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pH Dependence of the Coiled-Coil Structure of Keratin Intermediate Filament in Human Hair by ^{13}C NMR Spectroscopy and the Mechanism of Its Disruption

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ABSTRACT: ^{13}C NMR spectra of the low sulfur fraction in *S*-(carboxymethyl)keratine (SCMKA) which corresponds to the hard-keratin intermediate filament (KIF) in human hair have been observed as a function of pH to clarify its disruption mechanism. The assignment was performed by the amino acid composition, both the distortionless enhancement by polarization transfer (DEPT) spectra and the chemical shift values of the SCMKA sample in 8 M urea solution. ^{13}C NMR spectra at pH 5.0 and 6.0 contain essentially no peaks from the amino acid residues in the rod domain, which is due to its coiled-coil structure having highly restricted mobility. The coiled-coil structure was disrupted between pH 6.0 and 7.0 along with great great increase in peak intensities, which indicated the random coil structure occurred. During this disruption process, single chains with a helical form could not exist because there were no helical peaks in the spectra, or they existed for only a very short time, even if they did. Especially, the peak intensities of the side chains of the negatively charged amino acids, Glu and Asp, and those of the positively charged amino acids, Lys and Arg, increased abruptly at around pH 7.0, and these side chains formed ion-pairing interactions maintaining the coiled-coil structure in the rod domain. The peak intensities of the side chains of Leu and Ile also increased abruptly, indicating that hydrophobic interactions among these side chains in the coiled-coil structure were weakened. When the pH of the SCMKA solution was readjusted to pH 6.0 from 9.6, the ^{13}C NMR spectrum was almost identical to that obtained originally at pH 6.0. Thus, both interactions are considered to contribute to the stability of the coiled-coil structure in the rod domain.

KEY WORDS Keratin Intermediate Filament / Low Sulfur Fraction in *S*-(Carboxymethyl)keratine (SCMKA) / Coiled-Coil Structure / ^{13}C NMR Spectroscopy /

Like wool, nail, and horn, human hair is one of several mammalian structural components formed from α-keratin.[1,2] The histological structure of hair fiber consists of two components, the cortex and the cuticle. The cortex, comprising 85 to 90% of hair,[3-5] seems to be responsible for most physical and mechanical properties of hair. This component consists of spindle-shaped macrofibrils having two main structures, microfibril and matrix, which are distinguished by their structures and amino acid compositions.[1-3,6-8] The microfibril is a crystalline fibrous protein which is mainly composed of α-helical proteins with a low content of cystine. These structures are aligned along the fiber axis and embedded in an amorphous matrix with a high content of cystine. Therefore, hair fiber is regarded as an oriented fiber in which the crystalline filaments are aligned, and such a structure must be correlated with the mechanical properties of the keratin fiber.

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.[9-11] Hence, the microfibril has been recently called a keratin intermediate filament (KIF). All sorts of IF proteins consist of a central rod domain, and N- and C-terminal domains.[3,12-20] The structure of the N- and C-terminal domains has been assumed to be disordered according to amino acid sequence analysis. On the other hand, the rod domain has four helical segments, in which two α-helix chains associates with each other to form a double-stranded coiled-coil rope. This coiled-coil structure was proposed by Crick,[21] and Pauling and Corey[22] as shown in Figure 1, and the detailed structure has been studied by analyses of amino acid sequence, X-ray and NMR.[9,23-28]

The amino acid sequence of the coiled-coil structure shows a well-conserved, common heptad repeat of **(a-b-c-d-e-f-g)**$_n$ (Figure 1). About 75% of all **a** and **d** positions is occupied by apolar residues, which stabilize the arrangement of the two strands by hydrophobic interactions.[29] This regular disposition of the apolar residues was observed in tropomyosin[30] and then recognized also in KIF.[2,3,9,25,26] Recent sequence analyses of KIF have revealed that both positively and

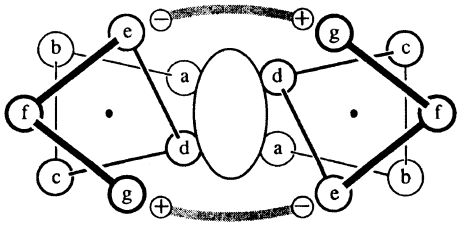

Figure 1. Model of coiled-coil structure, drawn schematically.[28,33] The view which is down the helical axis of a KIF and shows one pair of heptad repeats for each polypeptide chain. Positions **a** and **d** are apolar residues, and an oval lying in the center indicates the hydrophobic interaction area. The oppositely charged amino acid residues in positions **e** and **g** form ion pairing (gray bold line).

