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Carbon-13 solid-state n.m.r. study of ^{13}C-enriched human hair keratin

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Abstract

The ^{13}C-enriched human hair was prepared by labeling human hair with ^{13}C-enriched methyl iodide for the thiol groups formed by reduction of the disulfide bonds. In the ^{13}C CP/MAS n.m.r. spectrum, the Cys S-$^{13}CH_3$ peak split into two peaks with a chemical shift difference of 1.7 ppm. With increasing reduction time in the process of the sample preparation, the relative intensity of the down field peak is increased from 0.17 (20 min reduction time) to 0.53 (20 h reduction time), although that of the up field peak stays almost constant. Here the main peak of the carbonyl carbon was used for the reference of the peak intensity (= 1.00). On the other hand, the residual ratio of the disulfide bond is decreased from 15% at the reduction time of 20 min to approximately 0% at that of 20 h. These results indicate that the down field peak is assigned to the Cys S-$^{13}CH_3$ carbons in the domain with the ordered structure where the disulfide bond is relatively difficult to reduce during the sample preparation. Besides, the up field peak is assigned to the Cys S-$^{13}CH_3$ carbons in the amorphous domain where the reduction occurs completely for at least 20 min. These two Cys S-$^{13}CH_3$ peaks are remarkably different in the half-height width. From the determination of the ^{13}C spin-lattice relaxation times of two peaks, this difference is due to a difference in the chemical shift distribution rather than the difference in the molecular motion.

Keywords: ^{13}C-enriched human hair; ^{13}C CP/MAS n.m.r; Disulfide bond

1. Introduction

Like wool, nail and horn, human hair is one of several mammalian structural components formed from α-keratin [1]. The histological structure of the hair fiber consists of two components, cortex and cuticle. The cortex, comprising 85–90% of hair consists of spindle-shaped macrofibril which have two main structures, microfibril and matrix [2]. The microfibril is a crystalline fibrous keratin protein which is composed of mainly α-helix structure with low content of cystine, while the matrix is an amorphous keratin protein with high content of cystine. The microfibril molecules are aligned along the fiber axis and embedded in an amorphous matrix.

Based on the amino acid sequence and structural homologies, the microfibril of keratin is classified into a member of intermediate filaments (IF) such as vimentine, desumin, glial filaments and neurofilaments [3] and recently labeled as a keratin intermediate filament (KIF) [1–4]. In our previous studied [5,6], the stability of the coiled coil structure in KIF and its disruption mechanism with changing in pH were discussed by means of ^{13}C solution n.m.r. spectroscopy. The coiled coil structure was a rigid structure with very restricted motion at pH 5–6.5, although the structure changed rapidly to random coil above pH 6.5. On the other hand, the largest fraction of the cortex is a matrix which is referred as keratin intermediate filament associated proteins (KIFap) [2]. The matrix contains significant amounts of disulfide bonds which can form the cross-linked structure, though intra- and/or intermolecular covalent bond formation [1,7–9]. The KIFap is basically an amorphous structure, and only limited studies on the structure have been reported. The presence of a well-conserved common pentapeptide repeat of -(Cys-X-Pro-Y-Cys)-$_n$ has been pointed out by the amino acid sequence analysis [1,10]. The conformation is considered to be β-bend, which is stabilized by the disulfide bond between the side chains of Cys residues.

High resolution solid-state n.m.r. spectroscopy has been established as a powerful tool for obtaining information about the secondary structure, the higher ordered structure and the molecular motion of keratin. The earliest ^{13}C CP/MAS n.m.r. studies for horse horn, horse hair,

