Determination of the Through-Bond Carbon−Carbon and Carbon−Proton Connectivities of the Native Celluloses in the Solid State

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ABSTRACT: Using the two-dimensional (2D) refocused CP-INADEQUATE spectra of natural abundance *Cladophora* and tunicate celluloses, we determined the ^{13}C homonuclear through-bond correlations of cellulose I_α and I_β, respectively. Two sets of the $^{13}C-^{13}C$ connectivities from C1 through C6 were observed in the 2D INADEQUATE spectrum of the respective cellulose where two directly bonded carbons share the common frequency in the double quantum dimension, which indicated that both cellulose I_α and I_β contain two magnetically nonequivalent anhydroglucose residues in the unit cells. After the ^{13}C assignment of each carbon of the cellulose I_α and I_β, assignments of the ^1H chemical shifts of protons attached to each carbon of the both allomorphs were performed by use of the 2D MAS-*J*-HMQC spectra of the cellulose samples for the first time. These spectra gave the through-bond $^{13}C-^1H$ correlations, which allowed the assignment of the ^1H chemical shifts of protons that bind to C1, C3, C4, and C6 of the cellulose I_α and I_β. From the differences in the ^{13}C and ^1H shifts of cellulose I_α and I_β, it was revealed that the primary difference between two forms of cellulose I was in the conformations of anhydroglucose residues contained in the cellulose chains. In addition, the conformational difference in the torsion angle around the β-1,4 linkage between cellulose I_α and I_β was suggested by the notable differences in their ^1H chemical shifts of protons attached to C1.

Introduction

Since Atalla and VanderHart[1,2] established first by CP/MAS ^{13}C NMR techniques in 1984 that cellulose I is a composite two allomorphs, namely cellulose I_α and I_β, a number of studies employing CP/MAS ^{13}C NMR have been performed to establish the structures of the two allomorphs in cellulose I. As a result, it was revealed that the proportion of the two phases of cellulose I differs greatly from species to species;[2−13] the cellulose I_α is the dominant in algae and bacterial celluloses, while the cellulose I_β the dominant form in higher plant and animal celluloses.

In the CP/MAS ^{13}C spectra of the native celluloses, the region between 67 and 63 ppm is assigned to C6 of the primary group, resonance lines at 91−80 ppm are associated with C4, and those at 107−102 ppm are associated with C1 anomeric carbon.[2,3,6] The cluster of resonance lines observed in the region of 76−69 ppm is attributed to C2, C3, and C5. Since the respective line shapes of the C1, C4, and C6 signals are relatively simple triplets, the ^{13}C resonance lines for these three carbons were well assigned by the fitting analysis of the spectra of the native celluloses;[6] cellulose I_α has a singlet in C1 and C6 and a doublet in C4, while cellulose I_β has all doublets for C1, C4, and C6. On the other hand, although assignment of the resonance lines for C2, C3, and C5 regions had been carried out on the basis of a contribution of the ^{13}C spin−lattice time measurements[14] and spin-diffusion experiments,[15] the results of the assignment differed according to the methods used for the signal assignments. Recently, we prepared ^{13}C-enriched *Acetobacter* celluloses from D-[1,3-$^{13}C_2$]glycerol and D-[2-^{13}C]glucose as carbon sources and performed the assignment of the C2, C3, and C5 signals of both I_α and I_β allomorphs by the detailed comparison of the ^{13}C amount of each carbon in the ^{13}C-enriched celluloses and the intensity of the resonance lines observed in the solid-state ^{13}C NMR spectra of the ^{13}C-enriched celluloses.[16] As a result, it was revealed that all carbons of cellulose I_α and I_β except for C1 and C6 of cellulose I_α and C2 of cellulose I_β are shown in equal intensity of doublet in the CP/MAS spectrum of the native cellulose. This finding suggested that there are two magnetically nonequivalent sites in the unit cells of the both allomorphs although there is not absolute proof.

In a liquid-state NMR spectroscopy, 2D through-bonds correlation experiments such as COSY,[17] HMQC,[18] and INADEQUATE[19,20] usually constitute the first step toward the assignment of signals and the determination of the structure of unknown compounds. These techniques provide unambiguous through-bond connectivities between the nuclei such as ^1H and ^{13}C by using the magnetization transfer between the nuclei through the internuclear scalar couplings. In the case of the solid-state NMR spectroscopy, the signals can be made sufficiently narrow by the MAS so that the scalar coupling can be exploited for the correlation spectroscopy in much the same way as in liquid-state NMR spectroscopy. However, virtually no techniques had existed to obtain the through-bond correlation spectra in the solid-state NMR since the scalar couplings between the adjacent nuclei are much smaller than the through-space dipolar couplings in the solid state. Multitudes of techniques based on magnetization transfer through the homonuclear and heteronuclear dipolar couplings, therefore, have been proposed to obtain through-space chemical shift correlations, yielding the

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information about internuclear distances.[21−25] Nevertheless, through-bond connectivities had been generally necessary to establish unambiguous assignment of the solid-state NMR spectra, before through-space interactions can be used to determine the structure. Recently, the group of Meier et al.[26−28] reported first that it is possible to use the scalar couplings in the solid state in the total through-bond correlation experiment. The group of Emslay et al. successively developed the new pulse techniques that mediated the scalar couplings in order to obtain through-bond $^{13}C-^{13}C$[29,30] and $^{13}C-^{1}H$[31,32] correlation spectra in the solid state and demonstrated that these methods can be practicable on the natural abundance compounds as well as ^{13}C-labeled products.

