J|A|C|S COMMUNICATIONS

Published on Web 11/06/2009

Time-Resolved Dehydration-Induced Structural Changes in an Intact Bovine Cortical Bone Revealed by Solid-State NMR Spectroscopy

Peizhi Zhu,[†] Jiadi Xu,[†,‡] Nadder Sahar,[§] Michael D. Morris,*[,†,§] David H. Kohn,[§,||] and Ayyalusamy Ramamoorthy*[,†,‡]

Department of Chemistry, Biophysics, Biological and Materials Sciences, and Biomedical Engineering, University of Michigan, Ann Arbor, Michigan 48109

Received September 23, 2009; E-mail: ramamoor@umich.edu

Water is an important component of cortical bone, accounting for ~20% by volume.[1] It is well-known that interstitial water molecules play a major role in stabilizing both collagen and mineral through enthalpic stabilization and hydrogen bonding.[2] The water content of bone tissue decreases with age and is associated with a reduction in biomechanical properties that are critical to the function of bone.[3] The loss of water in collagen decreases the toughness of bone, and the loss of water associated with the mineral decreases both bone strength and toughness.[4] However, the underlying mechanisms, including structural changes in the matrix protein and mineral and changes in their interactions brought about by dehydration, are unclear. Understanding this mechanism could provide insights into the susceptibility of bone to fracture, especially in the osteoporotic tissues of many elderly people. While previous solid-state NMR experiments on bone and related materials have provided valuable structural information,[5−10] an intact bone still remains as a complex system for high-resolution structural studies. In this study, we report dehydration-induced effects on an intact bovine cortical bone using magic-angle-spinning (MAS) NMR experiments.

^{13}C chemical shift spectra of a fresh cortical bone, dehydrated cortical bone, and a H_2O/D_2O (H/D)-exchanged cortical bone are shown in Figure 1 along with the resonance assignments from previous studies.[11,12] The dehydrated cortical bone was prepared by placing a piece of cortical bone in a vacuum desiccator for 3 days, while the H/D-exchanged cortical bone was prepared by immersing the fresh intact cortical bone in deuterated phosphate buffer for 3 days. The ^{13}C chemical shift spectrum of a fresh cortical bone exhibits well-resolved spectral lines and is dominated by resonances from collagen (Figure 1a). The assignment of peaks originating from most amino acids present in the cortical bone matrix is indicated in Figure 1a (more detailed assignment of peaks is given in Tables S1−S3 in the Supporting Information). The spectra of a dehydrated cortical bone (Figure 1b) and a H/D-exchanged cortical bone (Figure 1c) are similar and exhibit much broader spectral lines than the fresh cortical bone (Figure 1a). The only major difference between the two spectra in Figure 1b,c is the disappearance of the peak at ~78 ppm representing glycosaminoglycans (GAGs) as a result of H/D exchange (Figure 1c).

Since there are considerable differences between ^{13}C spectra of fresh (Figure 1a) and dehydrated (Figure 1b) bones, we performed NMR experiments to study the water-dependent structural and dynamical changes in the intact bone caused by slow dehydration of a cylindrical specimen of bovine cortical bone in an NMR rotor. The sample was spun by a constant velocity stream of air against the tip of the rotor, creating a constant reduced pressure at the distal end, and dehydration of the bone was induced by means of a 0.1 mm diameter hole in the distal end cap of the rotor. At the reduced pressure, the water content of the specimen decayed exponentially with time (τ = 7.34 h; Figure S1) over 24 h. Since the ^{13}C spectrum shown in Figure 1 contains well-resolved spectral lines, dehydration-induced changes in the bone were monitored as the change in the intensities and line widths of ^{13}C signals (Figure 2A). The extent of water in the cortical bone (Figure S1) was monitored from the decreasing water ^{1}H NMR peak intensity from a series of ^{1}H MAS spectra of the intact bone (Figure 2B); differences in the ^{1}H NMR spectra of intact cortical bone and ground cortical bone powder are highlighted in Figure S2. In control experiments, no spectral changes were observed even after 24 h of spinning the sample as long as the end cap was tightly sealed to prevent evaporation.