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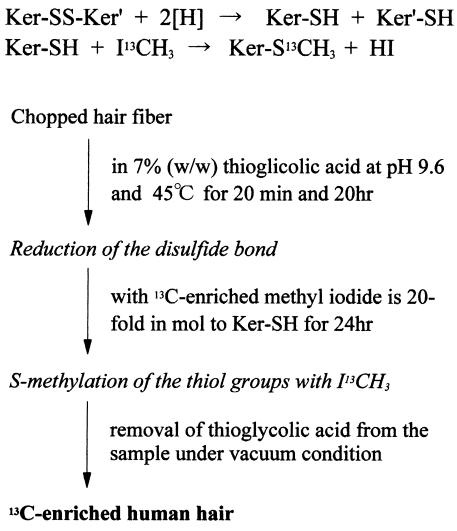

Fig. 1. Reaction scheme for preparation of ^{13}C-enriched human hair.

human hair and parrot feather by Kricheldorf and Müllar showed that this method was useful for the structural characterization of the intact keratin substances [11]. Yoshimizu and Ando measured ^{13}C CP/MAS n.m.r. spectra of several kinds of fractions extracted from wool, and revealed the change of secondary structure and molecular motion with change in the degree of stretching of the samples. The conformation-dependent ^{13}C chemical shift values and ^{13}C relaxation times were used for the purpose [12–15]. In our recent study [16], the damage of human hair by permanent waving as a cosmetic treatment was examined by ^{13}C CP/MAS n.m.r. spectroscopy; the permanent waving treatment caused the partial disruption of helical structure constituting the coiled coil structure in KIF.

Our previous ^{13}C n.m.r. study showed the structure of the matrix was random coil judging from the ^{13}C chemical shift of the carbonyl carbon peaks and gave no detailed information. In this work, we aim to obtain further information on the structure of matrix of human hair basically consisting of amorphous structure. Thus, we will prepare the ^{13}C-enriched human hair with ^{13}C-enriched methyl carbon of the thiol group formed by reduction of the disulfide bond in matrix because the selectivity in the specific site of the molecules and the increased sensitivity are required to discuss the detailed structure with ^{13}C solid-state n.m.r. The chemical shifts, half-height widths and the relaxation times of the given ^{13}C carbon peaks in hair will be used for the study.

2. Experimental

2.1. Materials

Commercial black Asian hair purchased from Staffs (Tokyo) was used for experiments. Hair bundles were washed with a 1% solution of sodium dodecyl sulfate (SDS), rinsed thoroughly with deionized water, air-dried at room temperature and chopped to about 0.5 cm length. In the Raman spectral experiment, natural grey Asian hair was used instead of the black hair in order to decrease the fluorescence.

2.2. Preparation of ^{13}C-enriched human hair

Methylation of Cys residue in hair with ^{13}C-enriched methyl iodide was performed, according to the procedure shown in Fig. 1 [17,18]. The disulfide bond in the chopped hair was reduced in 10 ml of 7.0% (wt/wt) ammonium thioglycolic acid (pH 9.6) for 20 min or 20 h at 45°C in a sealed vial. After rinsing sufficiently by excess deionized water, they were soaked in 5 ml of 0.1 M potassium nitrate and the pH was adjusted at 4.0 with 0.1 N hydrochloric acid. The thiol groups formed by reduction treatment were methylated with ^{13}C-enriched methyl iodide at room temperature, with stirring for 24 h and in the dark. The amount of the ^{13}C-enriched methyl iodide used for the reaction was 20-fold that of the thiol groups estimated from the amino acid composition [1,2,11]. The ^{13}C-enriched methyl iodide was purchased from ISOTEC (Miamisburg, OH). The ^{13}C-enriched human hair was cleaned by repeating the centrifugation and washing, and then the thioglycolic acid penetrated into hair fiber in the reduction process was removed under vacuum condition (10^{-3} torr) at 50°C for 5 days and then at 75°C for 4 days.