In this paper, we presented the through-bond ^{13}C homonuclear correlation spectra of natural abundance *Cladophora* and tunicate celluloses by using the refocused CP-INADEQUATE experiment[30] for the elucidation of the backbone structure of the both cellulose I_α and I_β allomorphs, respectively. From the analysis of the refocused CP-INADEQUATE spectra, we directly demonstrate that the unit cells of the both allomorphs contain two magnetically nonequivalent anhydroglucose residues, which were previously suggested by ^{13}C-labeling analysis of the CP/MAS spectra of the ^{13}C-labeled *Acetobacter* celluloses.[16] In addition, chemical shifts of protons attached to ring carbons of cellulose I_α and I_β were assigned for the first time by use of MAS-*J*-HMQC[31] spectra, which give the scalar coupled through-bond $^{13}C-^{1}H$ heteronuclear correlations, of the *Cladophora* and tunicate celluloses, respectively.

Experimental Section

Cellulose Samples. The samples of natural abundance cellulose for the NMR measurements were prepared from air-dried *Cladophora sp*. and outer skin of tunicate (*Halocynthia sp*.) according to the method described previously.[16] Air-dried *Cladophora sp*. cut into small pieces was soaked with 1% aqueous HCl overnight at room temperature, washed thoroughly with water, heated at 60 °C for 1 h in a 1% NaOH solution, and finally washed thoroughly with water. The whole procedure was repeated twice. The lipid extraction from the cellulose specimen was carried out with acetone and followed by bleaching with 4% $NaClO_2$ solution under acetic conditions at room temperature for 24 h. The completely white particles were sampled and washed thoroughly with water, followed by freeze-drying. The purified cellulose was incubated in 40% H_2SO_4 solution at 37 °C for 8 h to hydrolyze the amorphous part of the cellulose sample. It was then filtered and washed thoroughly with a continuous stream of cold water, followed by freeze-drying. The tunicate cellulose sample was obtained by purification of the outer skin cut into small pieces similar to *Cladophora* cellulose. Estimation of mass fractions of cellulose I_α (f_α) and I_β (f_β) in the crystalline part of cellulose I was performed by the fitting analysis of the triplet of C4 signal (91−87 ppm) in the CP/MAS ^{13}C NMR spectra, which were previously described.[12] The real parts of the CP/MAS ^{13}C spectra were transported from the spectrometer to a PC by use of a LAN and FTP and then imported into a Bruker Winfit program to perform the fitting of spectra. Nonlinear least-squares methods were engaged for the line-fitting with Lorenzian function. The f_α and f_β values were determined by the integral value of the three Lorenzian lines at 90, 89, and 88 ppm that were assigned to be I_α, (I_α + I_β), and I_β phases, respectively.

Solid-State NMR Experiments. All NMR experiments were performed on a Bruker AV300 wide-bore spectrometer (proton frequency 300 MHz). CP/MAS ^{13}C NMR spectra of cellulose samples were obtained using a Bruker 7 mm double-tuned MAS probe. The sample volume was about 280 μL, and the MAS frequency was 6 kHz. The proton radio-frequency field strength was set to 72 kHz during acquisition using a TPPM scheme.[33] The phase modulation angle for the TPPM decoupling[33] was set to 15.0°, and the flip-pulse length was optimized to 6.3 μs to yield optimal ^{13}C resolution of the α-carbon resonance of D-glycine. The contact time for cross-polarization and the recycle delay were set to 1 ms and 2 s, respectively. Refocused CP-INADEQUATE experiments were performed according to the method of Lesage et al.[30] using a Bruker 7 mm double-tuned MAS probe. The pulse sequence for the refocused CP-INADEQUATE experiment is shown in Figure 1A. The sample volume was about 280 μL, the MAS frequency was set to 6 kHz, and a ramped-amplitude cross-polarization (RAMP−CP) sequence[34] was used for protons. The τ delay was set to 3.6 ms, and the contact time for RAMP−CP was set to 1 ms. The proton decoupling field strength was 72 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 6.3 μs to yield optimal ^{13}C resolution. The 180° pulse on carbons was set to 7.6 μs. Quadrature detection was achieved using the TPPI method.[35] The recycle delay was set to 1.6 s. A total of 128 t_1 increments with 1536 scans each were collected. MAS-*J*-HMQC spectra were measured using a Bruker 4 mm double tuned MAS probe according to the method of Lesage et al.[31] The pulse sequence for the MAS-*J*-HMQC experiment is shown in Figure 1B. The sample volume was restricted to about 25 μL and set to the center of the rotor to improve the radio-frequency field homogeneity.[31,32] The MAS frequency was set to 12 kHz and the delay τ equal to 3.1 ms. The proton radio-frequency field strength was set to 81 kHz during the τ delay (FSLG decoupling[36,37]) and was set to 72 kHz during acquisition (TPPM decoupling). Two off-resonance pulses with opposite phases (i.e., $+x$, $-x$ or $+y$, $-y$) during the FSLG decoupling were set to 8.65 μs. The magic angle pulse length was 2.7 μs. For the cross-polarization step, the contact time was set to 1 ms. The recycle delay was set to 1.6 s. Quadrature detection was achieved using the States method.[38] A total of 96 t_1 increments with 800 scans each were collected. In all NMR experiments used in this study, the ^{13}C chemical shifts were calibrated through the carbonyl carbon resonance of glycine as an external reference at 176.03 ppm, and the ^{1}H chemical shifts were referenced by setting the Hβ resonance of L-[$^{13}C_3$]alanine to 1.0 ppm. In all MAS-*J*-HMQC spectra, the proton chemical shift scale was corrected by the scaling factor of $1\sqrt{3}$.

