Complete Assignment of the CP/MAS ^{13}C NMR Spectrum of Cellulose III_I

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Received June 27, 2002; Revised Manuscript Received January 15, 2003

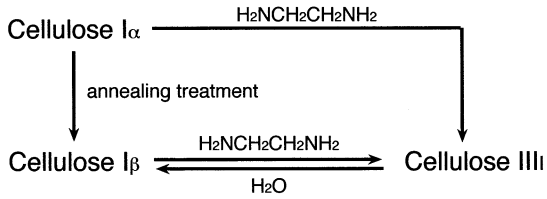
ABSTRACT: For the assignment of ^{13}C resonance for C2, C3, and C5 of cellulose III_I, ^{13}C-enriched celluloses were biosynthesized by *Acetobacter xylinum* (*A. xylinum*) ATCC10245 in the culture medium containing D-(2-^{13}C), D-(3-^{13}C), or D-(5-^{13}C)glucose. After the ^{13}C-enriched celluloses and *Cladophora* cellulose were converted into III_I allomorph by the ethylenediamine treatment, cross-polarization/magic angle spinning (CP/MAS) ^{13}C NMR spectra of these cellulose III_I samples were measured. By comparison of the CP/MAS ^{13}C NMR spectrum of *Cladophora* cellulose sample with those of the ^{13}C-enriched celluloses, ^{13}C resonance lines of the cellulose III_I were completely assigned for the first time. As a result, all carbons of cellulose III_I were shown in singlet in the CP/MAS spectrum of cellulose III_I, which suggests that cellulose III_I is made up of one kind of glucopyranose residue.

CP/MAS ^{13}C NMR has been widely applied for the structural analysis of cellulose, and it is well-known that the CP/MAS ^{13}C NMR can, because of differing spectral appearances, easily distinguish between cellulose I, II, III_I, III_{II}, IV_I, and IV_{II}.[1,2] Among these allomorphs, cellulose I was revealed to be a composite of different two allomorphs, namely cellulose I_α and I_β, by the line shape analysis of the triplets of C1, C4, and C6 signals in the CP/MAS ^{13}C NMR spectrum of the native cellulose.[3−6] However, resonance lines for C2, C3, and C5 of the cellulose I_α and I_β had not been assigned; ^{13}C signals of the C2, C3, and C5 of the cellulose I_α and I_β overlap in narrow regions (70−80 ppm) of the solid-state NMR spectrum of the native celluloses. Although the assignment of C2, C3, and C5 signals of cellulose I have been performed using spin-diffusion,[7] spin-relaxation,[8] and the two-dimensional refocused INADEQUATE measurements of the ^{13}C-enriched woods,[9] the results of the assignments obtained by the above solid-state NMR techniques differs from each other. Thus, complex line splitting observed in the region of C2, C3, and C5 signals of cellulose I had remained largely unexplained.

Recently, we applied the ^{13}C-labeled cellulose biosynthesized in the culture containing D-(2-^{13}C)glucose or D-(1,3-$^{13}C_2$)glycerol as a carbon source by *Acetobacter xylinum* ATCC10245 for the CP/MAS ^{13}C NMR measurement in order to assign the resonance lines of C2, C3, and C5 of the cellulose I_α and I_β.[10] The final metabolic distribution of labeled cellulose sites that arise from D-(2-^{13}C)glucose or D-(1,3-$^{13}C_2$)glycerol was determined by quantitative solution-state ^{13}C NMR measurements of glucose which was obtained by the complete hydrolysis from the biosynthesized ^{13}C-labeled cellulose. Knowledge of this distribution enabled us to interpret the CP/MAS ^{13}C NMR spectra of celluloses.