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negatively charged amino acid residues are present in positions **e** and **g** and distributed periodically in KIF molecules.[27,28,31-33] This ion-pairing interaction forming between **e** and **g** residues also stabilizes the coiled-coil structure like the hydrophobic interaction. These two interactions have been studied by X-ray and NMR for the GCN4 leucine zipper which forms the coiled-coil structure.[23,24,34]

It is generally known that the mechanical properties of hair fiber are changed by chemical cosmetic treatments such as permanent-waving, bleaching or dyeing. It is said that this decrease is caused by the incomplete re-oxidation of the disulfide bond existing in the matrix during these treatment processes. However, the structural change in KIF should be related to the decrease in mechanical properties because of the oriented structure of the hair fiber. Chemical reagents in alkaline aqueous solutions are often used for these treatments,[3,35-37] so it is important to examine the influence of various pHs on the coiled-coil structure of KIF aligned along the fiber axis. Moreover, the influence on the coiled-coil structure is interesting, because it is stabilized by the hydrophobic and ion-pairing interactions formed by several definite amino acid residues.

In this study, the *S*-carboxymethyl derivative of keratin protein corresponding to KIF was extracted from human hair and then analyzed by ^{13}C solution NMR spectroscopy in order to clarify the disruption mechanism of the coiled-coil structure.

EXPERIMENTAL

Materials

Commercial black Asian hair from Staffs Co., Ltd. (Tokyo) was used for all experiments. The carboxymethyl derivative of keratin protein (SCMK) was extracted from the human hair and then separated into low sulfur fraction (SCMKA) and high sulfur fraction (SCMKB), which correspond to microfibril and matrix, respectively,[38,39] as follows: The hair bundle was washed with 1% aqueous solution (w/w) of sodium dodecyl sulfate (SDS), rinsed thoroughly with deionized water, chopped, cleaned by extraction three times with hexane, ethanol, and acetone, and then air-dried at room temperature. This dried hair was wetted with 8 M urea, and reduced by 0.23 M 2-mercaptoethanol overnight at room temperature under nitrogen gas. Carboxymethylation of thiol groups was carried out by treatment with iodoacetic acid for 15 min, the amount of which was equivalent to that of 2-mercaptoethanol. The extract containing SCMK was filtered and dialyzed at least 24 h by exchanging with deionized water several times. This SCMK solution was adjusted to pH 4.4 with acetic acid and then SCMKA was sedimented by centrifugation. After this precipitate was dissolved in 50 mM sodium tetraborate solution, SCMKA was again sedimented at pH 4.4. These purification procedures were repeated twice more. The supernatant fraction containing SCMKB was dialyzed against deionized water, and then the solution was adjusted to pH 4.4 with 2 M acetate buffer. Upon continued centrifugation of this solution, the supernatant fraction containing SCMKB was obtained. This solution was dialyzed against deionized water again, and then these purification procedures were repeated twice more. The precipitate and supernatant fraction containing SCMKA and SCMKB, respectively, was lyophilized. The weight partitions of SCMKA and SCMKB among SCMK were estimated as 65 and 35%, respectively. This partition was similar to those of SCMKA and SCMKB obtained from wool.[40]

For assignment of the ^{13}C NMR spectra of SCMKA and analysis of its structure, helix-rich and amorphous-rich fractions of SCMKA were prepared by enzymic digestion. Both fractions were prepared by partial hydrolysis with α-chymotrypsin from bovine pancreas (Wako Pure Chemical Industries, Ltd., Tokyo) as described by Crewther and Dowling.[41] The digestion was terminated by precipitation at pH 4.0. The precipitate containing the helix-rich fraction was dissolved at pH 9.0 and re-precipitated at pH 4.0. The supernatant solution obtained by centrifugation contained the amorphous-rich fraction. These fractions were purified by repeating the precipitation and centrifugation, and then the final solution was lyophilized. The weight partitions of the helix-rich and amorphous-rich fractions in SCMKA were about 30 and 70%, respectively.

Methods

The amino acid compositions of cleaned hair, SCMKA, the helix-rich and amorphous-rich fractions of SCMKA were determined as follows; 4 mg of each sample was hydrolyzed in 6 *N* hydrochloric acid for 24 h at 110°C, dried under nitrogen gas flow and dissolved in 0.5 ml of citrate buffer (pH 2.2). Filtered solutions were analyzed with an amino acid analyzer (Mitsubishi Kasei Model-AA01, Tokyo). Experimental errors in the amino acid analysis were within 1%.