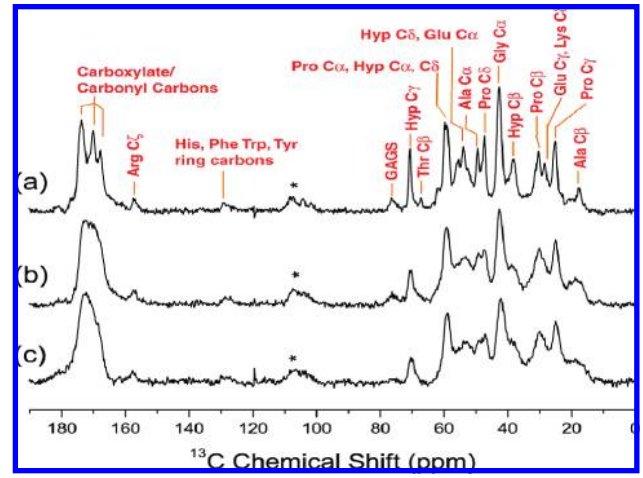

Figure 1. ^{13}C chemical shift NMR spectra of intact (a) fresh, (b) dehydrated, and (c) H/D-exchanged 60 mg bovine cortical bone samples. All of the spectra were obtained using a Varian VNMRS 600 MHz solid-state NMR spectrometer and a 4 mm double-resonance MAS probe with a 2 ms ramp cross-polarization, 80 kHz TPPM proton decoupling during acquisition, and a 3 s recycle delay under 10 kHz MAS at room temperature (25 °C). Spinning sidebands are marked with asterisks. The bone samples were shaped to a 2.5 × 2.5 × 12 mm size and inserted into a 4 mm zirconia MAS rotor (see the Supporting Information).

The appearance of three well-resolved peaks in the carbonyl carbon chemical shift region (~170 ppm) indicates that the native collagen structure in a fresh cortical bone is in agreement with a previous study on pure collagen;[11] a ^{13}C MAS spectrum of collagen type I is given in Figure S3. Interestingly, these well-resolved peaks broadened to a single peak as a result of dehydration, whereas the integrated area of these peaks did not change (Figure 2A). Peaks in the 10−80 ppm region, including the Hyp C_γ, Hyp C_β, Pro C_α, Gly C_α, Pro C_β, and Pro C_γ peaks, gradually decreased in intensity and broadened as the cortical bone was dehydrated with time. Some less intense peaks, including Thr C_β at 67.4 ppm, Ser C_β and Val C_α at 62.2 ppm, Val C_β

[†] Department of Chemistry.
[‡] Biophysics.
[§] Biological and Materials Sciences.
[||] Biomedical Engineering.

10.1021/ja9081028 CCC: $40.75

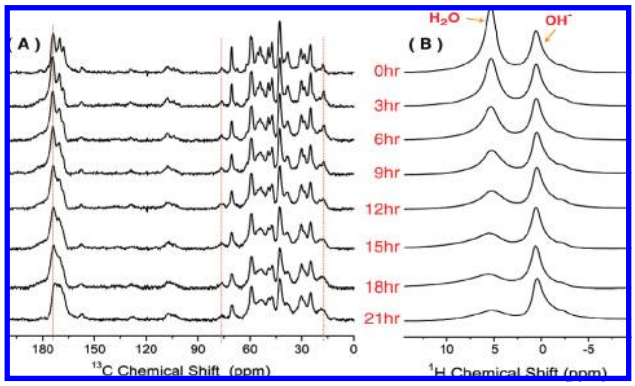

Figure 2. (A) ^{13}C and (B) ^{1}H chemical shift spectra of an intact bovine cortical bone obtained from a fresh bovine cortical bone (0hr) and after successive 3 h intervals of dehydration (spectra labeled 3hr to 21hr). The total acquisition times for each ^{13}C and ^{1}H spectrum were 3 h and 3 min, respectively. All other experimental details are as given in Figure 1 caption.

