^{13}C and ^{1}H Resonance Assignment of Mercerized Cellulose II by Two-Dimensional MAS NMR Spectroscopies

Hiroyuki Kono,*,† Yukari Numata,‡ Tomoki Erata,*,‡ and Mituo Takai‡

Bruker BioSpin Company Ltd., Tsukuba, Ibaraki 305-0051, Japan, and Division of Molecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo, Hokkaido 060-8628, Japan

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ABSTRACT: Through-bond $^{13}C-^{13}C$ and $^{13}C-^{1}H$ correlations in the crystal structure of cellulose II were determined by the two-dimensional (2D) refocused INADEQUATE and MAS-J-HMQC spectra of the mercerized cellulose, which was biosynthesized by *Acetobacter xylinum* ATCC 10245 from the uniformly labeled glucose (D-(U-$^{13}C_6$)glucose). Mercerization of the bacterial cellulose was achieved in a solution of 20% NaOH in water for 2 weeks. In the 2D INADEQUATE spectrum of cellulose II, two sets of the through-bond $^{13}C-^{13}C$ connectivities from C1 through C6 were clearly observed, which enabled us to completely assign the complex 1D CP/MAS ^{13}C spectrum of cellulose II. Following the ^{13}C assignment, the ^{1}H resonance for the proton(s) attached to each carbon of cellulose II could be assigned by use of the 2D MAS-J-HMQC spectrum of the mercerized cellulose.

Introduction

Cellulose is the most abundant biopolymer on earth and is widely used in commercial materials. The polymer, which is composed of β-(1→4)-D-glucopyranose repeating units, forms fibrous structures with high crystallinity. In nature, cellulose occurs in the cell walls of all plants and algae, and some bacteria produce cellulose extracellularly. As shown in Figure 1, the native cellulose is easily converted into a considerable number of allomorphs by certain chemical and thermal treatments.[1] Among the allomorphs of cellulose, the major forms are cellulose I and cellulose II; the crystalline part of the native celluloses is cellulose I, and cellulose II is obtained from cellulose I by a regeneration or mercerization process. Following the report of the first X-ray diffraction image of cellulose in 1913,[2] a number of structural models of cellulose I[3−5] and II[6−8] have been proposed on the basis of the results obtained by X-ray diffraction with model building and conformational analysis. The unit cell parameters and molecular structure for the cellulose chain in each allomorph, however, depended on the origins of the specimen used for the analysis. Therefore, considerable questions remain regarding the structures of cellulose I and II.

In 1984, the solid-state NMR technique allowed new insights into the crystal structure of cellulose I.[9,10] Widespread use of the cross-polarization/magic angle spinning (CP/MAS) ^{13}C NMR technique in cellulose science led to the revelation, by Atalla and VanderHart,[9,10] that cellulose I is a composite of two distinct crystalline allomorphs, namely, cellulose I_α and I_β. After the discovery of the dimorphism in cellulose I, reinvestigation of the structure of cellulose I, which had been determined before the discovery by Attala and VanderHart, was made using solid-state NMR, X-ray diffraction, and so forth. As a result, the unit cells of both allomorphs were experimentally determined by electron diffraction analysis,[11] and the exact atomic coordinates and hydrogen-bonding system in only cellulose I_β were revealed by the combination of fiber neutron diffraction data with model building and conformational analysis.[12]

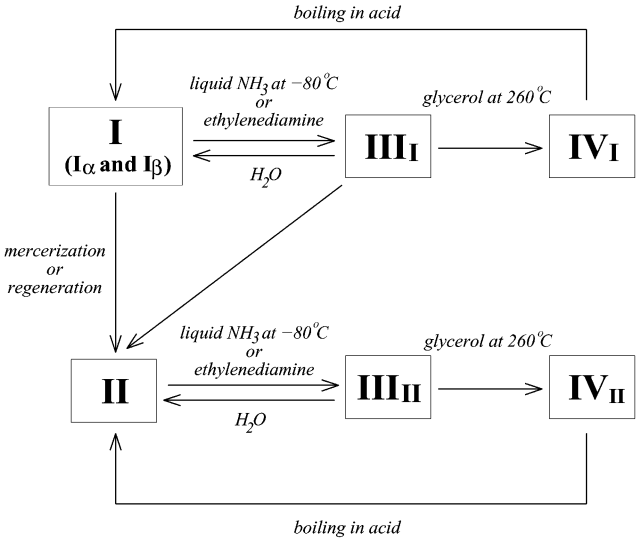

Figure 1. Polymorphy of cellulose.

In the case of cellulose II, the structure had been widely accepted to consist of a two-chain unit cell and a $P2_1$ space group where the two chains were in antiparallel arrangement and crystallographically independent.[6,7] The two chains had been considered to show identical backbone conformations but different conformations for the torsion angles around the C5−C6 bond; the chains located at the corner of the unit cell have a *tg* conformation while those at the center have a *gt*. On the other hand, the CP/MAS ^{13}C NMR spectrum of cellulose II suggested that the hydroxymethyl groups of both chains all had the *gt* conformation, since the ^{13}C resonance for C6 of this allomorph appears around 64 ppm.[13] The conflict between the NMR spectroscopic data and the structure of cellulose

† Bruker BioSpin Company Ltd.
‡ Hokkaido University.
* To whom correspondence should be addressed: Tel +81-298-52-1235, Fax +81-298-858-0322, e-mail hiroyuki.kono@bruker-biospin.jp (H.K.), Tel/Fax +81-11-706-6566, e-mail erata@dove-mc.eng.hokudai.ac.jp (T.E.).