In this work present herein, we performed the complete assignment of the CP/MAS ^{13}C NMR spectrum of cellulose III_I allomorph using the ^{13}C-enriched cellulose samples. As shown in Scheme 1, transformation between cellulose I and III_I are reversible; cellulose III_I is directly obtained from treatment of cellulose I with liquid ammonia or organic amines followed by removal of the chemicals,[11,12] while the conversion of cellulose III_I back into cellulose I is easily achieved by a warm-temperature treatment.[13] The structures of both cellulose I and III_I are, therefore, considered a parallel chain arrangement, which were supported by the X-ray diffraction analyses of both allomorphs.[14−16] However, notable contrasts appear in the CP/MAS ^{13}C NMR spectra of cellulose I and III_I; several more resonance lines appear in the spectra of cellulose I than in that of the III_I allomorph since the latter shows only six clearly resolved resonance lines.[1,2,16−18] Among six resonance lines of cellulose III_I, C2, C3, and C5 signals observed at 80−70 ppm were not assigned although C1, C4, and C6 signals were easily assigned on the basis of the chemical shifts. To investigate the structural difference between these cellulose allomorphs and the mechanism of the transformation of cellulose I into cellulose III_I, complete assignment of the CP/MAS ^{13}C NMR spectrum of cellulose III_I is required.

Scheme 1. Polymorphism Relationship between Cellulose I and Cellulose III_I



Herein, ^{13}C-enriched celluloses biosynthesized by *A. xylinum* ATCC10245 in the culture medium containing D-(2-^{13}C), D-(3-^{13}C), or D-(5-^{13}C)glucose as a carbon source. After a part of the ^{13}C-enriched celluloses was converted into cellulose triacetate derivatives (CTA), the redistribution of the specific glucose label to other ring carbon sites was monitored using quantitative ^{13}C intensities from CTA solution spectra.[19] These relative ^{13}C intensities were used to assign ^{13}C resonance lines

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10.1021/ma021015f CCC: $25.00
Published on Web 04/18/2003

of cellulose III_I. On the basis of the chemical shifts and the splitting of the ^{13}C signals of cellulose I and III_I allomorphs, the structural difference between these allomorphs is discussed. In addition, the structures of cellulose III_I previously proposed by diffraction analyses[14−16] were reevaluated, which is also described herein.

Cladophora cellulose was prepared as described previously.[10] ^{13}C Enriched cellulose was biosynthesized by *A. xylinum* ATCC10245 from a Hestrin and Schramn medium[20] containing 1% (v/v) of ethanol and 7.5% of D-(2-^{13}C), D-(3-^{13}C), or D-(5-^{13}C)glucose (Cambridge Isotope Laboratories, Inc., Cambridge, MA, the isotropic purity of all the labeling compounds is 99%) in unlabeled glucose.[19] Biosynthesis of the ^{13}C-enriched celluloses was carried out at 28 °C for 7 days under static conditions. The formed cellulose pellicle was purified by boiling in 1 (w/v) % aqueous NaOH for 8 h, washed with distilled water, and freeze-dried. After the purified cellulose pellicle was cut with a scissors into small pieces by scissors, the cellulose sample was incubated in a 40% H_2SO_4 solution at 37 °C for 8 h to hydrolyze the noncrystalline region.[10] It was then filtered and washed thoroughly with a continuous stream of cold water, followed by freeze-drying. After the conversation of a part of the dried ^{13}C-enriched celluloses to their triacetate derivatives by using the method of Tanghe et al.,[21] the redistribution of the specific glucose label to the other carbon sites was monitored using the quantitative ^{13}C intensities from inverse-gated decoupling ^{13}C NMR spectra of these triacetate derivatives, which was previously reported in detail.[19]

