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# Communications

## Investigation of the Nature of the Protein−Mineral Interface in Bone by Solid-State NMR

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Bone is a complex composite material[1] composed of an organic matrix (predominantly collagen[2,3] but with some 200 other noncollagenous proteins[4−8]) with inorganic crystals (a complex calcium phosphate phase dominated by hydroxyapatite[9]) deposited on it. However, how these components are bound together is largely unknown.[10,11] That they must be bound together is beyond dispute.[12] Bone has remarkable material properties, having the stiffness not to deform under normal use, yet it is relatively light and robust too.[13] The key to understanding how these properties come about is a knowledge of the structure of the interface between the various components, in particular, the inorganic crystallites and the organic matrix.[12−14] Not only would such knowledge go a long way to understanding the material properties of bone, it also would greatly assist in the design of new synthetic bone materials and other materials aiming to emulate the material properties of bone.[12,13]

In undertaking a study to identify the structure of the organic−inorganic interface in bone, it is imperative to use a naturally occurring system rather than a model. Model systems may be an excellent starting point for a study, but in a composite material as complex as bone, they can only ever be that.

Solid-state NMR is one of the few techniques that allow quantitative structural measurements on a heterogeneous system, such as bone presents. The dipolar coupling between NMR-active nuclei has a strength proportional to $1/r^3$ where $r$ is the internuclear distance. The Rotational Echo DOuble Resonance (REDOR) experiment allows quantitative deter-

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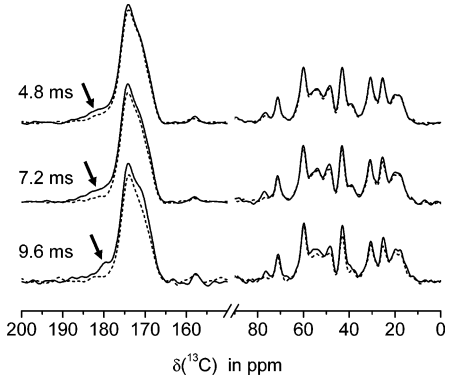


**Figure 1.** Nondephased (solid line) and dephased $^{13}$C-{$^{31}$P} REDOR NMR spectra (broken line) of bone as a function of dephasing time.

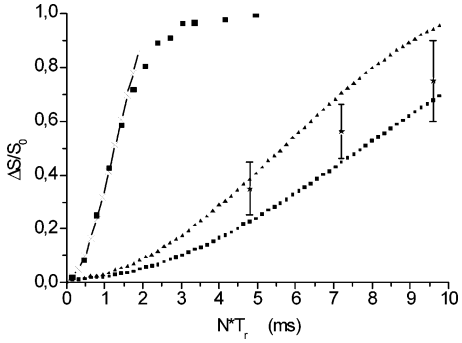


**Figure 2.** Experimental and simulated $^{13}$C-{$^{31}$P} REDOR dephasing curves for the $\beta$-$^{13}$C of *o*-phospho-L-serine and bone. Large squares represent the experimental REDOR dephasing curve for $\beta$-$^{13}$C of *o*-phospho-L-serine and crosses the curve simulated for a $\beta$-$^{13}$C−P internuclear distance of 0.270 nm. Stars represent the experimental curve for the $^{13}$C at 182 ppm of bone, small squares the curve simulated for a $^{13}$C-P internuclear distance of 0.50 nm, and triangles the curve simulated for a $^{13}$C-P internuclear distance of 0.45 nm.

mination of the dipolar coupling and is described in detail elsewhere, including its specific dependence on the dipolar coupling.[15,16] In this work we employ a $^{13}$C-{$^{31}$P) REDOR experiment. In such an experiment, a $^{13}$C NMR experiment is recorded both with and without a train of radio frequency (rf) pulses being applied to the $^{31}$P spins prior to acquisition of the $^{13}$C spectrum, forming the so-called non-dephased and dephased $^{13}$C spectra, respectively. The $^{13}$C spectrum recorded without $^{31}$P pulses acts as a reference experiment. In the spectra recorded with a $^{31}$P pulse train prior to acquisition, $^{13}$C signals due to $^{13}$C spins which are in close spatial proximity to $^{31}$P spins in the sample will show a reduction in intensity. Following the reduction in intensity of a given $^{13}$C signal as a function of the length of time for which the $^{31}$P pulse train is applied makes quantitative determination of the $^{13}$C-$^{31}$P distance possible. The beauty of such an experiment for bone is that $^{13}$C spins are very largely confined to the organic matrix while $^{31}$P spins are very largely confined to the inorganic component and thus any $^{13}$C-$^{31}$P distances we measure will be relevant to the organic−inorganic interface.

The non-dephased and dephased $^{13}$C-{$^{31}$P} REDOR spectra for the bone sample are shown in Figure 1 for dephasing times of 4.8, 7.2, and 9.6 ms. There are several signals in the spectrum which show a clear reduction in intensity upon application of the $^{31}$P dephasing pulses, most notably the signals in the range 180−185 ppm which show significant dephasing after 4.8 ms of $^{31}$P dephasing and almost complete dephasing after 9.6 ms. Clearly, the $^{13}$C sites that give rise to the signals in this region are in close proximity to $^{31}$P. This signal corresponds (from its chemical shift) to the carboxylate ($\delta$) carbon of glutamic acid residues, with possibly a contribution from the amide carbon of glutamine residues in the lower end of this frequency range. There is also dephasing of the signal around 175 ppm (carbonyl groups in a protein backbone), at 170 ppm (carbonate within the calcium phosphate phase[17]) and significant dephasing of the relatively sharp signal observed at 75 ppm.[18] This latter signal can be assigned to a secondary alcohol, either in an amino acid residue, or in the polysaccharide side chain of one of the glycoproteins which exist in bone.[7,8,12]