The ^{13}C NMR spectra were recorded on a JEOL α-500 NMR spectrometer operating at a ^{13}C frequency of 125 MHz at 50°C. Typical NMR conditions for observation are 11000 scans, 34 kHz spectral width and 3.0 s delay between pulses. Distortionless enhancement by polarization transfer (DEPT) spectra were also recorded for the spectral assignment. The concentration of the sample solution was prepared to 5% in 10% (w/w) D_2O, and its pH was adjusted with hydrochloric acid and sodium hydroxide. The pH value of each sample solution was again reconfirmed after NMR measurement. The chemical shifts were measured relative to an internal reference of dioxane and then converted to the tetramethylsilane (TMS) reference.[42]

RESULTS

Amino Acid Composition

The amino acid compositions of hair, SCMKA, the helix-rich and amorphous-rich fractions of SCMKA determined by amino acid analysis are summarized in Table I. Although the relative amounts of α-helix forming amino acids such as Leu, Ile, Ala, Lys, Arg, Glu, and Asp occupied about two-thirds of the total in SCMKA, those of α-helix-breaking amino acids were small in proportion. These compositions coincide with those of hair and wool which have been reported.[1,2,40] The helix-rich and amorphous-rich fractions of SCMKA prepared by partial digestion with α-chymotrypsin were

roughly regarded as the rod domain and the N- and C-terminal domains in KIF, respectively.[40,41,43] The portions such as Leu, Ile, Ala, Lys, Arg, Glu, and Asp in the helix-rich fraction were larger than those in the amorphous-rich fraction, contrarily the portions such as Thr, Ser, Pro, CMCys, and Gly in the helix-rich fraction of SCMKA were small.

Table I. Amino acid compositions of human hair, SCMKA, helix-rich fraction, and amorphous-rich fraction of SCMKA, which are expressed as percentage of total amino acid residues

Amino acid	Human hair[a]	SCMKA		
		Whole	Helix-rich fraction	Amorphous-rich fraction
Lys	2.7	3.7	4.8	2.4
His	0.9	0.1	0.9	0.5
Arg	5.8	7.7	6.2	4.7
CMCys[b]	—	7.7	3.4	9.1
Asx[c]	4.9	9.6	9.6	7.8
Thr	6.8	6.0	3.9	6.6
Ser	11.7	10.6	7.7	10.8
Glx[d]	11.4	19.1	19.9	12.5
Pro	8.4	1.9	2.0	10.7
Gly	6.4	5.9	2.9	6.6
Ala	4.6	6.8	6.8	5.7
1/2Cys[e]	17.8	0.2	—	—
Val	5.8	6.5	5.8	5.8
Met	0.6	0.6	0.3	0.2
Ile	2.6	4.2	5.2	3.5
Leu	5.8	11.0	15.4	9.2
Tyr	2.0	3.2	3.2	2.4
Phe	1.6	2.2	1.9	1.6

[a] Gillespie (1983)[1]. [b] Means *S*-(carboxymethyl)cysteine. [c] Means Asp and Asn. [d] Means Glu and Gln. [e] Means half-cystine.

Assignment of the ^{13}C NMR Spectra of SCMKA

The ^{13}C NMR spectrum of SCMKA in aqueous solution at pH 6.0 showed broad peaks with low intensities, but it was strongly intensified with high resolution upon the addition of 8 M urea (Figures 2 and 3). In the previous CD experiments on SCMKA,[43] the α-helix content has been evaluated to be about 40% in aqueous solution. This α-helix structure changed to the random coil structure completely in 8 M urea solution. Thus, the ^{13}C NMR spectrum of SCMKA in 8 M urea solution must reflect the random coil structure. Indeed the chemical shifts of all observed peaks in NMR spectrum indicated the random coil values; for example, in C^{α} which is especially susceptible to conformational change, the chemical shift of Glu C^{α} was 54.7 ppm, which was not consistent with the value of the α-helix (57.5 ppm) but with that of the random coil structure (54.9 ppm).[44]

Therefore, peak assignment of the ^{13}C NMR spectra of SCMKA was performed by comparison to that in 8 M urea solution based on; (i) random coil ^{13}C NMR chemical shift values of the amino acid residues of proteins,[44,45] (ii) DEPT spectra for identification of individual function groups, and (iii) a comparison of the amino acid composition estimated from the ^{13}C NMR peak area of SCMKA in 8 M urea with that determined

