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Direct Evidence of Imino Acid−Aromatic Interactions in Native Collagen Protein by DNP-Enhanced Solid-State NMR Spectroscopy

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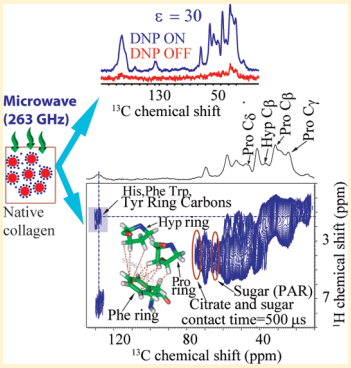
Supporting Information

ABSTRACT: Aromatic amino acids (AAAs) have rare presence (∼1.4% abundance of Phe) inside of collagen protein, which is the most abundant animal protein playing a functional role in skin, bone, and connective tissues. The role of AAAs is very crucial and has been debated. We present here experimental results depicting interaction of AAAs with imino acids in a native collagen protein sample. The interaction is probed by solid-state NMR (ssNMR) spectroscopy experiments such as $^{1}H-^{13}C$ heteronuclear correlation (HETCOR) performed on a native collagen sample. The natural abundance ^{13}C spectrum was obtained by dynamic nuclear polarization (DNP) sensitivity enhancement coupled with ssNMR, providing ∼30-fold signal enhancement. Our results also open up new avenues of probing collagen structure/dynamics closest to the native state by ssNMR experiments coupled with DNP.



SECTION: Biophysical Chemistry and Biomolecules

Collagen is a triple helical protein having functions such as providing strength and flexibility in bone, ligaments, structural component of connective tissues, skin, extracellular matrix (ECM), basement membranes, and cartilage.[1] It has also the functions of entrapment, local storage, and delivery of growth factors and cytokines, playing a key role at the time of organ development, wound healing, and tissue repair.[1,2] The collagen protein is composed of repeating units of Gly−Xaa−Yaa, where Gly is glycine and Xaa and Yaa can be any amino acid, but mostly hydroxyproline (∼38% abundance) and proline (∼28% abundance) imino acids occupy these positions.[1] Gly comes at every third residue and fits well in its triple helical structure due to the small size. Collagen is a well-studied system by different spectroscopic techniques, and most of the structural studies have been performed on synthetic, overexpressed, and purified collagen.[3] The structural study in the native state has been very limited.[4,5] The crystal structure of collagen has been solved by X-ray diffraction, with a resolution of 1.9 Å (Å).[3]The collagen present in bone provides template for mineralization (deposition of apatite crystals).[6] This mineralized collagen provides rigidity and flexibility to the bone.[7] In addition to apatite deposition, bone collagen interacts with noncollagenous proteins (NCPs) as well as citrate.[8] The presence of these interactions is the main reason for the native collagen structure being considerably different inside of bone from the extracted form of collagen.

Aromatic amino acids (AAAs) (having π electrons) form a very small fraction of total amino acids present in collagen. Phe and Tyr are 1.4 and 0.3% of total amino acid residues present inside of collagen of mammalian bone.[9] The role of AAAs in stabilizing protein structure via CH/π interaction in globular protein has been debated until detected recently by applying NMR spectroscopy.[10] Another report has described the role of these interactions in the organization of the collagen triple helix.[11] The kinetics of synthetic collagen-like peptides with AAAs shows a higher rate of aggregation promoted by the presence of AAAs in the helix.[11] The study indicated participation of CH/π interaction in the process of aggregation. However, to the best of our knowledge, there has been no report of direct detection of this interaction in native collagen protein. Various intermolecular interactions between aromatic residues (Phe, Tyr, and Trp) and Hyp/Pro are considered as major factors for promoting self-association in collagen triple helices.[11] These aromatic residues reside within the $(Gly-Xaa-Yaa)_n$ domain of type-I collagen. On the basis of the high-resolution crystal structure of model collagen-like peptide (PDB id: 1Q7D), CH/π interactions of Phe and imino acids were predicted between antiparallel pairs of triple helices.[11] These CH/π interactions played an important role in self-association of collagen triple helices, hydration, hydrogen bonding, and Hyp-mediated interaction.[11] In the native environment, collagen interacts with other proteins, glyco-