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II determined by X-ray diffraction analysis[6,7] prompted a reinvestigation of the crystal structure of this allomorph. Recently, a new structure was proposed on the basis of neutron[14] and synchrotron[15] diffraction data.

As described above, CP/MAS ^{13}C NMR has contributed to elucidation of the crystal structures of cellulose allomorphs and conformations for cellulose chains. However, the structures of cellulose crystals could not be determined on the basis of only 1D CP/MAS ^{13}C NMR spectra because some of the ^{13}C resonance lines in the spectra had not been completely assigned. In the CP/MAS ^{13}C spectra of the cellulose allomorphs, the region between 67 and 62 ppm is assigned to C6 of the primary group, the resonance lines at 91−80 ppm are associated with C4, and those at 109−103 ppm are associated with C1 anomeric carbon.[16] Since the ^{13}C spectral line and splitting of the C1, C4, and C6 lines are relatively simple, research on the structure of cellulose by employing the solid-state ^{13}C NMR generally focused on the analysis of these carbon spectral lines. On the other hand, since the remaining C2, C3, and C5 lines overlap each other in the narrow region between 80 and 70 ppm, these carbons were not assigned precisely. For cellulose I, although the ^{13}C resonance assignment of the C2, C3, and C5 regions was carried out on the basis of the contribution of the ^{13}C spin−lattice time measurements[17] and diffusion experiments,[18] the results of the assignment differed according to the methods used for the signal assignments. With the development of NMR instruments, many solid-state multidimensional techniques, mostly 2D, have been proposed for providing through-bond and through-space correlation between a pair of nuclei such as (^{13}C, ^{13}C)[19,20] and (^{13}C, ^{1}H),[21−23] thereby yielding information about the conformation and internuclear distance of solid materials. Very recently, we applied the solid-state 2D INADEQUATE technique[20] to natural abundance *Cladophora sp.* (I_α-rich) and tunicate (I_β-rich) celluloses and assigned all ^{13}C resonance lines observed in the 1D spectra of cellulose I_α and I_β by use of the 2D through-bond ^{13}C−^{13}C correlation spectra of these celluloses.[24] As a result, it was revealed that both cellulose I_α and I_β contain two magnetically nonequivalent anhydroglucose residues in the unit cells and that the primary difference between cellulose I_α and I_β was in the conformations of anhydroglucose residues contained in the cellulose chains.

In this paper, we provide the complete assignment of the ^{13}C resonance of cellulose II by using the 2D INADEQUATE technique[20] for the elucidation of the structure of this allomorph. Herein, ^{13}C-enriched cellulose II, which was biosynthesized using *Acetobacter xylinum* (*A. xylinum*) ATCC10245 strain from D-($^{13}C_6$)-glucose followed by mercerization treatment, was used for the NMR experiment. After the complete assignment of the ^{13}C resonances for cellulose II, the ^{1}H chemical shift of proton(s) attached to each carbon was assigned by use of the MAS-J-HMQC[21] spectrum of the ^{13}C-enriched cellulose II. From the data on the ^{13}C and ^{1}H chemical shifts obtained from these 2D NMR spectra, the structures of the cellulose II proposed on the basis of X-ray[6,7] and synchrotron data[15] were revaluated, and these results are also described herein.

Experimental Section

Cellulose Sample. Purification of *Valonia* cellulose was performed according to the method reported previously.[25−27] An air-dried *Valonia sp.* sample specimen was cut into small pieces, soaked with 4% aqueous HCl overnight at ambient temperature, washed thoroughly with water, heated at 37 °C for 4 h in a 1% NaOH solution, and thoroughly washed with water a final time. The whole procedure was repeated twice. The complete lipid extraction from the cellulose specimen was performed with acetone. Bleaching with 0.5% $NaClO_2$ solution at ambient temperature for 10 h followed. The completely white particles were sampled, washed thoroughly with deionized water, and then freeze-dried. The purified cellulose was incubated in 40% sulfuric acid at 60 °C for 2 h to hydrolyze the amorphous part of the cellulose sample under continuous stirring. It was then filtered, washed thoroughly with a continuous stream of cold water, dialyzed against deionized water for several days, and freeze-dried.

^{13}C-enriched cellulose was biosynthesized by *A. xylinum* ATCC10245 from a Hestrin and Schramn medium[28] containing 1% (v/v) of ethanol and 10 mol % of D-($^{13}C_6$)glucose (Cambridge Isotope Laboratories, Inc., Cambridge, MA; the isotropic purity of all the labeling compounds was 99%) in unlabeled glucose. Biosynthesis was carried out at 28 °C for 7 days under static conditions. The formed cellulose pellicle was purified by boiling in 1% aqueous NaOH for 8 h, washed with distilled water, and freeze-dried. After the purified cellulose pellicle was cut into small pieces with scissors, the cellulose sample was incubated in 40% H_2SO_4 solution at 37 °C for 8 h to thoroughly hydrolyze its noncrystalline region under continuous stirring. It was then filtered, washed thoroughly with a continuous stream of cold water, dialyzed against deionized water for 7 days, and freeze-dried.