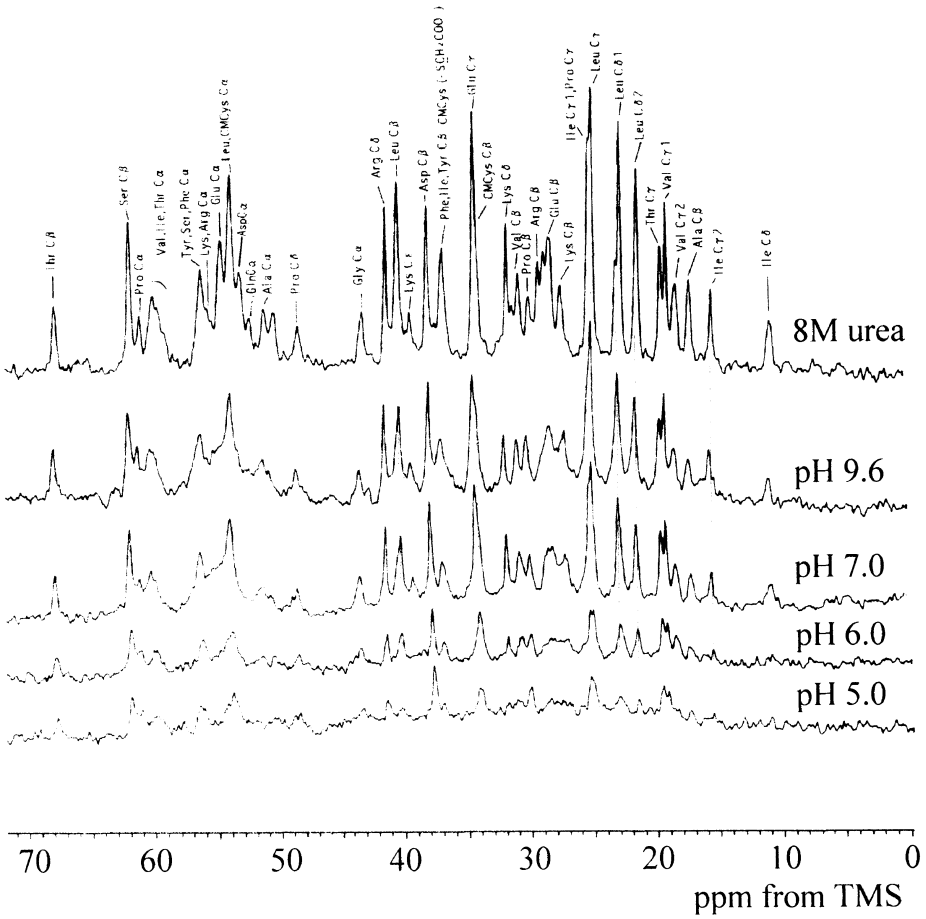

Figure 2. High field region of ^{13}C NMR spectra of SCMKA at various pHs and in 8 M urea solution.

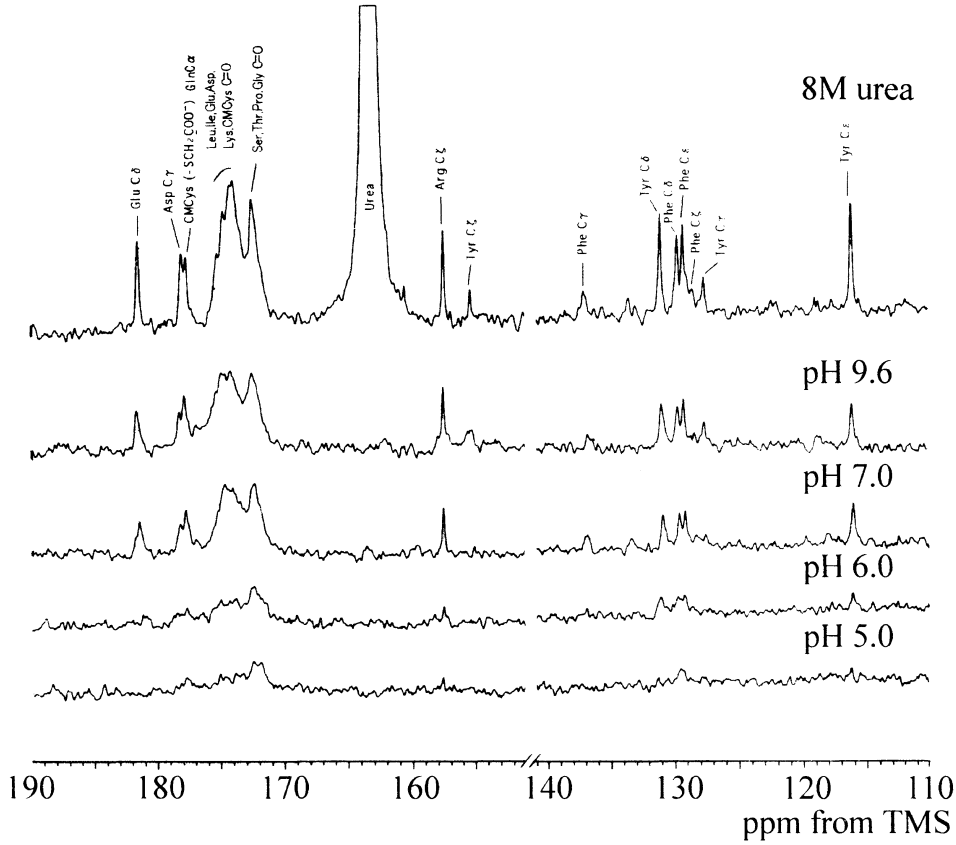

Figure 3. Low field region of ^{13}C NMR spectra of SCMKA at various pHs and in 8 M urea solution.