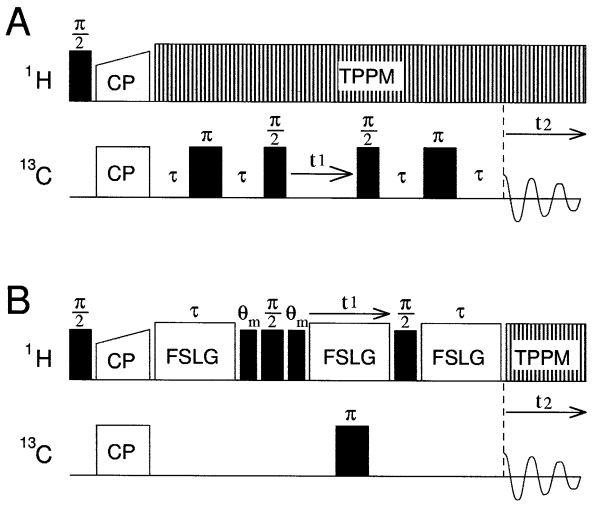

Figure 1. Pulse sequences for refocused CP-INADEQUATE (A) and MAS-*J*-HMQC (B). A 32- and 16-phase cycle were used for refocused CP-INADEQUATE and MAS-*J*-HMQC experiments, respectively. The phase cycle for refocused CP-INADEQUATE and that for MAS-*J*-HMQC were adapted from refs 30 and 32, respectively. θ_m is a magic angle (54.7°) pulse.

Results and Discussion

The native celluloses contain three components: crystalline I_α and I_β and amorphous cellulose. The mass fractions of the components can be estimated by the fitting analysis of the C4 signals in the CP/MAS ^{13}C NMR spectra of the native cellulose: C4 resonance for cellulose I_α appears as a doublet at 90 and 89 ppm, that for cellulose I_β as a doublet at 89 and 88 ppm, and that of amorphous cellulose as a singlet with a broad line width between 87 and 82 ppm.[12] Solid lines in Figures 2A and 3A show the CP/MAS ^{13}C NMR spectra of the purified *Cladophora* (I_α-rich, $f_\alpha/f_\beta = 64/36$) and tunicate (I_β-rich, $f_\alpha/f_\beta = 8/92$) celluloses, respectively. In the both spectra, since the intensity of C4 signals at 87−82 ppm deriving from amorphous phase was considerably lower than that at 91−87 ppm deriving from crystalline part, the both samples were considered almost crystalline phase. As previously described, the respective subspectra of cellulose I_α and I_β phases could be derived from the CP/MAS ^{13}C spectra of the highly crystallized I_α-rich and I_β-rich celluloses by the following mathematical treatment[16]

$$\text{subspectrum (the pure } I_\alpha \text{ phase)} = \text{spectrum } (I_\alpha\text{-rich}) - a \times \text{spectrum } (I_\beta\text{-rich})$$

and

$$\text{subspectrum (the pure } I_\beta \text{ phase)} = \text{spectrum } (I_\beta\text{-rich}) - b \times \text{spectrum } (I_\alpha\text{-rich})$$

where the constants a and b were calculated from f_α and f_β of the I_α- and I_β-celluloses as follows:

$$a = f_\alpha(I_\alpha\text{-rich})/f_\alpha(I_\beta\text{-rich})$$

and

$$b = f_\beta(I_\beta\text{-rich})/f_\beta(I_\alpha\text{-rich})$$

Figures 2B and 3B show the subspectra of the pure I_α and I_β phases, respectively, which were obtained by the mathematical treatment of the CP/MAS spectra of the *Cladophora* (Figure 2A) and tunicate (Figure 3A) celluloses. In this derivation, 0.39 and 0.09 were used for the constants a and b, respectively. As shown in these figures, ^{13}C resonance lines for the both I_α and I_β allomorphs had been assigned by the detailed analysis of the CP/MAS NMR spectra of *Acetobacter* celluloses in which certain carbons were selectively ^{13}C enriched, respectively.[16] According to the assignment, all carbons of cellulose I_α and I_β except for C1 and C6 of cellulose I_α and C2 of cellulose I_β are doublet in the subspectra, suggesting that two magnetically nonequivalent anhydroglucose residues were contained in the both cellulose I_α and I_β allomorphs. However, the ^{13}C resonance line for each carbon of respective anhydroglucose residues contained in cellulose I_α and I_β had not been revealed. For the elucidation of the ^{13}C subspectra of two magnetically nonequivalent anhydroglucose residues in the both cellulose I_α and I_β, refocused CP-INADEQUATE spectra of the *Cladophora* and tunicate celluloses were measured, respectively.

Refocused CP-INADEQUATE Experiments. Since the completely pure cellulose I_α and I_β could not be obtained from any species of plants and animals, we measured the refocused CP-INADEQUATE spectra of *Cladophora* and tunicate celluloses for the characterization of the structures of cellulose I_α and I_β, respectively. Figure 4 shows the refocused CP-INADEQUATE spectrum of tunicate cellulose. In the spectrum, a clearly resolved C1 doublet of cellulose I_β was observed at 106.1 and 104.0 ppm. In the refocused CP-INADEQUATE spectrum, two directly bonded carbons share a common double quantum frequency and the identical carbon shares a common single quantum frequency.[30] It is, therefore, possible to sequentially assign each carbon of the two magnetically nonequivalent anhydroglucose residues in the cellulose I_β straightforwardly using the refocused CP-INADEQUATE spectrum of tunicate cellulose and starting from the anomeric C1 doublet of cellulose I_β at 106.1 and 104.0 ppm. As indicated by the solid lines in the spectrum of tunicate cellulose, the ^{13}C signal appeared at 106.1 ppm was correlated with that appeared at 71.3 ppm in the double quantum frequency of 178 ppm. This indicated that the signal at 71.3 ppm was assigned to C2 that binds to the C1 at 106.1 ppm. As displayed by the dotted lines in this figure, the other C1 signal at 104.0 had a clear correlation with the 71.0 ppm line in the double quantum frequency of 176 ppm, which indicated that C1 at 104.0 ppm bonded to the C2 appeared at 71.0 ppm. In addition, the C2 signal at 71.3 ppm was correlated with the signal appeared at 74.9