Cellulose III_I samples were prepared by successive treatments of cellulose I in anhydrous ethylenediamine alternating washing in anhydrous methanol.[2,17] The cellulose III_I samples were the dried in under high vacuum at room temperature. Removal of ethylenediamine from the cellulose III_I samples was estimated by the disappearance of ^{13}C signal of ethylenediamine observed at 46.4 ppm in the CP/MAS ^{13}C NMR spectra of the samples. The conversion from cellulose I to III_I was confirmed by X-ray diffraction pattern of the cellulose samples.[1] The X-ray diffraction patterns of the samples were recorded on a Rigaku Rint-2000 diffractometer equipped with the refraction-type goniometer and the pulse-height discriminator system according to the method of described previously.[19] CP/MAS ^{13}C NMR spectra of the cellulose samples were recorded on a Bruker MSL300 and AVANCE 300 spectrometers at 300.1 MHz for proton frequency. CPMAS ^{13}C NMR measurements and the line shape analysis of the NMR spectra were performed according to the method described previously.[10,19] For the quantitative discussion of ^{13}C resonances of the CP/MAS ^{13}C NMR spectra, the $T_{CH} - T_{1\rho(H)}$ measurements were performed. The contact time in the CP/MAS measurements were varied from 100 μs to 100 ms. The obtained ratio of $T_{CH}/T_{1\rho(H)}$ were from 0.01 to 0.1, which means that rather quantitative discussions are possible for cellulose when the contact time is properly chosen. Nonlinear least-squares methods were engaged for the line shape analysis with Lorentzian function, which was previously reported.[10] ^{13}C Chemical shifts were calibrated through the carbonyl carbon resonance of glycine as an external reference at 176.03 ppm and converted to the values from tetramethylsilane.

Figure 1 shows the CP/MAS ^{13}C NMR spectrum of cellulose III_I prepared by the ethylenediamine treatment of *Cladophora* cellulose. The transformation of cellulose I into III_I was accompanied by a dramatic change in spectral appearance. Interestingly, CP/MAS ^{13}C NMR spectrum of the III_I allomorph shows the only six clearly resolved resonance lines at 106.6, 89.6, 77.7, 75.3, 74.6, and 64.0 ppm. Among these lines, signals at 106.6, 89.6, and 64.0 ppm had already been assigned to C1, C4, and C6 signals, respectively,[17] while assignment of the other resonances were unexplained. For the assignment of C2 C3, and C5 signals, ^{13}C-enriched celluloses biosynthesized from D-(2-^{13}C), D-(3-^{13}C), and D-(5-^{13}C)glucoses were converted into the III_I allomorph and followed by the CP/MAS ^{13}C NMR measurements.

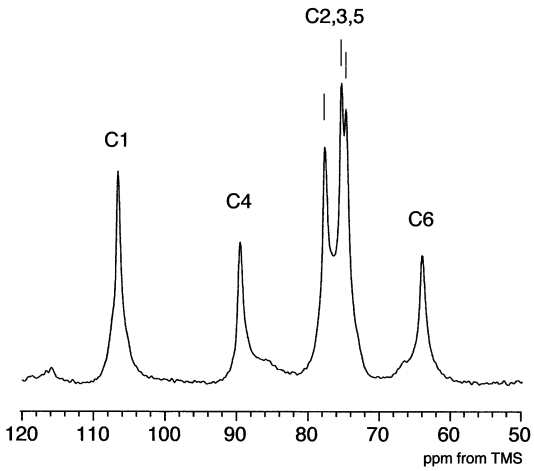

Figure 1. CP/MAS ^{13}C NMR spectrum cellulose III_I obtained from *Cladophora* cellulose at 75 MHz.

Table 1. ^{13}C Integral Ratio[a] for Each Carbon in ^{13}C Enriched Cellulose Samples

	carbon					
sample	C1	C2	C3	C4	C5	C6
cellulose from D-[2-^{13}C]glucose	16.2	40.7	14.6	8.3	12.3	7.9
cellulose from D-[3-^{13}C]glucose	13.8	24.7	36.3	7.3	11.0	6.9
cellulose from D-[5-^{13}C]glucose	9.4	9.0	11.1	7.4	55.7	7.4

[a] These values were determined by estimating the ^{13}C integral value of each carbon signal in the quantitative ^{13}C NMR spectra of the triacetate derivatives of the samples in $CDCl_3$. Experimental procedures for the determination of these values were described in ref 19 in detail. The sum of the integral ratio over all carbons is 100 for each sample.