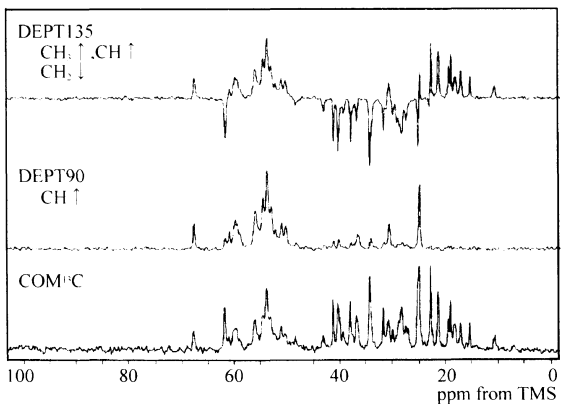

Figure 4. DEPT spectra of SCMKA in 8 M urea solution.

by the amino acid analyses.

DEPT measurement was used to distinguish four types of carbons among the quarternary, methine, methylene, and methyl carbons (Figure 4). From the general chemical shift values of the random coil, the peaks of Val C^{α}, Ile C^{α}, Thr C^{α}, Pro C^{α}, and Ser C^{β} were predicted to overlap around 58 to 63 ppm. The DEPT spectrum clearly indicated that one peak at 61.8 ppm should be assigned to the Ser C^{β}, because only this peak was assigned to a methylene carbon and the others to methine carbons. Judging from the peak shape and the general chemical shift data, the peak observed around 25 ppm was assigned to be overlapped peaks of the C^{γ}s of Leu, Ile, and Pro. The high field side of this peak was identified as the methine carbon of Leu C^{γ}, because the upward peak was detected in both DEPT135 and DEPT90 spectra on the high field side. Other components detected on the lower field side were thus assigned to be Ile $C^{\gamma}1$ and Pro C^{γ}.

The backbone carbonyl carbons from 170 to 176 ppm were difficult to assign exactly because of overlapping of several peaks (Figure 3). On the other hand, the carbonyl carbons in the side chain of Glu and Asp residues, Glu C^{δ} and Asp C^{γ}, were clearly assigned by the general chemical shift data.[44] The peak at 160 ppm was the carbonyl carbon of urea.

Table II shows the amino acid composition estimated from the ^{13}C NMR spectrum of SCMKA in 8 M urea solution. The well-resolved peaks were used for the estimation of amino acid composition from the ^{13}C NMR spectrum, and then the composition was calculated from the area of these peaks. This composition agreed with that from the results of amino acid analysis (Table II). Therefore, the exact assignment of the ^{13}C NMR spectrum of SCMKA was substantiated by compositions obtained from two different methods. The chemical shifts are summarized in Table III along with the assignment.

The Change in ^{13}C NMR Spectra of SCMKA under Various pHs

In the previous study the signals observed in the spectrum of SCMKA were speculated to show only the random coil structure, and the helical part of the coiled-coil domain of the SCMKA could not be observed because of its highly restricted motion.[43] The coiled-coil structure formed by two α-helix chains in SCMKA was disrupted into the random coil structure with high

Table II. Comparison of amino acid compositions of SCMKA obtained by amino acid analysis and ^{13}C NMR analysis

The total contents of these amino acids were considered 100.0%. Experimental errors were within 5%.

Amino acid	Amino acid analysis	^{13}C NMR analysis
Lys	4.5	6.2
His		
Arg	9.3	8.0
CMCys[a]		
Asx[b]	11.6	12.2
Thr	7.3	5.8
Ser	12.8	13.6
Glx[c]	23.0	20.2
Pro	2.3	4.4
Gly		
Ala	8.2	8.7
1/2Cys[d]		
Val	7.9	5.7
Met		
Ile		
Leu	13.3	15.3
Tyr		
Phe		
	100.0	100.0

[a] Means *S*-(carboxymethyl)cysteine. [b] Means Asp and Asn. [c] Means Glu and Gln. [d] Means half-cystine.

Table III. ^{13}C NMR chemical shifts of amino acids consisting of SCMKA

Blanks are unassigned peaks.