2.3. Raman measurements

The NIR-1000 spectrometer (JASCO, Tokyo) was used for Raman measurements using 514.5 nm light from an Ar ion laser (NEC, Tokyo) and the data were collected at a power level of 100 mW. A piece of hair was mounted in the fiber cramp. The typical condition of the Raman spectrometer was as follows: entrance slit: 700 μm; intermediate slit: 750 μm; exit slit: 700 μm; slit height: 4 mm; scan speed: 2 s/div; and repeat time: 16 times. In the Raman experiment, the fluorescence due to the impurities in the samples is often emitted during the measurement and obscures the normal spectra. Tanaka et al. were able to obtain the normal spectrum under decreased fluorescence condition with natural grey hair [19] and, therefore, we used the natural grey hair instead of the black hair.

2.4. ^{13}C CP/MAS n.m.r. measurements

^{13}C CP/MAS n.m.r. spectra were obtained with a GSX-400 n.m.r. spectrometer (JEOL, Tokyo) operating at a ^{13}C frequency of 100.1 MHz equipped with a CP/MAS accessory for solid-state n.m.r. Samples (ca. 110 mg) were filled into a cylindrical zirconia rotor. The spinning rate, contact time and repetition time were 6 kHz, 2 ms and 5 s, respectively. A spectral width of 27 kHz and sampling point of 2 k were used for the spectral acquisition. Spectra were accumulated 3–6 k times to achieve a reasonable

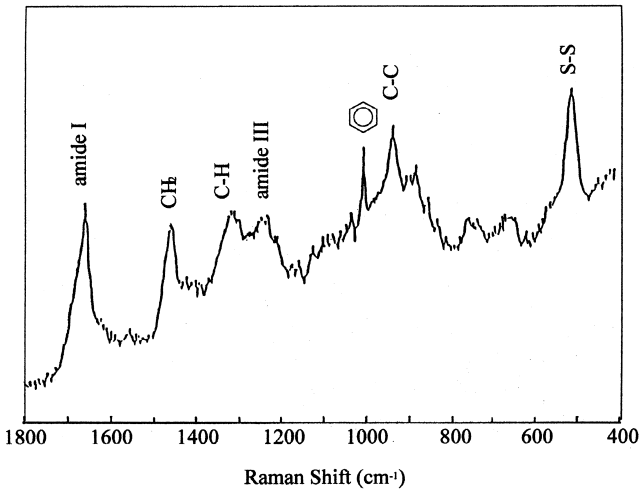

Fig. 2. Raman spectrum of untreated natural grey hair along with the assignment.

signal-to-noise ratio. The ^{13}C chemical shifts were referenced to the methyl peak of hexamethylbenzene and converted to ^{13}C peak of tetramethylsilane (TMS) by adding 17.3 ppm.

The ^{13}C spin-lattice time (T_1) experiment was performed by Torchia's pulse sequence [20]. The T_1 value was determined from the slope of the log plots of the ^{13}C CP/MAS n.m.r. peak intensity against the delay time. In order to obtain the T_1 values of Cys S-$^{13}CH_3$ methyl and C^{α} methine peaks, the delay time was changed in the range from 0.05 to 6 s.

3. Results

3.1. Change in the residual ratio of disulfide bond with reaction time

The fraction of the disulfide bond in human hair was monitored with change of the reduction time by Raman spectroscopy. The Raman spectrum of untreated natural grey hair was shown in Fig. 2. Details of the peak assignment were given in previous papers [19,21]. The peak assigned to the S–S stretching vibration in the Cys residue appeared at 510 cm^{-1}. The peaks of the C=O stretching vibration (amide I), the C–N stretching, NH deformation vibration (amide III) and the C–C stretching vibration in the main chain were detected at about 1660, 1240 and 940 cm^{-1}, respectively. Amide II peaks cannot be usually detected by Raman method because of very weak scattering. Moreover, the phenyl ring of Phe residue was observed at around 1000 cm^{-1}. The fraction of the relative peak intensity at 510 cm^{-1} of the disulfide bond against that of the sample without the reduction treatment was calculated with increasing the reduction times in the process of sample preparation. In the calculation, the intensity of the amide I peak was used for the reference. The change in the fraction of disulfide bond in human hair was plotted against the reduction time in Fig. 3. The fraction is decreased rapidly to 20% during the first 15 min reduction, and then changed slowly with the reduction time. The peak of the disulfide bond was no longer detected after the reduction treatment for 20 h. Naito et al. have pointed out from the amino acid analysis that the complete cleavage of the disulfide bond in hair has been required of the reduction for 19 h [22]. Our results from the Raman experiment agreed with their conclusion.