Mercerization of Cellulose. The purified *Valonia* cellulose was incubated in 20% (w/w) aqueous NaOH solution at ambient temperature for 2 h. The suspension of cellulose was diluted with a large amount of deionized water, and the suspended cellulose was neutralized by repeated washing and centrifugation. The purified bacterial cellulose was soaked with a small amount of deionized water for 1 day. 25% (w/w) NaOH solution was added to the bacterial cellulose suspension to form the final concentration of 20% (w/w). After 2 weeks of treatment at ambient temperature, the bacterial cellulose suspension was neutralized using a method similar to that for the neutralization of *Valonia* cellulose. These mercerized cellulose specimens were incubated in 40% H_2SO_4 solution at 37 °C for 4 h to enhance their crystallinities under continuous stirring. They were then filtered, washed thoroughly with a continuous stream of cold water, dialyzed against deionized water for 7 days, and freeze-dried. The mercerized celluloses were stored in a desiccator until NMR measurements.

Solid-State NMR Experiments. All NMR experiments were performed on a Bruker AV 300 wide-bore spectrometer (proton frequency, 300.1 MHz). CP/MAS ^{13}C NMR spectra were obtained using a Bruker 7 mm double-tuned MAS probe. The sample volume was about 300 μL, and the MAS frequency was set to 6 kHz. The ^{1}H radio-frequency field strength was set to 78 kHz during acquisition using a TPPM[29] scheme. The phase modulation angle for the TPPM decoupling was set to 15.0°, and the flip-pulse length was optimized to 5.8 μs to yield the optimal ^{13}C resolution of the α-carbon resonance of D-glycine.[24] The contact time for cross-polarization and the recycle delay were set to 1 ms and 2 s, respectively. The carbon−carbon 2D refocused INADEQUATE spectrum of ^{13}C-enriched cellulose II was recorded according to the method described previously[24] using a Bruker 7 mm double-tuned MAS probe. The pulse sequence for the refocused INADEQUATE experiment is shown in Figure 2A and has been described in detail by Lesage et al.[20] The sample volume was about 300 μL, the MAS frequency was set to 7 kHz, and a ramped-amplitude cross-polarization sequence[30] was used for protons. The τ delay was set to 3.4 ms and the contact time to 1 ms. The proton decoupling field strength was 78.1 kHz using a TPPM scheme. The phase modulation angle for the TPPM decoupling was set to 15.0°, and the flip-pulse length was optimized to 5.8 μs to yield the optimal ^{13}C resolution. The 180° pulse on carbons was set to 6.4 μs. Quadrature phase detection was achieved using the TPPI method.[31] The recycle delay was set to 2 s. A total of 384 t_1 acquisitions with 32 scans each were collected. The carbon−proton 2D MAS-J-HMQC spectrum of ^{13}C-

