in amino acids containing methyl groups (Ala, Ile, Leu, Met, and Val) will give rise to signals which are distinct from each other, and from other sp^3 carbon atoms, which are distinct from aromatic carbons (His, Phe, Tyr, and Trp), which in turn are distinct from backbone and sidechain amide carbons, and acidic carboxylate carbons. Similarly in phosphatic biominerals different phosphate environments, such as the PO_4^{3-} groups in apatite, give rise to different ^{31}P signals from HPO_4^{2-}, for instance brushite. Under favourable circumstances NMR can yield complete atomic level molecular and even crystal structures, although this is far from realization for complex mixtures such as pathological calcifications. The potential of NMR in the study of the composition of renal calculi has been demonstrated by Bak et al. [11] who distinguished from each other the common organic (calcium oxalates and uric acid) and inorganic (apatite, struvite, and brushite) constituents. Apatitic stones in particular have also been shown by ssNMR to contain polysaccharides and proteins of highly variable apparent composition [12] and citrate [13] in variable proportions. Using the ssNMR technique, REDOR atomic level (sub-nanometre) interactions between urolith phosphates, and the polysaccharides, proteins and (when present) citrate, were also demonstrated, prompting the suggestion that such interactions might reflect processes central to urinary calculus biogenesis [12].

In this paper, we apply ssNMR to extend the characterization of the composition of, and some aspects of the mineral–organic interactions present in, salivary stones (sialoliths) [14,15], to our knowledge for the first time in these pathological materials. We show that this technique constitutes a unique tool, complementary to the other techniques described above, to address many aspects of the organic constituents of pathological calcifications. The necessary background to the methods we use – cross-polarization (CP), magic angle spinning (MAS) and ^{13}C $\{^{31}P\}$ REDOR [12], and dipolar dephasing (DD) [13] – is described in the respective references just cited. Amino acid analyses were also performed on four of the samples, and the salivary protein statherin was identified as the most likely major organic component of the stones, consistent with the signals observed in the ^{13}C ssNMR. The function of the statherin is widely believed to be, at low concentrations, the inhibition of propagation of calcium phosphate crystallization and crystal growth, exerted by binding strongly with the nucleating mineral surface. However at high protein concentrations such proteins can solidify and actually act as crystallization promoters *via* heterogeneous nucleation processes. It is evidently the latter process which predominates in the stone materials we have studied.

1. Materials and methods

Eight sialoliths were from the Wharton's ducts of patients undergoing surgical sialolithotomy and they were initially examined by FTIR spectroscopy at the Hôpital Tenon, Paris. They were used in all analytical procedures with informed patient consent and institutional ethics approval.

Each one was studied using a stereomicroscope to define morphological type [16] and a Spectrospin Vector 22 Fourier transform infrared spectrometer (Bruker, Karlsruhe, Germany) to determine its mineral composition and detect the presence of proteins and lipids [17].

All solid-state NMR measurements were performed on a Bruker 400 MHz Avance II spectrometer, at frequencies of 400.42, 162.1, and 100.6 MHz for ^1H, ^{31}P, and ^{13}C, respectively, using standard Bruker double and triple resonance magic angle spinning (MAS) probes. Samples were powdered using a pestle and mortar, packed into 4 mm zirconia rotors and spun at 12.5 kHz, except for the DD experiments where a spin rate of 6.25 kHz was used. Samples were characterized using cross-polarisation magic angle spinning (CP-MAS; ^1H 90° pulse length 2.5 μs, ^{31}P CP contact time 10 ms, $^1H–^{13}C$ CP contact time 2.5 ms, spin lock field strength 70 kHz, SPINAL64 broadband decoupling at 100 kHz ^1H field, recycle time 2 s), DD (same $^1H–^{13}C$ CP parameters, ^{13}C refocussing π-pulse at the centre of the DD period, DD time 100 μs) and REDOR (same $^1H–^{13}C$ CP parameters, 10 ms dephasing time, interpulse spacing in ^{31}P π pulse train 80 μs synchronized with the MAS period). ^{31}P and ^{13}C chemical shifts were externally referenced using commercial macrocrystalline HAp (Fluka) at a shift of 2.6 ppm relative to 85% phosphoric acid at 0 ppm, and the methylene signal of the α-polymorph of glycine (Sigma) at 43.1 ppm relative to TMS at 0 ppm, respectively. Sample masses available for the study varied between ca. 100 and only a few milligrams; accordingly acquisition times for the most demanding experiment, the REDOR, varied from overnight to three days. Other experiments could be correspondingly shorter, typically a few hours to overnight.