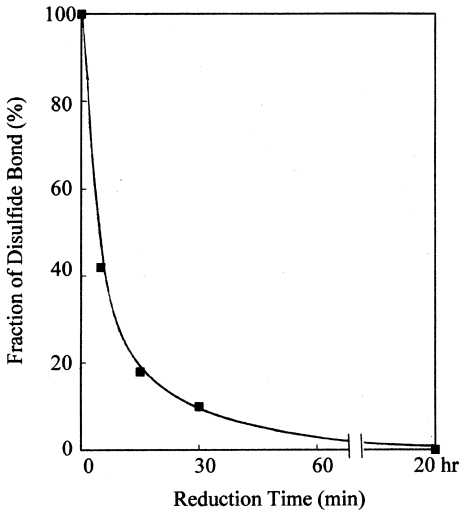

Fig. 3. The plot of fraction of disulfide bonds in human hair monitored with Raman spectroscopy against reduction time.

3.2. ^{13}C CP/MAS n.m.r. spectra of ^{13}C-enriched human hair

^{13}C CP/MAS n.m.r. spectra of untreated and ^{13}C-enriched human hairs after 20 h reduction treatment are shown in Fig. 4. The peak assignment of the spectrum of untreated human hair was given elsewhere in detail [15]. The ^{13}C peaks of the carbonyl, aromatic, C^{α} methine and side-chain aliphatic carbons are observed at about 172–180, 115–158, 45–65 and 10–40 ppm, respectively. Two peaks at 114 and 232 ppm were the spinning side bands in the ^{13}C CP/MAS observation. The conformation of keratin protein did not change by the reduction of disulfide bond and the subsequent S-methylation because the line-shape of carbonyl carbon at 172–180 ppm was the same as that of untreated hair. On the other hand, the spectrum of ^{13}C enriched human hair gave a strong peak at 12–20 ppm (Fig. 4(b)), which was assigned to the Cys S-$^{13}CH_3$ carbon in the ^{13}C-enriched human hair.

The expanded spectra in the Cys S-$^{13}CH_3$ peak region of ^{13}C-enriched human hair with changing the reduction time for 20 min or 20 h were shown in Fig. 5. In the spectrum of the sample after 20 h reduction treatment, the shoulder was clearly detected at a higher field, and therefore, the Cys S-$^{13}CH_3$ peak is considered to be two peaks. The spectral simulation by assuming Gaussian was performed in order to determine the relative intensities of these peaks quantitatively. A broad peak was observed around 26 ppm which was assigned to the natural abundance

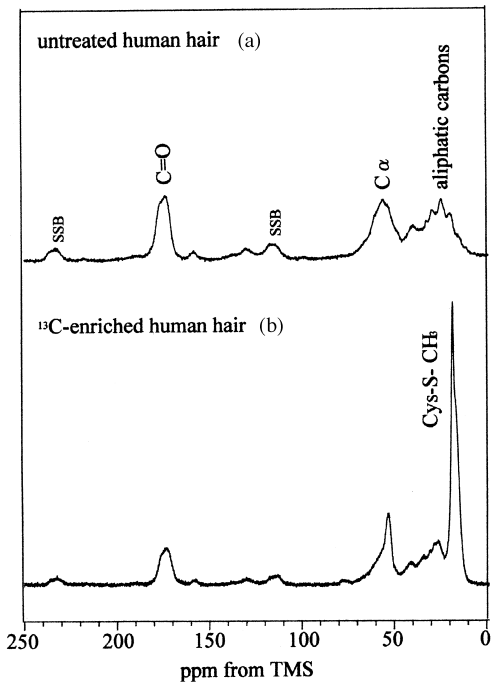

Fig. 4. ^{13}C CP/MAS n.m.r. spectra of untreated and ^{13}C- enriched human hair after 20 h reduction treatment.