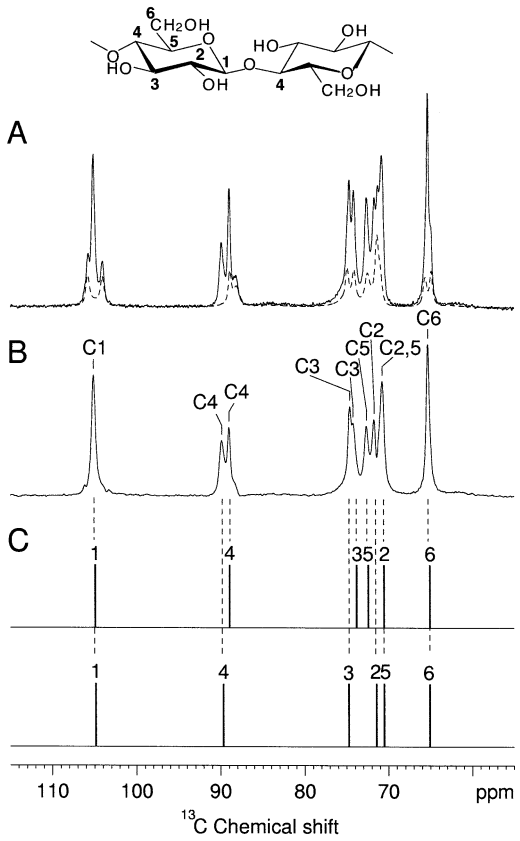

Figure 2. (A) CP/MAS ^{13}C NMR spectra of *Cladophora* (I_α-rich, $f_\alpha/f_\beta = 64/36$, solid line) and tunicate (I_β-rich, $f_\alpha/f_\beta = 8/92$, dotted line) celluloses. (B) The ^{13}C subspectrum of the pure cellulose I_α. The ^{13}C subspectrum of I_α phase was derived by subtracting the ^{13}C spectrum of *Cladophora* cellulose (solid line in Figure 2A) from that of tunicate cellulose (dotted line in Figure 2A) cellulose. (C) ^{13}C Line spectra of the two magnetically nonequivalent anhydroglucose residues composing cellulose I_α. The lines indicate the ^{13}C chemical shifts of each carbon of the anhydroglucose residues. The line spectra were determined by the 2D refocused CP-INADEQUATE spectrum of *Cladophora* cellulose (Figure 5).

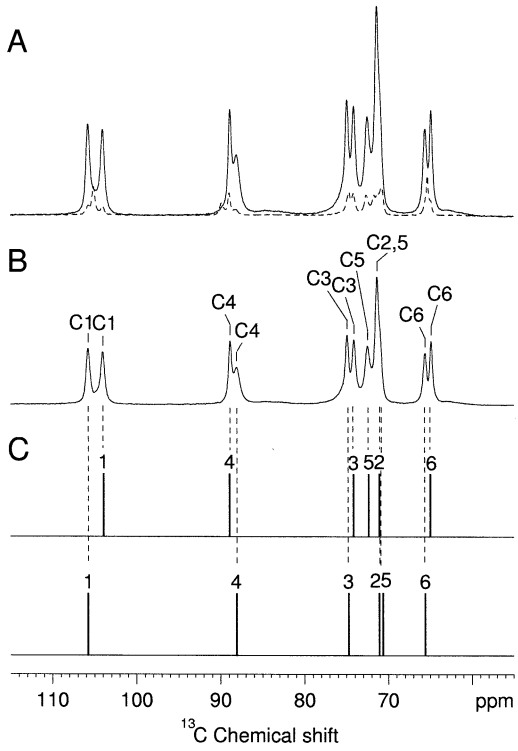

Figure 3. (A) CP/MAS ^{13}C NMR spectra of tunicate (I_β-rich, f_α/f_β = 8/92, solid line) and *Cladophora* (I_α-rich, f_α/f_β = 64/36, dotted line) celluloses. (B) The ^{13}C subspectrum of the pure cellulose I_β. The ^{13}C subspectrum of I_β phase was derived by subtracting the ^{13}C spectrum of tunicate cellulose (solid line in (A)) from that of *Cladophora* cellulose (dotted line in (A)) cellulose. (C) ^{13}C Line spectra of the two magnetically nonequivalent anhydroglucose residues composing cellulose I_β. The lines indicate the ^{13}C chemical shifts of each carbon of the anhydroglucose residues. The line spectra were determined by the 2D refocused CP-INADEQUATE spectrum of tunicate cellulose (Figure 4).

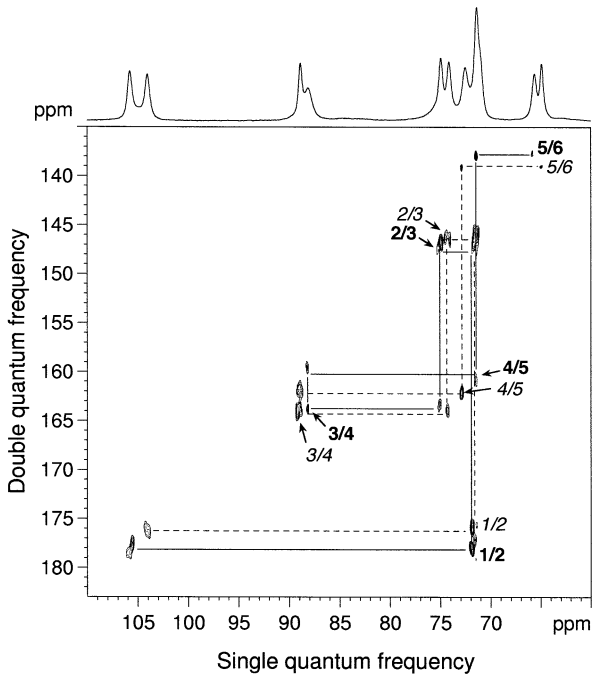

Figure 4. 2D refocused CP-INADEQUATE spectrum of tunicate cellulose. The through-bond connectivities between carbons of two magnetically nonequivalent anhydroglucose residues composing cellulose I_β are indicated by the solid and dotted lines.