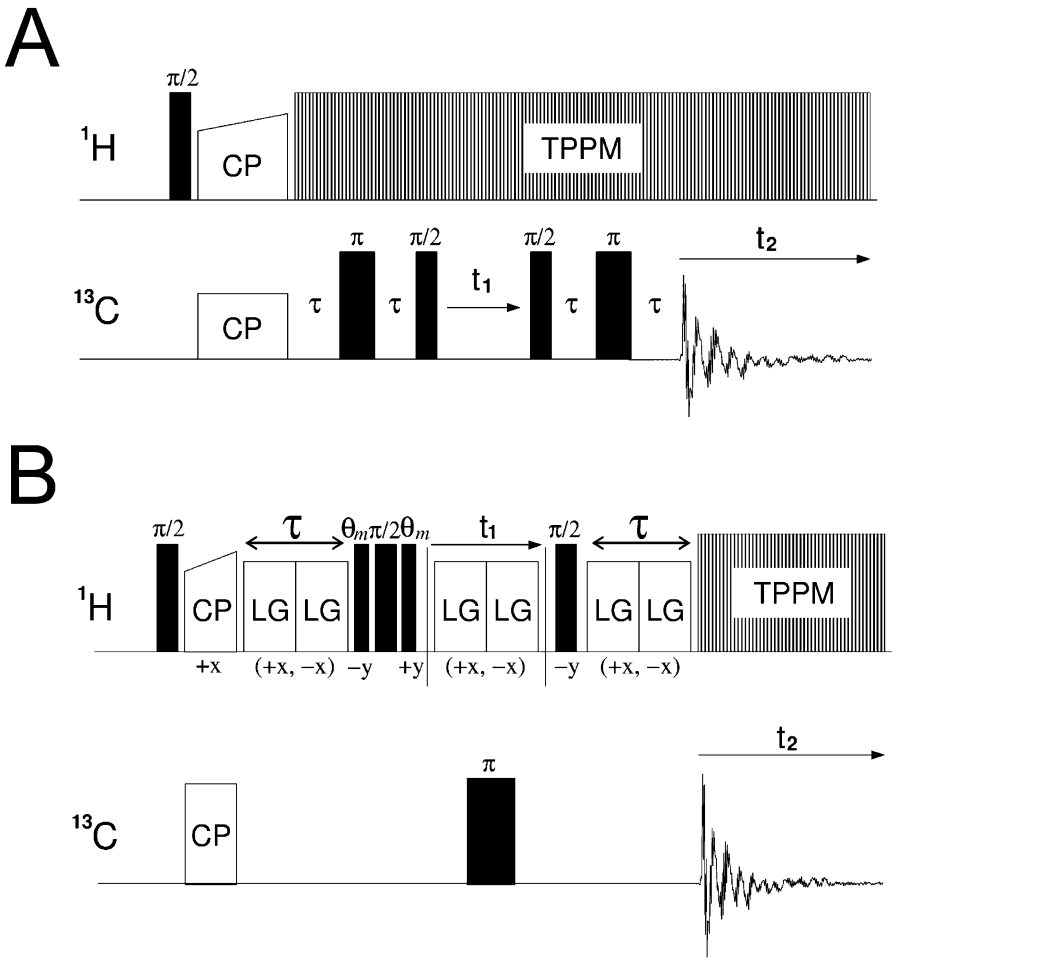

Figure 2. Pulse sequences for the 2D (^{13}C, ^{13}C) refocused CP INADEQUATE (A) and 2D (^{13}C, ^{1}H) MAS-J-HMQC experiments. The phase cycle for the INADEQUATE and that for MAS-J-HMQC were adapted from refs 20 and 21, respectively. θ_m is a magic angle (54.7°) pulse.

enriched cellulose II was obtained by use of a Bruker 4 mm double-tuned MAS probe according to the method of Lesage et al.[21] The pulse sequence for the MAS-J-HMQC experiment is shown in Figure 2B, and the details of the experiment are described in ref 21. The sample volume was restricted to about 10 μL and set to the center of the rotor to improve the radio-frequency field homogeneity.[32] The MAS frequency was set to 17.5 kHz, and the delay τ was set to 2.4 ms. The proton radio-frequency field strength was set to 86.2 kHz during the τ delay (FS-LG decoupling[33,34]) and was set to 92.6 kHz during acquisition (TPPM decoupling). Two off-resonance pulses with opposite phases (i.e., $+x$, $-x$ in Figure 2B) during the FS-LG decoupling were set to 5.8 μs. The magic angle pulse length was 1.8 μs. For the cross-polarization step, the radio-frequency field strength was set to 64.0 kHz for carbon, while a ramped radio-frequency field was applied on protons and matched to obtain the optimal signal. The contact time was set to 500 μs, and the recycle delay was 2 s. Quadrature phase detection was achieved using the States method.[35] A total of 256 t_1 acquisitions with 32 scans each were collected. In all NMR experiments, the ^{13}C chemical shifts were calibrated through the carbonyl carbon resonance of d-glycine as an external reference at 176.03 ppm. In the refocused INADEQUATE experiments, the double quantum frequency (DQ) scale (vertical axis) was calibrated by use of an L-alanine mixture prepared by dissolving and recrystallizing 20% fully ^{13}C-enriched L-($^{13}C_3$)alanine together with 80% nonenriched L-alanine. Since the assignments for the alanine ^{13}C spectrum are CH_3 (20.2 ppm), CH (50.7 ppm), and COOH (178.1 ppm), DQ of correlation between CH_3 and CH and that between CH and COOH in the INADEQUATE spectrum of the alanine mixture were set to 70.9 and 228.8 ppm, respectively. In the MAS-J-HMQC experiment, ^{1}H chemical shifts were referenced by setting the H_β resonance of the alanine mixture to 1.0 ppm. In addition, the proton chemical shift scale was corrected by the scaling factor of $1\sqrt{3}$, since the ^{1}H chemical shift dispersion is scaled by the factor during the FS-LG periods.[33]

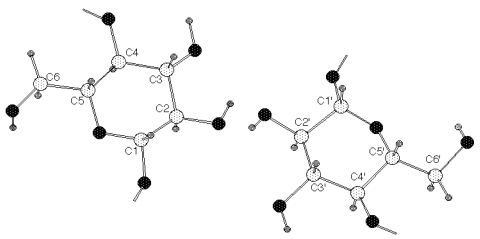

Figure 3. Two chains in the unit cell of cellulose II. The asymmetric unit consisting of two anhydroglucose units is shown in this figure.

Results and Discussion

Assignment of ^{13}C Spectrum of Cellulose II. Figure 4A shows the CP/MAS ^{13}C NMR spectrum of cellulose II that was obtained by mercerization treatment of *Valonia* cellulose. In the ^{13}C spectrum of cellulose II, a doublet at 107 and 105 ppm was easily assigned to C1 anomeric carbon, that at 89 and 87 ppm was associated with C4, and those at 63 and 62 ppm were associated with C6 of the primary group. The cluster of resonance lines observed in the region of 78−70 ppm, which form a triplet at 77, 75, and 73 ppm, are attributed to C2, C3, and C5, although the resonance