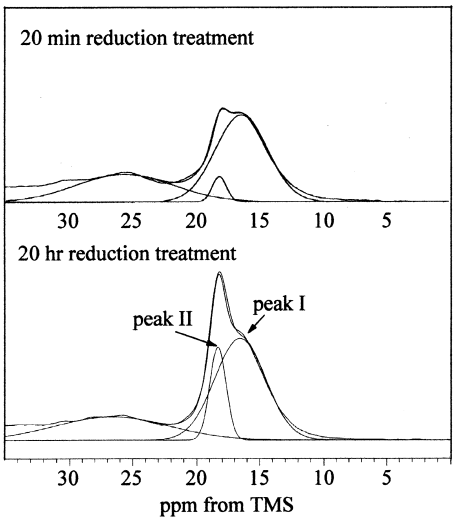

Fig. 5. ^{13}C CP/MAS n.m.r. spectra of ^{13}C-enriched human hairs after 20 min and 20 h reduction treatment (the region, 0–35 ppm, was expanded). The spectral simulation was performed assuming Gaussian line shape.

aliphatic carbons of the amino acid residues in keratin protein. The curve fitting was satisfactory, as seen in Fig. 5. The ^{13}C chemical shift values of these decomposed peaks were 16.6, 18.3 and 25.6 ppm, and the half-height width was observed to be 459, 146 and 889 Hz, respectively. For convenience, the two Cys S-$^{13}CH_3$ peaks at 16.6 ppm and 18.3 ppm are noted to be peak I and peak II, respectively. The relative intensities of peak I and peak II were 2.27 and 0.53, respectively, when the intensity of the main peak of the carbonyl carbon at 173 ppm as shown in Fig. 4 is assumed to be 1.00. The simulated results are summarized in Table 1. When the reduction time was 20 min, the relative intensity was 2.11 for peak I and 0.17 for peak II, although the half-height widths were almost the same between 20 min and 20 h reduction treatments. Thus, it is noted that the intensity of peak II is increased from 0.17 to 0.53 with increasing reduction time from 20 min to 20 h, although the intensity of peak I remains almost constant. This change in the relative peak intensities will be useful information in order to assign these two peaks, as will be discussed later.

Another difference between these two Cys S-$^{13}CH_3$ peaks was difference in the line width. As the origin of this difference, there are two possibilities. One is the difference in molecular motion and another the difference in the chemical shift distribution reflecting difference in the local electronic state. In order to clarify this origin, the ^{13}C spin-lattice relaxation times were measured as described in the next section.

3.3. ^{13}C spin-lattice relaxation times

The partially relaxed ^{13}C CP/MAS n.m.r. spectra of ^{13}C-enriched human hair were obtained using Torchia's pulse sequence at various delay times. The log plots of these peak intensities against the delay time are shown in Fig. 6 for three carbon peaks at 16.6 (peak I), 18.3 (peak II) and

Table 1
^{13}C chemical shifts, half-height widths, spin-lattice relaxation times and relative peak intensities of two Cys S-$^{13}CH_3$ peaks in the ^{13}C CP/MAS NMR spectra of ^{13}C-enriched human hair

		Chemical shift[a] (ppm)	Half-height width (Hz)	Relative intensity[b] (%)	Relaxation time (s)
Reduced for 20 min					
	peak I	16.6	460	2.11	–
	peak II	18.3	127	0.17	–
Reduced for 20 h					
	peak I	16.6	459	2.27	3.6
	peak II	18.3	146	0.53	3.7

[a]The chemical shift was represented from TMS.
[b]The relative peak intensity was determined when the intensity of the main peak of carbonyl carbon at 173 ppm in Fig. 4 was assumed to be 1.00.