ppm in the double quantum frequency at 147 ppm, and the other C2 at 71.0 ppm was correlated with the 74.2 ppm line in the double quantum frequency at 146 ppm. This finding indicated that the two signals at 74.9 and 74.2 ppm could be assigned to C3 doublet; the former directly attached to the C2 appeared at 71.3 ppm and the latter bonded to C2 at 71.0 ppm. By the similar way to assign the C2 and C3 signals sequentially, through-bond correlations of C4, C5, and C6 doublets could be determined. As displayed by solid line in this figure, the C3 signal appeared at 74.9 ppm was correlated with the C4 signal at 88.0 ppm, the C4 signal was correlated with C5 at 70.6 ppm, and the C5 signal is correlated with the C6 signal at 65.6 ppm. As displayed by dotted line in the figure, on the other hand, the other C3 signal at 74.2 ppm was correlated with the C4 signal appeared at 88.9 ppm, the 88.9 ppm line was correlated with C5 line at 72.2 ppm, and the 72.2 ppm line was correlated with C6 signal at 65.0 ppm. Since the refocused CP-INADEQUATE spectrum provides the correlations between two directly bonded carbons, it was concluded that the six carbons connected by the dotted line and the six connected by solid line each corresponds to the ^{13}C nuclei of a magnetically nonequivalent anhydroglucose residue. As summarized in Table 1, the ^{13}C chemical shift data of cellulose I_β assigned by the refocused CP-INADEQUATE spectrum of tunicate cellulose completely agreed with those assigned previously assigned.[16] From these findings, it was revealed that there are two magnetically nonequivalent anhydroglucose residues in the unit cell of cellulose I_β by the 2D correlation spectrum of the tunicate cellulose. The ^{13}C chemical shifts of each anhydroglucose residue of cellulose I_β were shown by the lines in Figure 3C.

In the case of the 2D refocused CP-INADEQUATE spectrum of *Cladophora* cellulose (Figure 5), three cross-peaks were observed at C1 region. Among these cross-peaks, the two cross-peaks at the 106.1 and 104.0 ppm in the single quantum frequency were assigned to C1 doublet of cellulose I_β and the center peak at 105.0 ppm could be assigned to C1 of cellulose I_α. The C1 cross-peak at 105.0 ppm in the single quantum frequency showed the broad line width (ca. 2.5 ppm) against the column direction because the C1 signal was correlated with two cross-peaks at 71.5 and 70.8 ppm in the double quantum frequency at 177 and 176 ppm, respectively. This indicated that the resonances at 71.5 and 70.8 ppm could be assigned to a doublet of C2 of cellulose I_α. Starting from the C2 signals at 71.5 and 70.8 ppm, two sets of the ^{13}C signals from C2 to C6 could be assigned by the similar way to sequentially assign the each cross-peak observed in the 2D refocused CP-INADEQUATE spectrum of tunicate cellulose. The two sets of the ^{13}C signal connectivities were displayed by the solid and dotted lines in the spectrum of *Cladophora* cellulose. As summarized in Table 1, ^{13}C chemical shifts of the two sets of the signals from C1 to C6 were in complete agreement with those of cellulose I_α that were assigned previously.[16] Therefore, it was revealed two magnetically nonequivalent anhydroglucose residues were contained in cellulose I_α by the refocused CP-INADEQUATE spectrum of the *Cladophora* cellulose. The ^{13}C chemical shifts of each anhydroglucose residue of cellulose I_α were shown by the lines in Figure 2C.

MAS-*J*-HMQC Experiments. Next, MAS-*J*-HMQC experiments of *Cladophora* and tunicate celluloses were performed to assign the chemical shifts of protons that

Table 1. ^{13}C and ^{1}H Chemical Shifts of the Cellulose I_α and I_β

		^{13}C shifts/ppm (^{1}H shifts/ppm)					
allomorph		C1 (H1)	C2 (H2)	C3 (H3)	C4 (H4)	C5 (H5)	C6 (H6)
cellulose I_α	ref 16	105.0	71.6, 70.1	74.7, 73.9	90.0, 89.1	72.6, 70.1	65.2
	solid line[a]	105.0 (4.0)	71.6	74.7 (3.2)	90.0 (3.1)	70.1	65.2 (2.9, 5.2)[b]
	dotted line[a]	105.0 (4.0)	70.1	73.9 (3.5)	89.1 (3.4)	72.6 (3.4)	65.2 (2.9, 5.2)[b]
cellulose I_β	ref 16	106.1, 104.0	71.0	74.9, 74.2	88.9, 88.0	72.2, 70.6	65.6, 65.0
	solid line[c]	106.1 (4.5)	71.3	74.9 (3.2)	88.0 (3.4)	70.6	65.6 (4.5, 4.5)
	dotted line[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)

[a] Two magnetically nonequivalent anhydroglucose residues composing cellulose I_α, which were indicated in Figure 5. [b] ^{1}H chemical shifts of protons attached to the respective C6 carbons could not be assigned since the C6 carbons of the two magnetically nonequivalent anhydroglucose residues overlap at the same chemical shift of 65.2 ppm. [c] Two magnetically nonequivalent anhydroglucose residues composing cellulose I_β, which were indicated in Figure 4.