Table 1 shows the relative ^{13}C intensity ratios at each carbon site of the ^{13}C-enriched cellulose sample. These intensity ratios were determined by the quantitative solution-NMR spectra of the triacetate derivatives of the enriched samples, which were previously reported.[19] According to this table, 41, 36, 56% of the labeling were remained in the C2, C3, and C5 of the ^{13}C-enriched celluloses biosynthesized from D-(2-^{13}C), D-(3-^{13}C), and D-(5-^{13}C)glucoses although the dilution of the labeling of the original position in the introduced glucose as carbon source and the redistribution of the label from the original position to other positions occur during the biosynthesis of cellulose by *A. xylinum*.[22−24] The intensity ratio of each carbon in the solid-state NMR spectra of these ^{13}C-enriched celluloses should be in complete agreement with the intensity ratio of the corresponding carbon as shown in Table 1.

Figure 2 shows the expansion of the C2, C3, and C5 signal region of the solid-state ^{13}C NMR spectra of cellulose III_I allomorph derived from *Cladophora* and

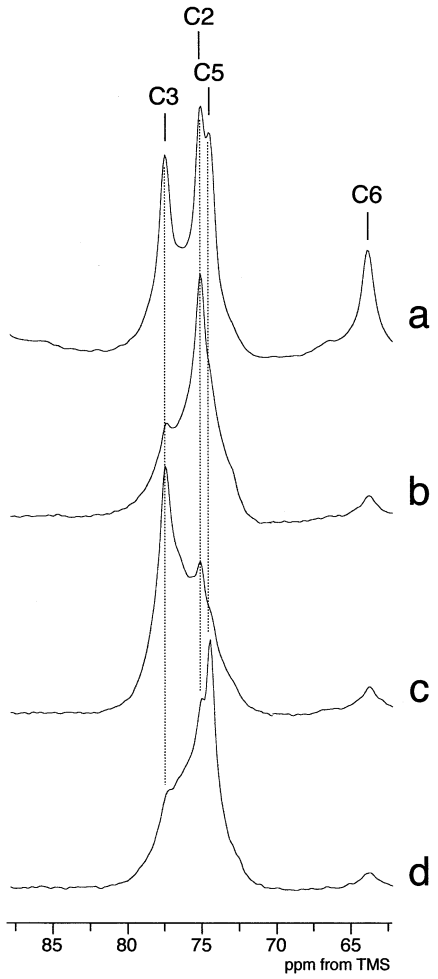

Figure 2. Expansion C2, C3, and C5 region in the CP/MAS ^{13}C NMR spectra of cellulose III$_I$ at 75 MHz: (a) *Cladophora* cellulose; (b) ^{13}C-enriched cellulose biosynthesized from D-(2-^{13}C)glucose; (c) ^{13}C-enriched cellulose biosynthesized from D-(3-^{13}C)glucose; (d) ^{13}C-enriched cellulose biosynthesized from D-(5-^{13}C)glucose.