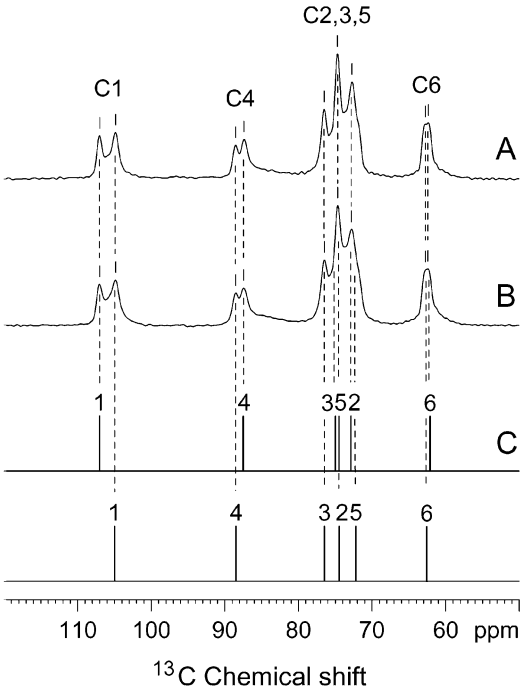

Figure 4. CP/MAS ^{13}C NMR spectra of the mercerized cellulose II obtained from *Valonia* (A) and ^{13}C-enriched bacterial cellulose (B). ^{13}C line spectra of the two magnetically nonequivalent anhydroglucose residues composing cellulose II (C). The lines indicate the ^{13}C chemical shifts of each carbon of the anhydroglucose residues. The line spectra were determined by the INADEQUATE spectrum of the ^{13}C-enriched bacterial cellulose (Figure 5).

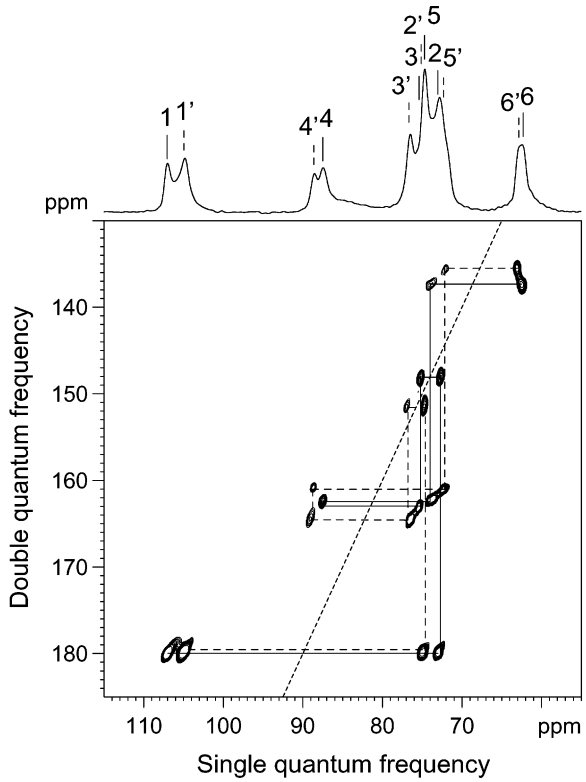

Figure 5. 2D INADEQUATE spectrum of the mercerized cellulose II obtained from ^{13}C-enriched bacterial cellulose. The through-bond connectivities between carbons of two magnetically nonequivalent anhydroglucose residues composing cellulose II are indicated by the solid and dotted lines.

lines for these carbons have not been assigned. For the complete assignment of this cluster of resonance lines for C2, C3, and C5 of cellulose II, we attempted to obtain 2D a scalar-coupled through-bond ^{13}C−^{13}C correlation spectrum of cellulose II by means of the refocused INADEQUATE technique reported by Lesage et al.[20] In the solid-state NMR, since the amount of natural abundance ^{13}C nuclei is about 1.11%, it is difficult to detect a correlation peak for a pair of through-bonded ^{13}C−^{13}C nuclei in natural abundance compounds. ^{13}C-enriched cellulose II was, therefore, prepared by mercerizing of bacterial cellulose, which was biosynthesized by *A. xylinum* ATCC10245 in a culture containing 10% of D-(U-$^{13}C_6$)glucose mixed with 90% of natural abundance D-glucose. As shown in Figure 4B, the mercerized bacterial cellulose showed the ^{13}C spectral pattern of highly crystallized cellulose II, and the chemical shift of each peak of the spectrum of bacterial cellulose II was in complete agreement with that of *Valonia* cellulose II. Therefore, the through-bond ^{13}C−^{13}C correlations in the cellulose II were determined by use of the ^{13}C-enriched cellulose II sample.

Figure 5 shows the 2D INADEQUATE spectrum of bacterial cellulose II. The corresponding 1D spectrum (Figure 4B) is indicated at the top of the 2D spectrum. In the spectrum, two directly bonded carbons share a common double quantum frequency (horizontal connection), and along a vertical direction corresponding to a given carbon's single quantum frequency, there are a number of features equal to the number of C−C bonds that the given carbon participates in. It is, therefore, possible, starting from the anomeric C1 doublet of cellulose II appearing at 107.1 and 105.0 ppm in the single quantum dimension, to assign sequentially each carbon resonance of an anhydroglucose residue using the INADEQUATE spectrum. In addition, the correlation peaks are spaced symmetrically about the diagonal with slope 2 in the INADEQUATE spectrum. It is, therefore, possible to unambiguously assign carbon connectivities by analyzing the shift difference from the diagonal with the slope 2 in the case that many couplings are very similar and cannot be completely resolved. As indicated by the solid lines in the 2D spectrum, the ^{13}C signal appearing at 107.1 ppm was correlated with that appearing at 72.8 ppm at the DQ frequency of 180 ppm. This indicated that the signal at 72.8 ppm should be assigned to C2. The 72.8 ppm position shows a second clear correlation with the C3 resonance at 75.1 ppm (DQ 148 ppm). Using a method similar to that for sequential assignment of C2 and C3 resonances, the scalar-coupled correlations of C3, C4, C5, and C6 resonances could be determined. As displayed by the solid lines in the 2D correlation spectrum, the C3 signal appearing at 75.1 ppm was correlated with the C4 signal at 87.4 ppm (DQ 163 ppm), the C4 signal was correlated with C5 at 74.3 ppm (DQ 162 ppm), and the C5 signal was correlated with the C6 signal at 62.4 ppm (DQ 137 ppm). In the INADEQUATE spectrum, the C5−C6 cross-peaks are weak compared with the other cross-peaks, probably because of a different carbon−carbon scalar coupling constant (J_{CC}) in cellulose II. The efficiency of conversion from single quantum coherence to double quantum coherence depends on the τ delay in the INADEQAUTE pulse program (Figure 2A), and the degree of conversion into double quantum coherence is maximum for $\tau = 1/(4J_{CC})$ and is zero for $\tau = 1/(2J_{CC})$. The τ delay was set to 3.4 ms for these INADEQUATE experiments. If another τ were used, the