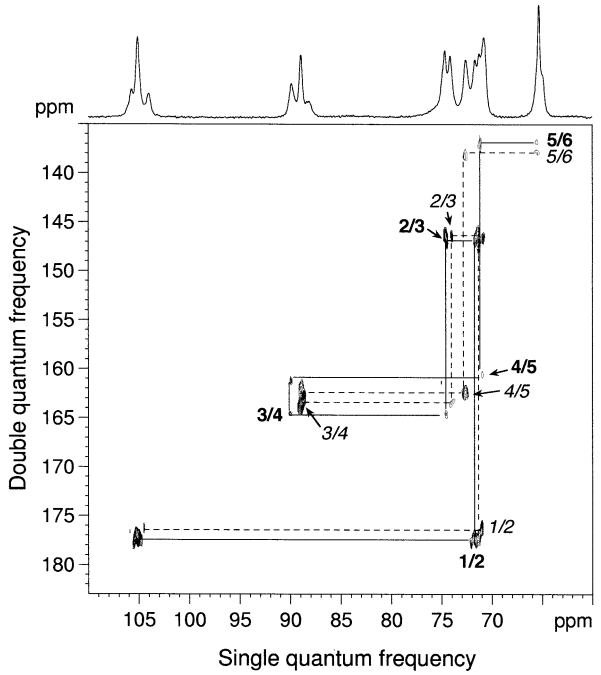

Figure 5. 2D refocused CP-INADEQUATE spectrum of *Cladophora* cellulose. The through-bond connectivities between carbons of two magnetically nonequivalent anhydroglucose residues composing cellulose I_α are indicated by the solid and dotted lines.

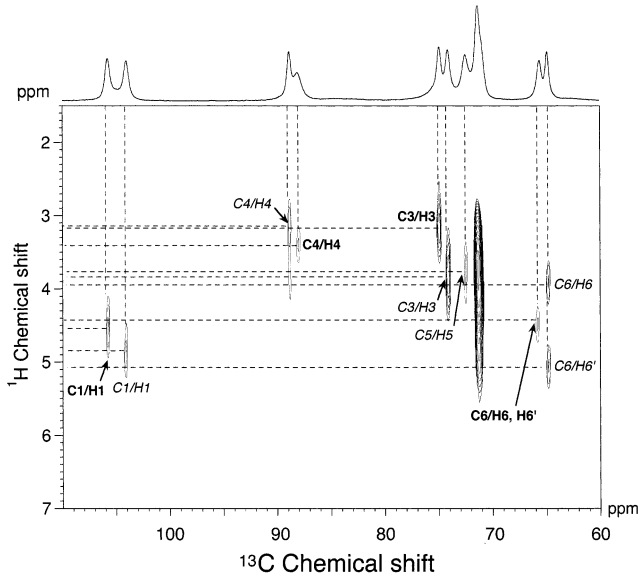

Figure 6. 2D MAS-*J*-HMQC spectrum of tunicate cellulose. Dotted lines indicate the ^{1}H and ^{13}C chemical shifts of the $^{13}C-^{1}H$ through-bond correlation peaks of the cellulose I_β.

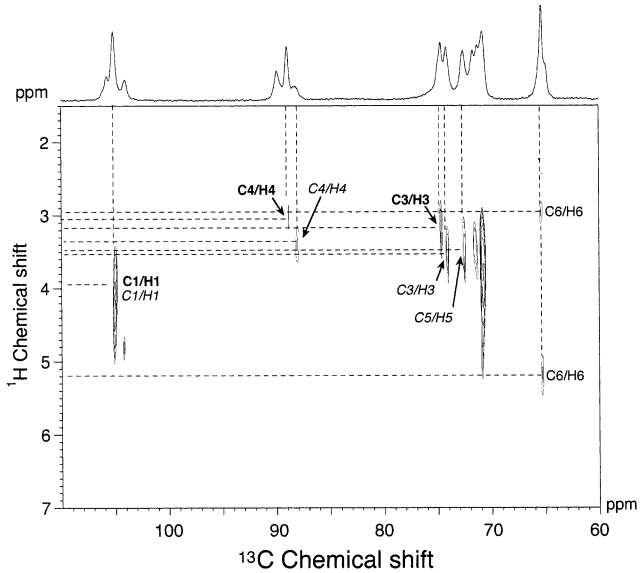

Figure 7. 2D MAS-*J*-HMQC spectrum of *Cladophora* cellulose. Dotted lines indicate the ^{1}H and ^{13}C chemical shifts of the $^{13}C-^{1}H$ through-bond correlation peaks of the cellulose I_α.

are directly attached to carbons of cellulose I_α and I_β. The spectra of tunicate and *Cladophora* celluloses are shown in Figures 6 and 7, respectively. In the MAS-*J*-HMQC experiments, since the proton chemical shift is scaled by $1\sqrt{3}$ under FSLG decoupling,[36,37] the scale of the ^{1}H chemical shift direction was corrected for this scaling. ^{1}H chemical shifts of the celluloses were determined by estimating the peak top of the proton traces extracted in the column direction from the MAS-*J*-HMQC spectra. In the spectrum of tunicate cellulose, a doublet for C1 of cellulose I_β at 106.1 and 104.0 ppm was correlated with their attached protons at the chemical shift of 4.9 and 4.5 ppm in the column direction, respectively. On the other hand, ^{1}H chemical shifts of protons attached to C1 of cellulose I_α differ from those of cellulose I_β. In the MAS-*J*-HMQC spectrum of *Cladophora* cellulose (Figure 7), a singlet for C1 of cellulose I_α at 105 ppm gave a correlation peak with 4.0 ppm of ^{1}H chemical shift, suggesting that the two protons attached to two magnetically equivalent C1 carbons overlapped at the same chemical shifts of 4.0 ppm. A difference between cellulose I_α and I_β was observed in the ^{1}H chemical shifts of protons that attach to C4 and C6. ^{1}H chemical shifts of protons attached to C4 signals of cellulose I_β at 88.9 and 88.0 ppm were assigned to 3.1 and 3.4 ppm (Figure 6), respectively, while two protons attached to C4 doublet of cellulose I_α at 90.0 and 89.1 ppm appeared at 3.1 and 3.4 ppm (Figure 7), respectively. In the case of protons that