^{13}C-enriched cellulose samples. Since the intensities of the ^{13}C signals at C2, C3, and C5 in the CP/MAS ^{13}C NMR spectra of the ^{13}C cellulose samples fluctuated according to the intensity ratios of the corresponding carbons in Table 1, intensities of C2 C3, and C5 signals were expected to be higher than those of the other signals in the CP/MAS ^{13}C NMR spectra of cellulose samples prepared from D-(2-^{13}C), D-(3-^{13}C), and D-(5-^{13}C)-glucose, respectively. In the spectrum of the cellulose III$_I$ obtained from D-(2-^{13}C)glucose, the ^{13}C signal at 77.7 ppm loses nearly half of its intensity in comparison with that of cellulose III$_I$ derived from *Cladophora* cellulose, and that at 74.6 ppm is missing. The enhanced remaining signal observed at 75.3 ppm should thus be assigned to C2. In the case of the spectrum of cellulose III$_I$ obtained from D-(3-^{13}C)glucose, on the other hand, the signals at 75.3 and 74.6 ppm have deficient intensities. Therefore, the remaining signal at 77.7 ppm is assigned to be C3 of cellulose III$_I$, which was confirmed in the spectrum of cellulose III$_I$ obtained from D-(5-^{13}C)glucose. In that spectrum, the signals at 77.7 and 74.6 ppm are weak in comparison with those of cellulose III$_I$ derived from *Cladophora* cellulose. Through the analysis of the CP/MAS ^{13}C NMR spectra of the ^{13}C-enriched cellulose III$_I$, C2, C3, and C5 of cellulose III$_I$ were completely assigned for the first time (Table 2), and it was revealed that all carbons appeared as singlets in the CP/MAS ^{13}C NMR spectrum of cellulose III$_I$ (Figure 2).

Table 2. ^{13}C Chemical Shifts (ppm) of Cellulose I$_\alpha$, I$_\beta$, and III$_I$

	atom					
polymorph	C1	C2	C3	C4	C5	C6
cellulose I$_\alpha$[a]	106.9	73.6	76.2	91.6	74.4	67.1
		72.6	76.5	90.8	72.6	
cellulose I$_\beta$[a]	107.6	73.2	76.8	90.6	74.2	67.5
	105.9		76.0	90.0	73.0	66.9
cellulose III$_I$	106.6	75.3	77.7	89.6	74.6	64.0

[a] See ref 10.

Table 2 summarizes the ^{13}C chemical shift data of cellulose I$_\alpha$, I$_\beta$, and III$_I$ allomorphs. The ^{13}C chemical shifts of cellulose I$_\alpha$ and I$_\beta$ were assigned previously.[10] In the case of cellulose I$_\alpha$ and I$_\beta$, the majority of resonances are doublets of equal intensity, which was determined by the line shape analysis of the CP/MAS spectrum of cellulose I. [10] Exceptions are singlets associated with C1 and C6 in the I$_\alpha$ allomorph and C2 in the I$_\beta$ allomorph. This indicated that two equally populated inequivalent glucose residues exist in both the I$_\alpha$ and I$_\beta$ phases. With respect to the structure of cellulose I, recent electron diffraction studies have revealed that the I$_\alpha$ phase can be assigned to the allomorph having a 1-chain triclinic unit cell containing two anhydroglucose units and that the I$_\beta$ phase can be assigned to another allomorph defined as the Meyer−Misch type[25,26] having a two-chain monoclinic unit cell with four such units.[27−29] Since the I$_\alpha$ phase has only two anhydroglucoses per unit cell, they must be magnetically inequivalent. This inequivalence likely points to an inequivalence of glycosidic linkages in the I$_\alpha$ phase. With respect to the structure of the I$_\beta$ phase, the doublet at the C1 and C4 signals is considered to be due to inequivalent anhydroglucose residues along the cellulose chain.[3,30] On the other hand, cellulose III$_I$ was quite different from the I$_\alpha$ and I$_\beta$ phases in spectral appearance, and six clearly resolved signals were observed in the spectrum of the III$_I$ allomorph. Since each carbon of cellulose III$_I$ appears as a singlet in the solid-state NMR spectrum, all glucose residues of cellulose III$_I$ are magnetically equivalent. Thus, all residues experience the same conformation and crystal packing form.