The only $^{31}$P spins in bone and thus those which must be responsible for the observed dephasing of the $^{13}$C spectra are those in the various phosphate groups of the mineral phase and phosphorylated side chains in serine and threonine residues in peptides within the protein matrix. To assess the possible role of the latter $^{31}$P sites in the observed dephasing of the $^{13}$C spectra, we investigated the $^{13}$C-{$^{31}$P} REDOR behavior of *o*-phospho-L-serine under the same experimental conditions as for the bone sample. The difference in the $\beta$-$^{13}$C carbon signal intensity between the non-dephased and dephased $^{13}$C spectra for this sample as a function of dephasing time is shown in Figure 2. The intramolecular $\beta$-$^{13}$C-$^{31}$P distance has been determined by X-ray diffraction to be 0.264 nm.[19] The simulation of the initial part of this curve (Figure 2) using $^{13}$C-$^{31}$P spin pair with a distance of 0.27 nm reproduces with good accuracy the experimental data for *o*-phospho-L-serine. Further simulations incorporating the more distant (intermolecular) $^{31}$P spins in *o*-phospho-L-serine give little change in the initial part of the curve as would be expected, as the only significant dephasing at shorter dephasing times will come from the closest $^{31}$P spin.

In the $^{13}$C spectra of the bone sample, there is no $^{13}$C signal that corresponds to the $\beta$-$^{13}$C of phosphorylated serine. This signal would appear at 63−64 ppm and would show similar very rapid dephasing to that of the *o*-phospho-L-serine in Figure 2. No such signal or dephasing is observed, indicating that the amounts of phosphorylated proteins in bone are too small to be measured by NMR. Thus, we can conclude that all the dephasing observed in our $^{13}$C-{$^{31}$P} REDOR experiments arises from $^{31}$P spins in the mineral component and therefore that all the dephased $^{13}$C signals arise from $^{13}$C spins close to the mineral component. Thus, it is interesting now to analyze the most strongly dephased signals, the signals in the region 180−185 ppm, the glutamate carbox-

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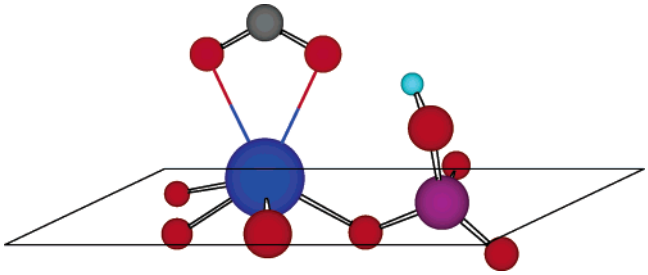

**Figure 3.** A structural model of a possible binding site for a carboxylate group on the surface of the calcium phosphate phase in bone. The Ca−O distances are taken as the typical distances found in calcium carboxylate complexes and the Ca−O−P distances the average of those found in crystalline calcium phosphate compounds (see text for details). The carboxylate carbon−P distance is constrained by the REDOR data recorded in this work. Within this constraint, the phosphate group can be located anywhere on a circle whose plane is centered on the Ca−C axis; just one position is shown in the figure. Ca (blue), C (gray), O (red), P (purple), and H (cyan). The surface of the inorganic phase in bone is known to contain protonated phosphate groups, i.e., P−OH. It is worth noting that, within the distance constraints provided by this model, surface P−OH groups are close enough to hydrogen bond to the binding carboxylate group or, alternatively, carbonyl groups in the backbone of the binding peptide. This latter observation would explain the observed dephasing of part of the carbonyl signal in the $^{13}$C-{$^{31}$P} REDOR spectra in this work.

ylate carbons, and to extract a $^{13}$C-$^{31}$P internuclear distance from the experimental data. The difference signal intensities (integrated over the range 180−185 ppm) are plotted in Figure 2 along with simulations for $^{13}$C-$^{31}$P distances of 0.5 and 0.45 nm (Figure 2). The experimental curve lies between these two simulated curves and so we may conclude that the $^{13}$C-$^{31}$P distances for these $\delta$-$^{13}$C of glutamate residues is of the order of 0.45−0.5 nm. This relatively short distance constraint strongly supports a binding model in which a glutamate-containing protein interacts strongly with $Ca^{2+}$ ions on the surface of the mineral phase. As collagen contains very little glutamate or glutamine, this protein must be in the noncollagenous part of the protein matrix. Moreover, we see no dephasing of the characteristic proline and hydroxyproline signals for collagen (60 and 71 ppm).

We have used the distance constraint provided by our experiment for the carboxylate $^{13}$C-$^{31}$P distance to produce a model of the possible surface binding site of a glutamate carboxylate group. In producing this model, we have used typical C−Ca distances taken from calcium carboxylate complexes for which crystal structures are known[20] and Ca−O and Ca−P distances which are the average of distances found in crystalline hydroxyapatite, octacalcium phosphate, dicalcium phosphate, and dicalcium phosphate dihydrate (since the surface structure of the calcium phosphate phase in bone is unknown). The resulting structure using these constraints is shown in Figure 3. Monodentate binding modes are also possible within these distance constraints.

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**Supporting Information Available:** Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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