bonded to C6, since one carbon nucleus attaches to two proton nuclei, one carbon signal was expected to give two cross-peaks. As shown in Figure 7, C6 resonance of cellulose I_β at 65.0 ppm correlated with two protons at the ^{1}H chemical shifts of 3.9 and 5.1 ppm. On the other hand, the other C6 signal appeared at 65.6 ppm gave a cross-peak at 4.2 ppm. In the case of C6 of cellulose I_α, a singlet at 65.2 ppm gave two cross-peaks at the ^{1}H chemical shifts of 4.1 and 4.9 ppm although two magnetically nonequivalent anhydroglucose residues were contained in cellulose I_α. In addition, ^{1}H shifts of protons attached to C3 of cellulose I_α and I_β could be assigned from these spectra. A doublet of C3 of cellulose I_β at 74.9 and 74.2 ppm gave clearly resolved two cross-peaks at the ^{1}H chemical shifts of 3.2 and 3.8 ppm, respectively. Protons attached to a doublet of C3 of cellulose I_α at 74.7 and 73.9 ppm appeared at the chemical shifts of 3.6 and 3.8 ppm, respectively. With respect to the protons attached C2 and C5 of the both cellulose I_α and I_β, the ^{1}H shift of the protons attached to C5 of cellulose I_β at 72.2 ppm and C5 of cellulose I_α at 72.6 ppm could be assigned to 3.7 and 3.4 ppm, respectively. However, since the cross-peaks correlated with the other C2 and C5 overlapped each other in the both spectra, ^{1}H chemical shifts of the proton nuclei attached to these carbons could not be correctly assigned. The results of the assignment of ^{1}H chemical shifts of cellulose I_α and I_β are summarized in Table 1.

In the recent paper described by the author,[16] ^{13}C chemical shifts of cellulose I_α and I_β were assigned by the analysis of ^{13}C-labeling intensity in the CPMAS ^{13}C NMR spectra of ^{13}C-enriched bacterial celluloses. The result of the assignment suggested two magnetically nonequivalent anhydroglucose residues were contained in the unit cells of both cellulose I_α and I_β. In this study, the two sets of the correlation networks of ^{13}C signals from C1 thorough C6 were observed in the both allomorphs by the analysis of the refocused CP-INADEQUATE NMR spectra of tunicate and *Cladophora* celluloses, and the chemical shifts of the ^{13}C signals of cellulose I_α and I_β assigned by the refocused CP-INADEQUATE NMR spectra were in complete agreement with those determined previously.[16] In addition, all carbons of both allomorphs except for C1 and C6 of cellulose I_α and C2 of cellulose I_β appeared as equal-intensity doublet, which was revealed by a previous study[16] on the fitting analysis of the ^{13}C subspectra of cellulose I_α and I_β. From these findings, it was revealed that ^{13}C subspectra of cellulose I_α and I_β could be characterized by overlapping of the ^{13}C subspectra of the two magnetically nonequivalent anhydroglucose residues, which are shown in Figures 2C and 3C, respectively, in the same population.

With respect to the assignment of the ^{13}C resonance for the native cellulose, Lesage et al.[30] reported that the similar 2D refocused CP-INADEQUATE spectrum of the ^{13}C-enriched wood chips from aspen (*Populus euramericana*) that was grown under a 20% $^{13}CO_2$ atmosphere in order to prove the efficiency of this NMR technique. As a result, one set of the ^{13}C−^{13}C connectivities from C1 through C6 was observed in the 2D correlation spectrum. However, the ^{13}C−^{13}C through-bond connectivities provided by Lesage et al. could not be observed in the refocused CP-INADEQUATE spectra of *Cladophora* and tunicate celluloses. It was considered that the difference between the result of Lesage et al. and that of this study arose from the cellulose samples used for the measurements. The ^{13}C-enriched aspen chip used for the NMR measurement was considered to contain a large amount of amorphous cellulose because the C4 signal at 87−82 ppm arising from amorphous cellulose was considerably high in intensity in comparison with those arising from crystalline parts. On the other hand, since the cellulose samples were exhaustively acid-hydrolyzed to enhance their crystallinity prior to the NMR measurement, no resonance lines derived from the amorphous cellulose were observed. Thus, we could reveal that the two sets of the ^{13}C correlation networks of two magnetically nonequivalent anhydroglucose residues contained in ^{13}C signals of cellulose I_α and I_β with high resolution.