at 33.0 ppm, and Glu C_γ and Lys C_δ at 28.3 ppm, disappeared because of dehydration-induced line broadening. The line broadening caused by dehydration can be attributed to a local conformational disorder of the bone matrix and also to the slow dynamics of side chains of collagen.[12] The sensitivity of the $^{13}C_\beta$ chemical shift of Ala to the backbone conformation of collagen can be used to understand the specific structural changes due to dehydration.[12−14] The presence of a 17.6 ppm peak from $^{13}C_\beta$-Ala confirms the triple-helix structure of collagen in the fresh wet cortical bone. Because of dehydration of the bone, the relative intensity of this peak gradually decreased, broadened, and shifted from 17.6 to 18.8 ppm. This observation most likely indicates a change in the triple-helical structure and dynamics of collagen as a result of dehydration. The observed 5.3 ppm difference between the $^{13}C_\beta$ (30.5 ppm) and $^{13}C_\gamma$ (25.2 ppm) chemical shifts of Pro of a fresh cortical bone in this study confirms the presence of a *trans*-Xaa-Pro conformation in collagen.[12] Most importantly, the dehydrated bone also exhibited the chemical shift difference value (Table S2), suggesting that the *trans*-Xaa-Pro peptide bond conformation is maintained during the dehydration process.[15,16] It should be noted that no significant differences were observed in the Raman spectra of these bone samples.

In the ^{13}C NMR spectra of a H/D-exchanged cortical bone, the GAG peak at 76 ppm[17] becomes weaker in intensity and almost disappears. The spectra of a dehydrated and H/D-exchanged bones are similar in the 0−60 ppm chemical shift region but differ from that of the fresh cortical bone. For example, a significant line broadening was observed for Ala $^{13}C_\alpha$ at 47 ppm and Ala $^{13}C_\beta$ at 17.6 ppm from both dehydrated (Figure 1b) and H/D-exchanged (Figure 1c) bones, while this effect was greater in the case of the H/D-exchanged bone. This observation could be attributed to relatively more structural changes due to the H/D exchange process, as D_2O forms weaker hydrogen bonds than H_2O and therefore can disrupt hydrogen bonding, leading to structural changes in the collagen fiber. The ring carbons of GAG molecules in bone are motionally restricted because of its rigid pyranose structure. The almost complete disappearance of the GAG peak at 76 ppm indicates significant disorder in the conformation, particularly in the H/D-exchanged bone. This observation implies that GAGs in the collagen fiber and mineral interface may chelate with a Ca^{2+} ion present on the surface of the mineral through sulfate or carboxylate groups, while on the outside they form hydrogen bonds with surrounding water molecules. H/D exchange disrupts its bonding with water and changes its aqueous environment, which further disorders its conformation and causes the line-broadening effect in the NMR spectra, as shown by the reduction in the intensity of the GAG peak.

Cortical bone is a complex biological system with an intricate structure and unique mechanical properties. It is more meaningful to study it as a whole intact bone than to look separately at its components such as collagen or mineral. Moreover, the cut large section of bovine cortical bone used in our experiments may give more accurate results than the cryogenically milled bone powder commonly used in most previous solid-state NMR studies, especially in experiments involving changing the physical dimensions of the tissue. In this study, we have demonstrated that solid-state NMR experiments on an intact bone can provide high-resolution spectral lines, that the effect of dehydration inside the NMR rotor can be monitored, and that the observed dehydration-induced spectral changes can provide insights into structural changes in bone. For cortical bone, a highly heterogeneous material with a complex hierarchical structure, high-resolution solid-state NMR spectroscopy can probe selected magnetic nuclei (as shown by our results) and give snapshot pictures of the cortical bone structure at the atomic level that are difficult to obtain by other methods. Our results further suggest that the use of higher magnetic fields and sophisticated RF pulse sequences under ultrafast MAS can provide more piercing atomic-level structural insights into an intact bone structure that would be useful for understanding some unclear aspects of the structural mechanism regarding the role that water molecules play in bone structure, toughness, and mechanical strength.

Acknowledgment. This research was supported by NIH (R01 AR052010 to M.D.M., RR023597 to A.R., and GM084018 to A.R.), CRIF-NSF (to Chemistry), DoD/U.S. Army (DAMD17-03-1-0556 to D.H.K), and NIH Training Grant R90-DK071506.

Supporting Information Available: Assignments and values of ^{13}C chemical shifts, ^{1}H NMR spectra, Raman spectra, and water content evaluation of cortical bone. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA9081028