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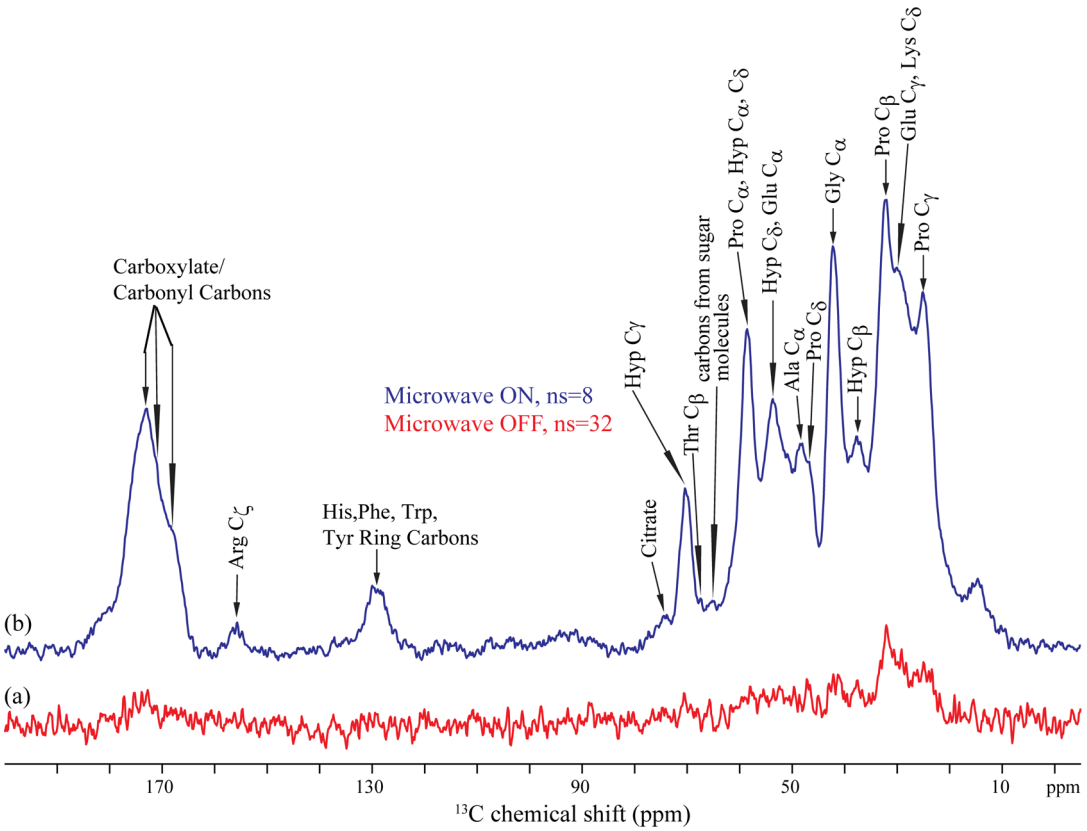

Figure 1. $^{1}H-^{13}C$ cross-polarization (CP) MAS spectra of native collagen, with (a) microwave OFF and (b) microwave ON. The native collagen sample was impregnated with a water solution of 12 mM AMUPol, using an 8 kHz spinning frequency, 108 K sample temperature, two dummy scans, and 8 s recycle delay. The number of scans is indicated on the figure (ns).

saminoglycans (GAGs), lipids, and inorganic surfaces. Hence, exclusive detection of this interaction in the native state becomes difficult.

Solid-state nuclear magnetic resonance (ssNMR) is one of the tools that have been used to elucidate native structural details of biological systems such as ion channels,[12] amyloid fibrils,[13] membrane proteins,[14] and so forth. All of these ssNMR methods have limited applicability for structural studies of native collagen due to the low concentration of organic components in bone and hence the low sensitivity of its natural abundance spectrum. Recently, sensitivity enhancements of the bone organic component spectrum have been attempted by incorporating relaxation agents such as Cu-EDTA[15] and gadolinium(III) diethylenetriaminepentaacetic acid (Gd-DTPA).[16] These methods improve the sensitivity of NMR signals from organic components of the bone matrix by 2−3 times. Another interesting approach is to incorporate ^{13}C and ^{15}N labeling in the organic part by enriching the entire animal model system.[17] While this approach is very demanding on animal handling and isotopic enrichment may adversely affect metabolism, it gives a well-resolved multidimensional NMR spectrum having the signature of an organic component inside of the bone matrix. This approach has also resulted in identifying the sugar-containing molecule polyadenosine diphosphate ribose (PAR) that may be implicated in calcification of the bone matrix.[17]