Amino acid	Chemical shifts/ppm					
	C^{α}	C^{β}	C^{γ}	C^{δ}	C^{ε}	C^{ζ}
Lys	55.7	27.9		31.8	39.5	—
Arg	55.7	28.8		41.4	—	150.4
CMCys[a]	53.9	34.0		—	—	—
Asp	53.1	38.1	177.8	—	—	—
Asn	52.0	37.6		—	—	—
Thr	59.9	67.6	19.5	—	—	—
Ser	56.3	61.8	—	—	—	—
Glu	54.7	28.2	31.3	181.6	—	—
Gln			31.4	177.5	—	—
Pro	61.2	30.0	25.2	48.6	—	—
Gly	43.4	—	—	—	—	—
Ala	50.8	17.2	—	—	—	—
Val		30.8	19.1/18.4	—	—	—
Ile		37.0	25.2/15.5	10.9	—	—
Leu	53.9	40.2	24.9	21.5/22.9	—	—
Tyr	56.3	37.0	127.7	129.4	116.2	150.4
Phe	56.3	37.0	136.9	129.8	129.3	127.7

[a] Means *S*-(carboxymethyl)cysteine.

mobility by 8 M urea, and therefore, the whole molecule of SCMKA was observed in the ^{13}C NMR spectra (Table II). The disruption and reconstitution processes were monitored by the changes in peak intensity. As shown in Figures 2 and 3, the ^{13}C NMR spectra of SCMKA in aqueous solutions were measured at various pHs, in which other conditions for NMR measurement such as sample concentration, accumulation time, and observed temperature were not changed. Because of the limited observation time, we concentrated on showing the difference in the pH dependence among the amino acid residues in the rod domain and the N- and C-domains of SCMKA clearly and on obtaining spectra with good S/N ratios. Thus, we observed each spectrum with changing pH value by one at a time. Overlapped peaks were neglected for analyses of the side chain and backbone of SCMKA.

Side Chain. The chemical shift values of the observed peaks did not change at all pH ranges examined, indicating random coil values similar to those of SCMKA denatured by 8 M urea (Figures 2 and 3). No chemical shift values indicating the α-helix structure were observed at all.

Peaks in the spectra at pH 5.0 and 6.0 were broad forms with low intensities. Although these two spectra were almost the same, peaks at pH 6.0 tended to be more distinct with slightly intense. On the other hand, the peaks of Thr (C^{β}, C^{γ}), Ser C^{β}, Pro (C^{α}, C^{β}, C^{δ}), and CMCys C^{β} were clearly observed even at pH 5.0. At pH 7.0, not only the intensities of most peaks rose markedly, but also several peaks of side chain carbons such as Lys (C^{β}, C^{γ}), Arg C^{β}, Glu (C^{α}, C^{β}, C^{δ}), and Asp (C^{α}, C^{γ}) appeared. In addition, the very low relative intensities of peaks at pH 6.0 such as Leu (C^{β}, C^{γ}, $C^{\delta 1}$, $C^{\delta 2}$), Ile ($C^{\gamma 2}$, C^{δ}), and Arg (C^{δ}, C^{ζ}) also increased at pH 7.0 remarkably. The increased ratios of peak intensity of these carbons between pH 6.0 and 7.0 were higher than those of Thr (C^{β}, C^{γ}), Ser C^{β}, Pro (C^{α}, C^{β}, C^{δ}), and CMCys C^{β}. Especially, remarkable increases were observed at Leu $C^{\delta 1}$ (21.5 ppm), Leu $C^{\delta 2}$ (22.9 ppm), Asp C^{γ} (177.5 ppm), and Glu C^{δ} (181.6 ppm). They are the carbons at the end of the side chain of amino acids responsible for formation of the coiled-coil structure, and therefore, these carbons must be reflected most markedly in the ^{13}C NMR spectrum with the increase in pH. The ratios of these peak areas in SCMKA at pH 5.0, 6.0, 7.0, and 9.6 were compared with those in 8 M urea (Figure 5).

The ratios of Leu $C^{\delta 1}$ and Leu $C^{\delta 2}$ markedly increased from 18% and 12% at pH 5.0, and 21% and 17% at pH 6.0, to 62% and 47% at pH 7.0, respectively. Those amino acids exist in the hydrophobic region of the rod domain corresponding to the helix-rich fraction of SCMKA. These findings suggest that the mobility of the Leu side chain forming hydrophobic interaction increases, when it was exposed in neutral or alkaline solution. Stability of the coiled-coil structure seems to be held restrictedly at low pHs under 6.0 and to decrease significantly at pH 7.0. A similar increase in the peak intensity at pH 7.0 was observed for the carbonyl carbons of two negatively charged amino acids, Glu C^{δ} (181.6 ppm) and Asp C^{γ} (177.8 ppm) (Figure 3). The carbonyl carbons of the side chains of Glu and Asp residues contribute to ion-pairing interactions between the helical chains at pH 5.0 and 6.0 because of low intensities of these peaks. The amino acids oppositely charged with respect to Glu and Asp holding an ion pair in the coiled-coil structure should be Lys and Arg. Like Glu C^{δ} and Asp C^{γ}, Lys C^{ε} (39.5 ppm), and Arg C^{ζ} (150.4 ppm) were scarcely detected at pH 5.0 and 6.0 but were clearly seen at pH 7.0 (Figures 2 and 3). No peaks of Glu C^{δ} and Asp C^{γ} were observed, and the ratios of Lys C^{ε} and Arg C^{ζ} at pH 5.0 were only 6% and 7%, respectively (Figure 5). They increased slightly from 10 to 15% at pH 6.0 but increased greatly from 40 to 60% at pH 7.0. These results suggest that the mobility of