Herein, through-bond ^{13}C−^{1}H correlation spectra of the native celluloses were presented for the first time. In general, ^{1}H NMR resonances of solids with high proton density such as cellulose are broadened by the strong ^{1}H homonuclear dipolar interactions. Regarding ^{1}H NMR in the solid state, the combination of magic-angle rotation and multipulse sequences,[39,40] i.e., CRAMPS, is a widely used technique to obtain high-resolution solid-state ^{1}H spectra. This experiment could remove the ^{1}H homonuclear dipolar interactions in the solid materials by use of precise phase cycling and multipulse cycling. It is, however, impossible to assign ^{1}H resonance of the organic materials by the CRAMPS experiment since the ^{1}H line width is considerably broad in comparison with ^{13}C spectral line width in the spectra obtained under MAS conditions. With the development of NMR probe, high MAS frequency (several tens of kilohertz) of sample rotation that removes the ^{1}H homonuclear dipolar interactions could be achieved in order to obtain very high-resolution ^{1}H NMR spectra. Recently, Gil et al.[41] isolated cellulose from *Eucalyptus globules* wood and measured the ^{1}H MAS spectrum of the cellulose at a MAS frequency of 32 kHz. Although they succeeded in the presentation of the ^{1}H spectrum of the native cellulose for the first time, the spectrum was insufficient in the resolution, which did not enable one to assign ^{1}H chemical shifts of cellulose. On the other hand, MAS-*J*-HMQC spectra of *Cladophora* and tunicate celluloses provided isotropic chemical shift correlations between pairs of directly bonded ^{1}H and ^{13}C nuclei of the native celluloses, which allowed the assignment of ^{1}H chemical shifts of cellulose I_α and I_β although some protons could not be assigned due to the overlap of their cross-peaks.

Beside the works of Lesage et al.[30] and Gil et al.,[41] some researchers have used woody specimens for the assignments of the ^{13}C and ^{1}H signals of the native cellulose in the solid. For example, Bardet et al.[15] applied the two-dimensional spin-exchange NMR measurements to aspen wood chip and observed the two broad ^{13}C signals at 76 and 74 ppm in the region of signals corresponding to C2, C3, and C5. They assigned the signal at 76 ppm to C2 and that at 74 ppm to C3 and C5 by comparing the intensities of these signals as a function of increasing mixing time. However, it is very difficult to assign each carbon of the cellulose I_α and I_β by using woody specimens because the spectral contribution of cellulose and that of the poly-β-(1→4)-D-xylopyranosyl polymer, the major components of hemicellulose, were completely overlapped.[42] On the other hand, since the *Cladophora* and tunicate celluloses used in this experiment have high crystallinity and be of exceptionally high purity, no resonance lines derived

from the impurities such as hemicellulose and lignin were observed. Thus, we could directly observe through-bond $^{13}C-^{13}C$ and $^{13}C-^{1}H$ correlations of cellulose I_α and I_β.

In this study, it was demonstrated by the analysis of the 2D refocused CP-INADEQUATE spectra of *Cladophora* and tunicate cellulose that cellulose I_α and I_β were characterized by two equally distributed magnetically nonequivalent anhydroglucose residues. In addition, as shown in Figures 2C and 3C, the four anhydroglucose residues in the cellulose I_α and I_β differed from each other in the spectral pattern, indicating that these anhydroglucose residues have different conformations and that there are two magnetically nonequivalent sites in the unit cells of both cellulose I_α and I_β. With respect to the structures of cellulose I_α and I_β, the electron diffractometric study of the native celluloses demonstrated that the cellulose I_α represents a triclinic phase with one-chain per unit cell while the cellulose I_β represents a monoclinic phase with two chains per unit cell with the space group $P2_1$.[43−45] On the other hand, Atalla and VanderHart strongly suggested that both cellulose I_α and cellulose I_β have only one chain per unit cell on the basis of the Raman spectroscopic observations.[46] In comparison of the Raman spectra of cellulose from *Valonia* (I_α-rich) and from *Halocynthia* (I_β-rich), they reported that the similarity was observed in the conformational-sensitive region of the spectra and that, in contrast, differences were significant in the OH stretching region. From these findings, they concluded that the primary difference between cellulose I_α and I_β was in the pattern of hydrogen bonding and that the difference between chain conformations was negligible. The discussions regarding the packing of cellulose chains are currently in progress. However, the result provided in this study supported that the cellulose I_α has one chain per unit cell since there must be two magnetically nonequivalent glucopyranose residues in the one-chain unit cell. In the case of the structure of cellulose I_β, it could not be determined by the NMR data presented herein whether cellulose I_β has one chain per unit cell or two chains per unit cell. Atalla and VanderHart proposed that the splitting of the C1 and C4 resonances into two in the CP/MAS ^{13}C spectrum of cellulose I_β might be due to magnetically nonequivalent glucopyranose residues along with the cellulose chain.[1,2,46] We also suggested previously that such a nonequivalence in cellulose I_β could possibly arise from a magnetically nonequivalence of the glycosidic linkage.[16] In the MAS-*J*-HMQC spectra of *Cladophor*a and tunicate celluloses, the notable differences between cellulose I_α and I_β were observed in the ^{1}H shifts of protons attached to C1 carbon. ^{1}H chemical shifts of two protons attached to C1 doublet of cellulose I_β were assigned to be 4.9 and 4.5 ppm, while two protons attached to C1 of cellulose I_α were appeared at the same ^{1}H chemical shift of 4.0 ppm. The noticeable difference in the ^{1}H shifts of cellulose I_α and I_β was considered be arisen from the conformational difference around β-1,4 linkages.

In conclusion, this study revealed that the ^{13}C subspectra of both cellulose I_α and I_β were characterized by overlapping of the ^{13}C subspectra of two magnetically nonequivalent anhydroglucose residues in the same population. In addition, the ^{1}H chemical shifts of protons attached to each carbon of cellulose I_α and I_β for the first time by using the MAS-*J*-HMQC spectra of *Cladophora* and tunicate celluloses, respectively. From the differences in the ^{13}C and ^{1}H shifts of the anhydroglucose residues in cellulose I_α and I_β, it was concluded that the primary difference between two forms of cellulose I was in the conformations of anhydroglucose residues contained in the cellulose chains. In addition, the conformational difference in the torsion angle around the β-1,4 linkage between cellulose I_α and I_β was suggested by the noticeable differences in their ^{1}H chemical shifts of protons attached to C1.

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