We have employed here sensitivity enhancement by the dynamic nuclear polarization (DNP)[18−24] based method in a native collagen sample.$^{1}H-^{13}C$ CP spectra of the native collagen sample show remarkable improvement in sensitivity of the ^{13}C spectrum when the microwave is ON (Figure 1b) over that when the microwave is OFF (Figure 1a). There is ∼30-fold enhancement in sensitivity of ^{13}C resonances. The sample particle size and surface area were sufficient to achieve enhancement (Supporting Information (SI) Figure S2). We have assigned most of the ^{13}C resonances from an organic matrix. Resonances from Gly, Hyp, and Pro from the backbone of collagen are similar to the spectrum recorded without DNP,[4,5,25] showing the structural integrity of collagen protein even at low temperature (SI Figure S1). Additionally, the DNP-enhanced ^{13}C spectrum shows a sharp peak at 14.6 ppm, which is assigned as Ala Cβ. The alanine Cβ chemical shift changes due to low temperature. We have also observed a significant increase in the intensity of ring carbon resonances from AAAs (Phe, Tyr, Trp) of collagen proteins, as well as side chain ring (His) resonances around 129 ppm Figure 1b. Similarly, we have observed resonances from citrates at 74−76 ppm, which is closer to the inorganic surface.[8] Resonances from ring carbons and citrate show very weak intensity when recorded without DNP, mainly due to low abundance, even with high signal averaging (SI Figure S1a and b). An increase in signal intensity due to DNP in the ring carbon (SI Figure S1) opens up the possibility to probe interactions present in collagen triple helices in the native state without any isotropic enrichment.

To probe the spatial proximity of ring carbons, DNP-enhanced two-dimensional $^{1}H-^{13}C$ heteronuclear correlation (FSLG-HETCOR) experiments were carried out at 150 and 500 μs contact times (Figure 2). Well-resolved $^{1}H-^{13}C$ spectra of native collagen are shown in Figure 2a. FSLG-HETCOR performed with a contact time of 150 μs showed one-bond C−

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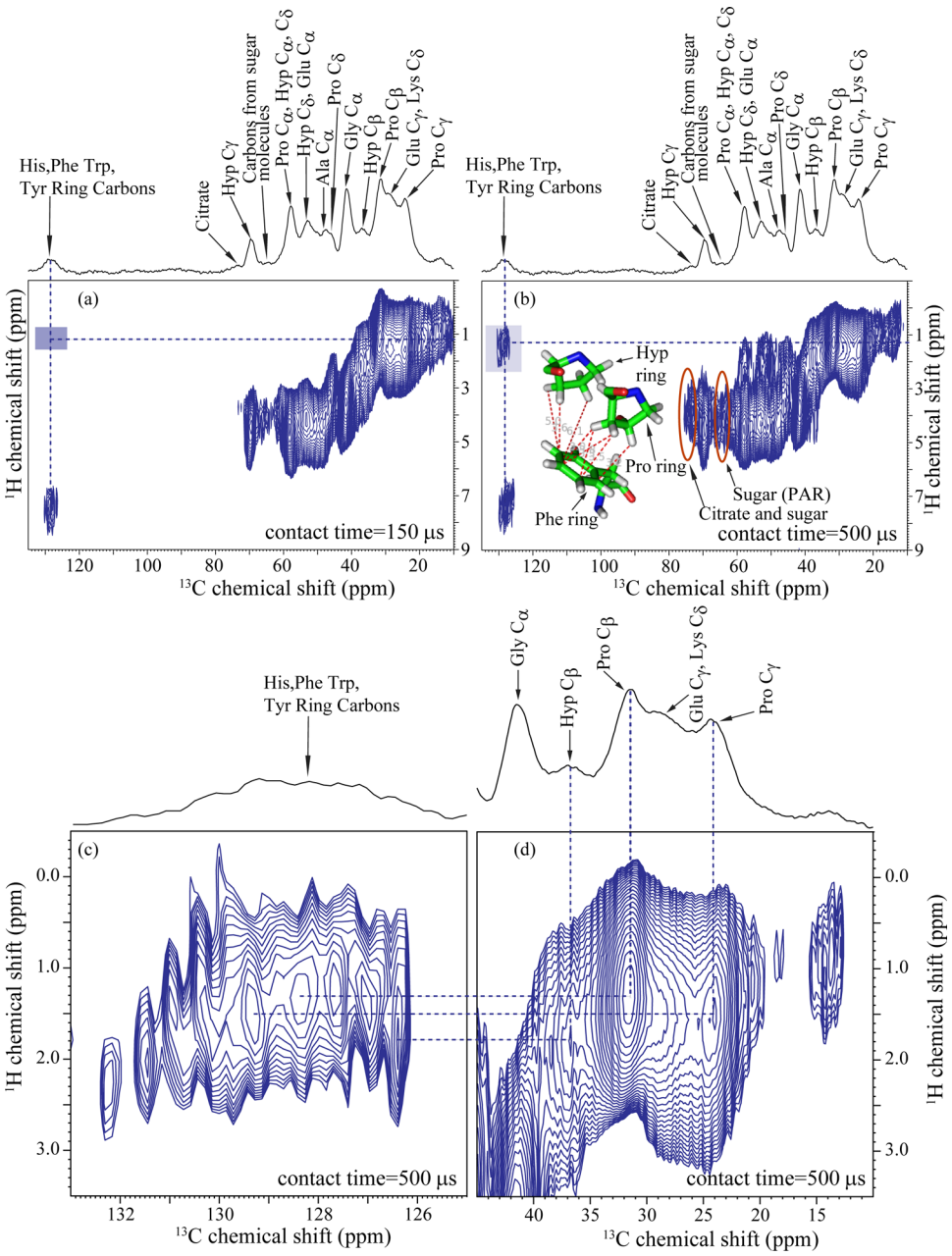