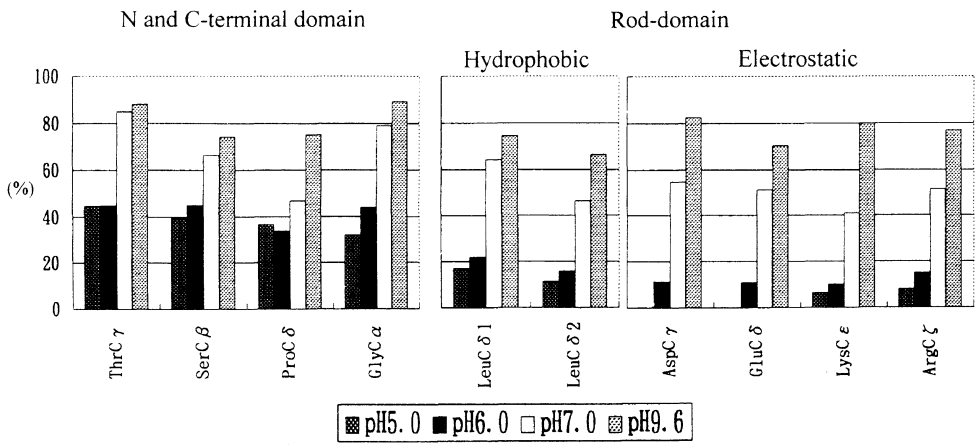

Figure 5. Intensity distribution of detected peaks at the end of the amino acid residue side chain. Thr, Ser, and Pro are amino acids constituting the non-helical N- and C-terminal domains. Other amino acids are preferentially located in the α-helical rod domain. The distribution was expressed as the ratio of peak area at a certain pH to that in 8 M urea solution.

charged amino acids is restricted at low pHs like the hydrophobic interaction between Leu residues. However, the intensities of all peaks at pH 9.6 were much lower than those in 8 M urea. The spectral baseline was somewhat broad at pH 9.6, and the peak intensities did not increase despite the high pH.

In contrast, the peak intensities of the carbons of the amino acids located selectively in the N- and C-terminal domains did not change independently of pH. At pH 5.0, not only the peaks of Thr C^{β} (67.6 ppm), C^{γ} (19.5 ppm), Ser C^{β} (61.8 ppm), Pro C^{β} (30.0 ppm), and C^{δ} (48.6 ppm) (Figure 2), but also about 40% of the carbon peaks at the end of the side chain of Thr, Ser, Pro, and Gly were detected reflecting molecular mobility (Figure 5). These peaks increased at pH 7.0, but the increased ratios were smaller than those of Leu, Glu, Asp, Lys, and Arg locating in the rod domain.

In the aromatic carbon region, the peaks of the C^{γ} (127.7 ppm), C^{δ} (129.4 ppm), C^{ε} (116.2 ppm), and C^{ζ} (150.4 ppm) of Tyr and the Cγ (136.9 ppm), Cδ (129.8 ppm), C^{ε} (129.3 ppm), and C^{ζ} (127.7 ppm) of Phe were not detected at pH 5.0 (Figure 3). Above pH 7.0, these carbon peaks became higher and sharper. Especially, the peak intensities of C^{δ} and C^{ε} of both amino acids showed a marked increase.

Back Bone. A conformational change in the peptide backbone at various pHs was observed in the carbonyl carbon region in ^{13}C NMR spectra (Figure 3). An overlapped carbonyl carbon peak at 172 ppm detected at pH 5.0 and 6.0 was assigned to the backbone carbonyl carbon of the amino acids, Thr, Ser, Pro, and Gly. On the other hand, a peak at around 174 ppm, which was scarcely observed at pH 5.0 and 6.0, was clearly observed at pH 7.0. This peak was assigned to the overlap of backbone carbonyl carbons of several species of amino acids except Thr, Ser, Pro, and Gly, which mainly corresponded to the amino acids constituting the rod domain. These findings indicate that the peptide backbone constituting the N- and C-terminal domains has high mobility at even pH 5.0 and 6.0, while that constituting the rod domain has relatively low mobility at these two pHs. The increase in the peak at around 174 ppm at pH 7.0 is interpreted as the peptide backbone of the coiled-coil structure was also being disrupted between pH 6.0 and 7.0 like the change in the side chain.