Table 1. ^{13}C and ^{1}H Chemical Shifts of the Anhydroglucose Residues in the Structure of Cellulose II and I_β Allomorphs

		^{13}C shifts/ppm (^{1}H shift/ppm)					
allomorph		C1 (H1)	C2 (H2)	C3 (H3)	C4 (H4)	C5 (H5)	C6 (H6)
cellulose II	residue 1[a]	107.1 (4.2)	72.8	75.1	87.5 (3.8)	74.3	62.6 (3.6)
	residue 2[a]	105.0 (4.4)	74.8	76.5 (3.4)	88.7 (3.8)	71.7	63.0 (3.2)
	shift difference[b]	1.9 (0.2)	2.0	1.4	1.2 (0)	2.6	0.4 (0.4)
cellulose I_β	residue 1[c]	106.1 (4.5)	71.3	74.9 (3.2)	88.0 (3.4)	70.6	65.6 (4.5, 4.5)
	residue 2[c]	104.0 (4.9)	71.0	74.2 (3.8)	88.9 (3.1)	72.2 (3.7)	65.0 (3.9, 5.1)
	shift difference[b]	1.9 (0.4)	0.3	0.7 (0.6)	0.9 (0.3)	1.6	0.4

[a] Two magnetically nonequivalent anhydroglucose residues composing cellulose II. [b] ^{13}C and ^{1}H chemical shift difference between residues 1 and 2. [c] ^{13}C and ^{1}H chemical shifts of two magnetically nonequivalent anhydroglucose residues composing cellulose I_β, which were assigned previously.[27]

C5−C6 cross-peaks might become stronger while the other signals would become weaker. For the second anhydroglucose residue, the C1 resonance appearing at 105.0 ppm correlates with C2 at 74.8 ppm (DQ 180 ppm). Dotted lines connect peaks for this residue in Figure 5. That C2 resonance has a clear correlation with the C3 resonance at 76.5 ppm (DQ 151 ppm), C3 also has a correlation with the downfield signal of the C4 doublet at 88.7 ppm (DQ 165 ppm), and this C4 resonance is correlated with C5 at 71.7 ppm (DQ 160 ppm). Finally, the ^{13}C signal of C6, which binds to the C5 appears at 71.7 ppm, is assigned to the downfield resonance of the C6 doublet at 62.8 ppm (DQ 135 ppm). From these findings obtained from the 2D $^{13}C-^{13}C$ correlation spectrum of cellulose II, the 1D CP/MAS ^{13}C NMR spectrum of cellulose II was completely assigned for the first time, as shown at the top of the 1D spectrum in Figure 5. In addition, since the 2D INADEQUATE spectrum provides the correlations between two directly bonded carbons, it was concluded that the group of six carbons connected by the dotted lines and the six connected by the solid lines correspond to the ^{13}C nuclei of magnetically nonequivalent anhydroglucose residues contained in the unit cell of cellulose II. The ^{13}C chemical shifts of each anhydroglucose residue contained in cellulose II are summarized in Table 1 and shown by the lines in Figure 4C.

Through-Bond $^{13}C-^{1}H$ Correlations in Cellulose II. Following the complete ^{13}C resonance assignment of cellulose II, ^{1}H chemical shifts of individual protons attached to carbon nuclei of this allomorph were assigned with the 2D MAS-J-HMQC spectrum. In this experiment, a relatively high MAS frequency of 17.5 kHz was used for the sample rotation in order to suppress the strong $^{1}H-^{1}H$ homonuclear dipolar interaction and to enhance ^{1}H resolution. In addition, the sample volume was restricted to about 10 μL and set to the center of the sample rotor for the enhancement of the radio-frequency field homogeneity.[32] Figure 6 shows the one-bond MAS-J-HMQC spectrum of ^{13}C-enriched bacterial cellulose II. In the 2D spectrum, the horizontal axis corresponds to ^{13}C, and the vertical axis corresponds to ^{1}H. Since the 2D MAS-J-HMQC spectrum provides the pure in-phase chemical shift correlations between pairs of bonded proton(s) and carbon in a manner analogous to the liquid HMQC[36] spectra, the proton traces extracted in the ^{1}H chemical shift dimension from the MAS-J-HMQC spectrum of cellulose II enabled us to easily assign ^{1}H chemical shifts of the protons attached to carbon nuclei. As indicated by the solid and dotted lines in the 2D spectrum, each doublet for C1, C4, and C6 of cellulose II provides a $^{13}C-^{1}H$ correlation peak. A doublet for C1 at 107.1 and 105.0 ppm was correlated with their attached protons at the ^{1}H chemical shift of 4.2 and 4.4 ppm in the column