Figure 2. DNP-enhanced $^{1}H-^{13}C$ heteronuclear correlation (FSLG-HETCOR) spectrum of native collagen sample with (a) 150 μs correlation time with the blue square depicting the absence of the cross peak, (b) with a 500 μs correlation time showing the cross peak at the position of the blue square in (a), and (c) showing the correlation of aromatic resonances with ^{1}H attached to Pro Cβ and Pro Cγ, CH/π interactions.

H correlation and that with 500 μs showed long-range correlation.[26] This is reflected in observation of additional cross peaks in Figure 2b. We observed additional resonances originating from ring carbons at 1.42 ppm in ^{1}H dimension, Figure 2b and c. These additional peaks arise due to transfer of polarization[26] from aliphatic protons of imino acids to ring carbon. In Figure 2c and d, the dotted lines show that these additional peaks correlate to ^{1}H attached to Pro Cγ, Pro Cβ, and Hyp Cβ. These cross peaks appear due to CH/π interactions of ring carbons to Pro Cβ and Hyp Cβ. This interaction is not intraresidue because Phe Cβ/Hβ resonates at (39.978 ± 2.352) ppm/(2.990 ± 0.386) ppm (source: BMRB, Biological Magnetic Resonance Databank).[27] In our $^{1}H-^{13}C$ HETCOR spectrum (Figure 2c and d), there is no cross peak in the region showing correlation of the Cβ/Hβ group of aromatic residues with ring carbons. We observed correlation of aromatic resonances with imino protons at a contact time of 500 μs, confirming the CH/π interaction. Further, to show that this interaction is interhelix in nature, we have carried out cross-polarization (CP) dynamic simulations for both inter- and intrahelix. The simulation results (given in SI Figure 5) show that with a 500 μs contact time, the interhelix correlation will be predominant. Earlier, this interaction was shown in the high-resolution crystal structure of collagen peptide (PDB ID: 1Q7D).[11] It has been shown that Hyp16 Cδ along with Pro Cα and Cβ participate in the CH/π interaction in crystal packing. In the inset of Figure 2, a portion from the stick model is shown depicting the spatial proximity of the Phe 9 aromatic ring to Pro 15 and Hyp 16 in an integrin binding collagen peptide (PDB ID: 1Q7D).[11] This proximity of these interchain

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residues paves the way to CH/π interaction between imino acid groups and the aromatic ring[11] (SI Figure 4). Our experimental results demonstrate that Hyp Cβ, Pro Cβ, and Pro Cγ are in close proximity with the aromatic ring.

It was also suggested that the catalysis of type-I collagen fibrillogenesis by nonhelical telopeptides owes to specific intermolecular CH/π interactions between aromatic residues in the telopeptides and Pro/Hyp residues within the triple-helix.[11] We could detect the CH/π interactions in our system due to enhanced sensitivity by the DNP method. Figure 2b and c shows that these aromatic residues are correlated with ^{1}H attached to Pro Cγ and Pro Cβ due to spatial proximity. Even with low abundance of AAA residues in collagen, this interaction might be playing an important role at the time of assembly of collagen.[11]

Apart from the CH/π interaction, other key molecules were observed due to enhanced sensitivity of the organic part of the bone matrix. Figure 2 shows cross peaks in the region of citrate[8] resonance (73−76 ppm), which is closest to the inorganic part. We also observed resonances from PAR carbons at 64−66 ppm. The enhancement in the sensitivity encourages the possibility of performing DNP-enhanced ^{13}C−^{13}C correlations,[28,29] ^{1}H−^{15}N correlation,[21] and various other 3D experiments in the absolute native environment of bone without any isotopic enrichment. DNP will pave the way to the design of experiments that can elucidate native structural details of the organic matrix, which may play a pivotal role in many biomaterial applications.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures, including the ^{1}H−^{13}C cross-polarization spectrum of bone powder, scanning electron microscopic image of bone powder, two-dimensional ^{1}H−^{13}C FSLG-HETCOR spectrum of bone powder, the spatial proximity of Phe 9 to Pro 15 and Hyp 16 in the crystal structure of integrin binding collagen, and the inter- and intrahelixCP dynamic simulation, and experimental details along with materials and methods and details of the simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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