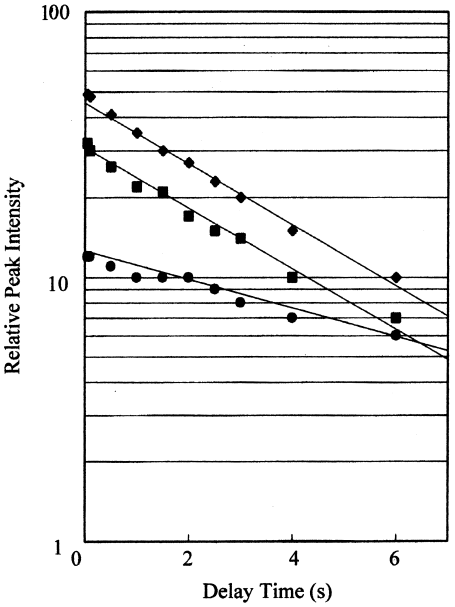

Fig. 6. The log plots of the ^{13}C intensities of two Cys S-$^{13}CH_3$ and C^{α} carbon peaks for ^{13}C-enriched human hair against the delay time. ■: Cys S-$^{13}CH_3$ peak at higher field (peak I); ◆: Cys S-$^{13}CH_3$ peak at lower field (peak II); ●: C^{α} carbon peak.

54 ppm. Each semi log plot indicates a single exponential decay, indicating one component for each carbon in the viewpoints of ^{13}C T_1 relaxation. The T_1 values of peak I and peak II were 3.6 and 3.7 s, respectively. These T_1 values were shorter than the value for the C^{α} carbon (8.3 s) as was expected. The T_1 values between two Cys S-$^{13}CH_3$ peaks have no significant difference, and therefore the molecular motion of two Cys S-$^{13}CH_3$ are almost same. It can be concluded that the difference in the half-height widths between the two Cys S-$^{13}CH_3$ peaks is due to the difference in the chemical shift distribution rather than the difference in molecular motion.

4. Discussion

4.1. Local structure of matrix in human hair keratin

The ^{13}C CP/MAS n.m.r. spectra of ^{13}C-enriched human hair were obtained, in order to clarify the local structure of matrix in human hair in detail. The introduction of the ^{13}C-enriched methyl groups into the disulfide bond was successfully performed by the method in the experimental section. Thus, the selectivity of structure information and increase of the sensitivity were accomplished in the n.m.r. spectrum of ^{13}C-enriched human hair. The Cys S-$^{13}CH_3$ methyl peak at 12–20 ppm split into two peaks with the chemical shift difference of 1.7 ppm. In addition, the higher field peak was considerably broader than the lower field peak (Fig. 5). These findings indicate there are at least two kinds of domains in the matrix region.

With increasing reduction time in the process of the sample preparation, the relative intensity of the lower field peak is increased from 0.17 (20 min reduction time) to 0.53 (20 h reduction time), although that of the up field peak stays almost constant. The residual ratio of the disulfide bond is decreased from 15% at the reduction time of 20 min to approximately 0% at that of 20 h as shown in Fig. 3. These results indicate that the lower field peak is assigned to the Cys S-$^{13}CH_3$ carbons in the domain with the ordered structure where the disulfide bond is relatively difficult to reduce during the sample preparation. On the other hand, the higher field peak is assigned to the Cys S-$^{13}CH_3$ carbons in the amorphous domain where the reduction occurs completely for at least 20 min, because the relative intensity remains constant by the further reaction.

There is a large difference in the half-height width between the two Cys S-$^{13}CH_3$ peaks. From the T_1 values of the two peaks, this difference is due to a difference in the chemical shift distribution rather than the difference in molecular motion. Thus, the broader peak at higher field implies that there is broad distribution in the electronic state for the carbons in the amorphous regions, while the narrower peak at low field corresponds to the presence of the relatively homogeneous local structure from the viewpoint of chemical shift. This suggestion is in agreement with the view that the lower peak is assigned to the ordered structure.