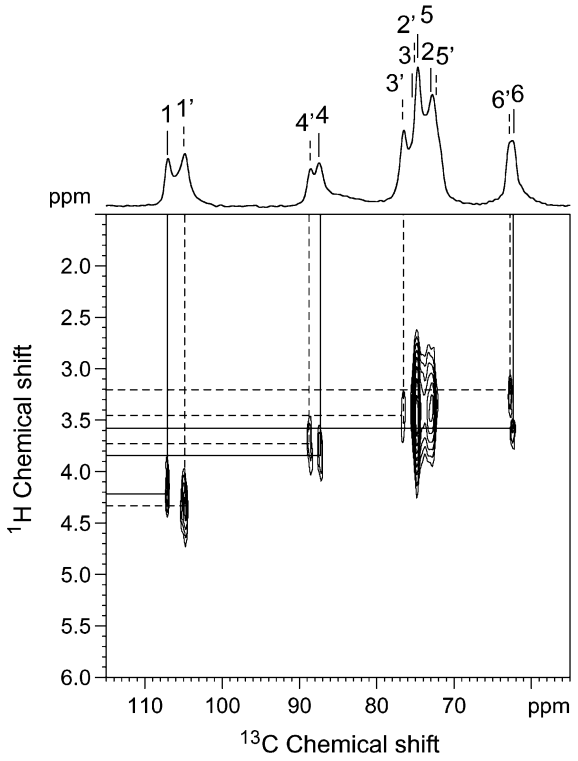

Figure 6. 2D MAS-J-HMQC spectrum of the mercerized cellulose II obtained from ^{13}C-enriched bacterial cellulose. The solid and dotted lines indicate the ^{1}H and ^{13}C chemical shifts of the $^{13}C-^{1}H$ through-bond correlation of the cellulose II.

direction, respectively, and ^{1}H chemical shifts of protons attached to C4 at 88.7 and 87.4 ppm could be assigned to 3.8 ppm. In the case of protons that bonded to a doublet of C6, each C6 resonance line provided one $^{13}C-^{1}H$ correlation signal, although each carbon signal binds to two proton nuclei. This indicated that two protons attached to each C6 have the same chemical shift. The downfield C6 signal appearing at 62.4 ppm correlated with two attached protons at the ^{1}H chemical shift of 3.6 ppm, and the other ^{13}C signal at 63 ppm correlated with attached protons at 3.2 ppm. With respect to the protons attached to C2, C3, and C5 of the cellulose II, the ^{1}H shift of the protons attached to C3 at 76.5 ppm could be assigned to 3.4 ppm. However, since the cross-peaks correlated with the other C3, C2, and C5 overlap each other in the 2D spectrum, the ^{1}H chemical shifts of the proton nuclei attached to these carbons could not be assigned precisely. The results of the assignment of the ^{1}H chemical shifts of cellulose II are summarized in Table 1. In general, ^{1}H resonances of solids with high proton density such as cellulose are considerably broadened by strong $^{1}H-^{1}H$ homonuclear dipolar interactions.

If these $^{13}C-^{1}H$ correlation experiments of cellulose II could be performed by use of the NMR instrument with higher magnetic field strength and higher MAS frequency of sample rotation which decreases the ^{1}H homonuclear dipolar interactions, ^{1}H chemical shift of protons at C2, C3, and C5 might be precisely assigned and $^{1}H-^{13}C$ correlation peaks associated with C6 carbons might be split into two.

As summarized in Table 1, the ^{13}C resonance lines for cellulose II were completely assigned by the 2D INADEQUATE experiment of the ^{13}C-enriched bacterial cellulose II that was biosynthesized from D-(U-$^{13}C_6$)-glucose. The results revealed that all the ring carbons of cellulose II were doublets. In addition, it was directly proven by analysis of the 2D through-bond $^{13}C-^{13}C$ correlation spectrum that there are two kinds of glucopyranose residues in the unit cell of cellulose II since two sets of the $^{13}C-^{13}C$ correlation signals from C1 through C6 could be observed in the 2D spectrum. As shown in Figure 4C, therefore, the 1D ^{13}C spectrum of cellulose II was simply characterized to be an overlap of the ^{13}C subspectra of the two kinds of glucopyranose residues, indicating that there are two magnetically nonequivalent sites in the unit cell of cellulose II. The existence of the two kinds of anhydroglucose residues contained in the unit cell of cellulose II was also confirmed by the MAS-J-HMQC spectrum, in which each ^{13}C doublet for C1, C4, and C6 of this allomorph provided a corresponding $^{13}C-^{1}H$ correlation peak with the proton nuclei attached to each carbon.