The crystal structure of cellulose III$_I$ proposed by Sarko et al.,[14] based on a combined X-ray fiber diffraction and stereochemical model analyses, is generally accepted. According to this model, the cellulose III$_I$ allomorph crystallizes in a $P2_1$ space group with two independent cellulose chains per unit cell, located on the 2_1 screw axes of the structure. The cell had the following parameters: $a = 1.025$ nm, $b = 0.778$ nm, c (chain axis) $= 1.034$ nm, and $\gamma = 122.4°$. The two chains in the unit cell show slightly different conformations for the torsion angles χ (O5−C5−C6−O6) around the C5−C6 bonds; the center chain has a *t* (trans)−*g* (gauche) conformation while the corner one has one that is slightly shifted from *t*−*g*. They also indicated that the corner chain is translated by −0.09 nm along *c* relative to the center chain in the unit cell. This movement should destroy the magnetically equivalence of the two chains and that is not supported by our data for cellulose III$_I$.

Recently, the refinement of the crystal structure of cellulose III_I was performed by Wada et al.,[15,16] based on combined X-ray, synchrotron, and electron diffraction analyses. These diffraction data indicated that the crystals of cellulose III_I could be described with a one-chain unit cell and a $P2_1$ space group, with the cellulose chain axis on one of the 2_1 axes of the cells and that the cell had the following parameters: $a = 0.448$ nm, $b = 0.785$ nm, $c = 1.031$ nm, and $\gamma = 105.1°$. The volume of the one-chain cell is half that of the two-chain cell reported by Sarko et al.[14] Therefore, they suggested that the asymmetric residue of cellulose III_I is reduced to one anhydroglucose unit. In this experiment, it was revealed that all glucose residues of cellulose III_I are magnetically equivalent. This supported the results of Wada et al., which showed one anhydroglucose residue is the crystallographic asymmetric unit in cellulose III_I.

With respect to the conformations for exocyclic carbon−carbon bonds, Horii et al.[31] reported that the relationships between ^{13}C chemical shifts of C6 of various carbohydrates in solid and the torsion angles χ around C5−C6 bonds determined by X-ray diffraction analysis. They concluded that chemical shifts of C6 fall into three groups of 62, 64, and 66 ppm, which are related to $g-g$, $g-t$, and $t-g$ conformations, respectively. As shown in Table 2, the ^{13}C chemical shift of the C6 singlet of cellulose I_α is 67.1 ppm, and those of the C6 doublet of cellulose I_β are 66.9 and 67.5 ppm. This correlation proposed by Horii et al. indicated that conformations for hydroxymethyl groups in cellulose I_α and I_β were all in the $t-g$ conformation, which was supported by X-ray[32−34] and recent neutron[35] diffraction analyses. On the other hand, since the C6 signal of cellulose III_I is shifted upfield to 64.0 ppm, the correspondence between ^{13}C chemical shift and conformation proposed by Horii et al. indicates that the C6 of cellulose III_I should be in the $g-t$ conformation, which conflicts with the $t-g$ conformation of the crystal structure of cellulose III_I proposed by Sarko et al.[14] Factors affecting the ^{13}C chemical shifts in the solid-state NMR spectroscopy have not yet been established, and the correlation between chemical shifts of C6 and the torsion angles χ does not always hold for some cyclodextrins.[36] However, the chemical shift differences observed are large enough to suggest a conformational difference between cellulose I and III_I in the exocyclic carbon−carbon bonds.

In conclusion, this study provided the complete assignment of the ^{13}C resonances of cellulose III_I through the detailed analysis of the CP/MAS ^{13}C NMR spectra of the cellulose III_I samples in which certain carbons were selectively ^{13}C-enriched. The results presented here indicated that all carbons of cellulose III_I are singlets. Hence, the anhydroglucose rings in the structure of cellulose III_I are magnetically equivalent and experience the same conformation and crystal packing form. In addition, conformational difference in the exocyclic bonds between cellulose I and III_I was suggested by the noticeable difference in their C6 chemical shifts.

Acknowledgment. The authors are indebted to Mr. Eiji Yamada, NMR Instruments Laboratory, Graduate School of Engineering, Hokkaido University, for his technical support of NMR measurements.

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MA021015F