It is well-known that the origin of the conformation-dependent ^{13}C chemical shifts of polyolefins such as polyethylene and polypropylene is due to ^{13}C γ-shielding effect coupled with the appearance of gauche conformation [23–28]. Therefore, if the fraction of gauche conformation around the C^{β}–S bond of the side chain of the Cys residue is larger, the corresponding peak tends to shift to a higher field. It is considered that such a situation in polyolefins is the case for the split Cys S-$^{13}CH_3$ peaks in this experiment. Namely, the broader peak at the higher field assigned to the carbons in amorphous domain indicates the larger fraction of the gauche conformation than the narrower peak at the lower field assigned to the carbons in the ordered domain. This seems reasonable because the presence of the gauche conformation basically means several kinds of disordered structure. These findings indicate that an ordered structure relating to the disulfide bond exists in human hair.

4.2. Possible ordered structure in human hair

As mentioned earlier, a part of the disulfide bond in the matrix forms a well-conserved pentapeptide repeat of -(Cys-X-Pro-Y-Cys)-$_n$. This sequence has been expected to form the β-bend structure, and thus, the ordered structure recognized from the n.m.r. experiment may be regarded as a repeat of such a β-bend structure. This ordered structure is considered to form a hydrophobic environment in matrix because the Cys-Cys (cystine) and Pro residues having relatively hydrophobic side chains are regularly arranged

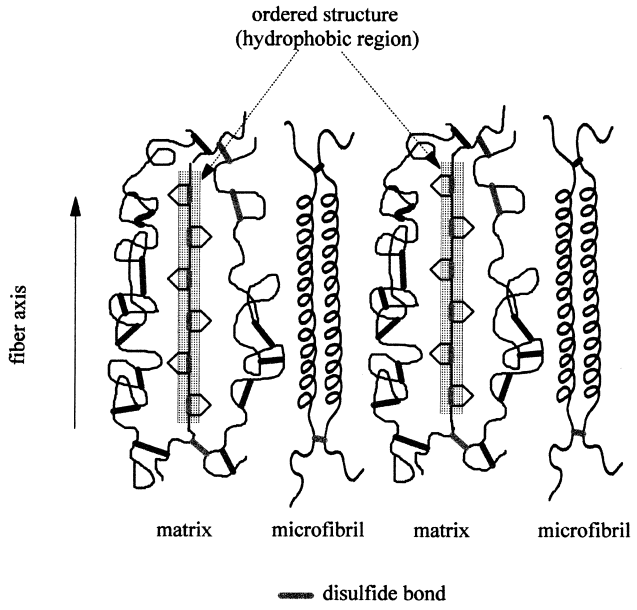

Fig. 7. The model of heterogeneous structure of hair keratin protein proposed on the basis of ^{13}C CP/MAS n.m.r. studies.

in this region. Thus, such a hydrophobic environment may prevent the penetration of the reducing agent in aqueous solution so that the complete reduction of the disulfide bond has required the reduction time of 20 h (Fig. 3 Fig. 5). In actual, the peak intensity of the Cys S-$^{13}CH_3$ corresponding to the ordered structure in 20 min reduction treatment was smaller than that in 20 h reduction. Arai and Naito reported that the hydrophobic region exists in a matrix, in which the disulfide bond was barely reduced for 19 h [22]. Our data from the n.m.r. experiment agree with their results, which clarified further that this hydrophobic region corresponded to the ordered structure repeating of β-bend. The heterogeneous structures proposed from the above results are shown in Fig. 7. From the standpoint of the crystalline structure of keratin protein, it is suggested that hair has not only the α-helical structure constituting microfibril but the ordered structure relating to disulfide bond. This ordered structure may be the repeat of β-bend structure stabilized by the intermolecule bonding between Cys residues, and thus, such structure must be correlated with the physical and mechanical properties of hair keratin fiber as well as microfibril.

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