The crystal structure of cellulose II has previously been refined by means of X-ray data and potential energy calculation.[6−8] According to the most famous X-ray structure proposed by Kolpack and Blackwell[6] and Stipanovic and Sarko,[7] cellulose chains in the cellulose II was considered to crystallize in the monoclinic unit cell of space group $P2_1$, which requires one anhydroglucose unit on each of the screw axes of the corner and center to form the antiparallel packing. The most important features of the X-ray structure are the different positions of O6 in the *gt* position at the center chain and O6*tg* at the corner chain and the similar chain conformations of the backbone of both chains. Recently, the refinement of the crystal structure of cellulose II was performed by Langan et al.[14,15] based on neutron scattering data,[14] and their results led them to propose a different placement of the primary hydroxyl group at C6 in the chain conformation of cellulose II. In addition, a very recent synchrotron investigation[15] on crystalline mercerized cellulose II led to further improvement of the structure of cellulose II with the O6 of both chains in the *gt* position, although with slight differences in the torsion angle around the C5−C6 bond of the two individual chains. As shown in Table 1, the shift difference of C6 in the two chains in cellulose II is only 0.4 ppm, while those of the other carbons are over 1 ppm. If the anhydroglucose inequivalence within the unit cell corresponds to difference between chains, the two chains in cellulose II may have nearly equivalent conformations for the hydroxymethyl group. However, the relation of the ^{13}C chemical shift of each carbon of the cellulose chains to the conformation around that carbon nucleus has not been clearly established, and the strength of and directions of hydrogen bondings are also expected to influence chemical shift.

In addition to the conformations for the hydroxymethyl groups of the two chains in cellulose II, Langan et al. reported that the glycosidic torsion angles of O5−C1−O1−C4 and C1−O1−O4−C3 in the center chain are different from those in the corner chain.[14] With respect to the structure of cellulose I_β, which contains two parallel cellulose chains in the monoclinic unit cell,[11] we previously suggested that the conformation for glycosidic linkage in one chain differs from that in the other chain.[24,25] As summarized in Table 1, the C1 and C4 chemical shifts of one chain of cellulose I_β are at 106.1 and 88.0, respectively. In the case of the other chain of cellulose I_β, the ^{13}C resonance line for C1 is shifted upfield at 104.0, while the C4 resonance is shifted downfield at 88.9 ppm. Since it had been already pointed out in the previous publications[37−39] that the C1 and C4 chemical shifts are related to the conformations of the β-1,4 linkage, we conjugated that the noticeable chemical shift change for C1 and C4 of the two chains in cellulose I_β might be due to two conformations for β-1,4 glycosidic linkages in the unit cell of cellulose I_β. This was recently confirmed by the neutron diffraction analysis of this allomorph.[12] If the two kinds of anhydroglucose residues observed in this study correspond to the independent two chains of cellulose II, that the ^{13}C chemical shift changes for C1 and C4 were similar to those of I_β for the two chains of cellulose II could be confirmed. As summarized in Table 1, the ^{13}C chemical shifts of C1 and C4 of one chain were assigned to 107.1 and 87.4 ppm, respectively, while the C1 of the other chain was shifted downfield at 105.0 ppm and the C4 was shifted upfield at 88.7 ppm. By analogy, the similarities of the two pairs of C1 and C4 chemical shifts in cellulose I_β and cellulose II suggest a difference in the β-1,4 linkage between the two chains of cellulose II.

Very recently, Sternberg et al. assigned the ^{13}C resonances appearing at 77, 75, and 73 ppm in the ^{13}C spectrum of cellulose II to C3, C5, and C2, respectively, based on their determination of the ^{13}C chemical shift anisotropy tensor values of each of the ^{13}C resonance lines of cellulose II[40] and performed crystal structure refinements of cellulose II by means of force field optimization with the ^{13}C chemical shift data of cellulose II.[41] The proposed structure has two antiparallel chains. The conformation for the hydroxylmethyl group of one chain is *tg*, and that of the other chain is *gt*, which favors the X-ray structure proposed by Kolpack and Blackwell[6] over the neutron diffraction structure proposed by Langan et al.[14] To the best of our knowledge, this is the first crystal structure refinement of cellulose II performed using solid-state ^{13}C chemical shifts, and the force field method[41,42] in combination with ^{13}C chemical shifts is expected to become a valuable technique for elucidation of the crystal structure of cellulose polymorphs. In the 2D $^{13}C-^{13}C$ correlation spectrum of cellulose II presented herein, however, it was revealed that the C2, C3, and C5 resonances for cellulose II appear as doublets with a chemical shift difference between 1.4 and 2.6 ppm (Table 1). Reinvestigation of the refinement by the ^{1}H as well as the ^{13}C chemical shift data provided by our 2D NMR experiments may lead to determination of the correct structure of cellulose II.

Conclusion

During the past two decades, CP/MAS ^{13}C NMR has been used as a powerful tool for the investigation of the chemical structure, conformation, polymorphism, and

crystallinity of cellulose. However, full exploitation of the CP/MAS ^{13}C NMR spectrum had been far from complete. This study has provided, for the first time, the ^{13}C and ^{1}H chemical shift data of cellulose II. The ^{1}H and ^{13}C chemical shifts of cellulose II assigned by the MAS-J-HMQC and INADEQUATE experiments of the mercerized bacterial cellulose will provided new information on the conformations of the chains in cellulose II.

The chemical shifts of cellulose in the solid are sensitive to the conformation of individual chains, chain packing, strength of hydrogen bonding, and so on. It is, therefore, difficult to separate, unambiguously, the contributions that give rise to small chemical shift differences. We are currently further investigating the conformation for the two kinds of anhydroglucose residues of cellulose II by the other NMR techniques. The shift data determined in this study are particularly useful for the further refinement of the crystal structure of cellulose II.

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