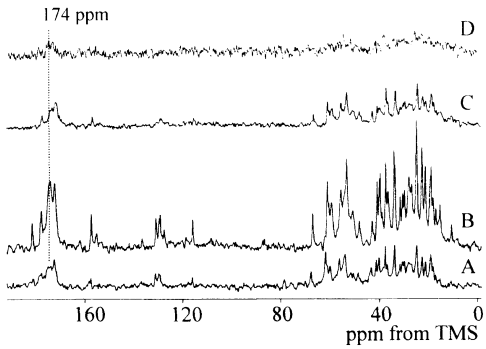

Figure 6. Changes in the spectrum at various pHs. A, SCMKA at pH 6.0, initial spectrum; B, SCMKA at pH 9.6; C, SCMKA restored to pH 6.0 from 9.6; D, difference spectrum A—C.

Reconstitution of the Coiled-Coil Structure in SCMKA

Figure 6 shows the ^{13}C NMR spectra of SCMKA at pH 6.0 (A), at pH 9.6 (B), and those after restoration from pH 9.6 to pH 6.0 (C). The spectra of SCMKA in aqueous solution showed high resolution and intensity on changing the pH from 6.0 to 9.6 because of the disruption of the coiled-coil structure. After SCMKA was kept at pH 9.6 for a few days at room temperature, the SCMKA solution was readjusted at pH 6.0. The spectrum of this SCMKA solution (C) was basically similar to the initial spectrum of SCMKA at pH 6.0 (A). However, the peak shape of the backbone carbonyl carbon region in the restored spectra were just slightly different from the initial spectrum. The peak intensity at around 174 ppm overlapped amino acids such as Leu, Glu, Asp, and Arg contained in the rod domain of the restored SCMKA was weaker than that of the initial SCMKA. The top of the carbonyl carbon peak in the difference spectrum of SCMKA (D) which was calculated from the initial spectrum (A) and the restored spectrum (C) is shown at 174 ppm. It was therefore interpreted that the coiled-coil structure in SCMKA was in any event reversible with the change in pH, although the reverse brought slight structural change.

DISCUSSION

The hair fiber is regarded as an oriented fibrous protein in which KIF is aligned along the fiber axis. Our previous study has suggested from the results of the ^{13}C solution NMR analysis of SCMKA corresponding to KIF that the N- and C-terminal domains consisting of a non-helical structure have high mobility, but that the rod domain forming the coiled-coil structure has highly restricted mobility.[43] Therefore, the properties of hair fiber must be correlated with such dynamic structure of the rod domain forming the coiled-coil structure. For hair cosmetics, its chemical treatments such as permanent-waving, bleaching and dyeing are usually provided in some alkaline solutions,[3,35-37] resulting in a decrease in the mechanical properties of hair. Because the coiled-coil structure is predicted to be more and less disrupted during these treatments, it is important to clarify the disruption process as a function of pH. To obtain a clear disruption mechanism, SCMKA was analyzed by ^{13}C solution NMR, and a site specific analysis was performed with due consideration for the arrangement of specific amino acids, as shown in Figure 1. The conformation of SCMKA in the solid state has already been studied with ^{13}C CP/MAS NMR by Yoshimizu and Ando.[40,46,47] However, the disruption process could not be fully monitored because of the low resolution of the spectra from the solid state NMR. In order to study the influence of various environments such as pH or chemical agents, the analysis in solution is preferred rather than that in solid state. Thus, ^{13}C solution NMR is adopted in this paper to monitor the disruption process of SCMKA relate to pH, which can be analyzed from the resulting changes in the chemical shift and intensity.

At pH 5.0 and 6.0, the spectra of SCMKA gave very the low resolution with low intensity and broad shape peaks (Figures 2 and 3). This observation indicates the low mobility of the SCMKA molecule at the low pHs. However, the peaks assigned to the side chain carbons of amino acids existing in the N- and C-terminal domains, such as Thr , Ser, Pro, and CMCys, were observed even at pH 5.0. Chemical shifts of these peaks indicated the random coil values similar to those of the coiled-coil structure of disrupted SCMKA in 8 M urea solution. It was thus clarified that the N- and C-terminal domains formed the disordered structure with high mobility, because these amino acids could be detected in the ^{13}C NMR spectra at pH 5.0 and 6.0. Indeed, they were classified as the α-helix-breaking amino acids, which consisted of the amorphous-rich fraction of SCMKA forming the non-helical structure. However, the detected peak areas at pH 5.0 and 6.0 were smaller than those in 8 M urea solution. If such amino acids constitute the N- and C-terminal domains, their area must be 100% compared with those in 8 M urea solution. This is caused by the distribution of amino acids. Such amino acids do not always exist in the N- and C-terminal domains, but a part of these amino acids is contained in the rod domain.

The rod domain is formed by the coiled-coil structure in which two α-helix chains are twisted together. These peptide chains contain the heptad repeats of a periodic amino acid sequence, $(\mathbf{a}\text{-}\mathbf{b}\text{-}\mathbf{c}\text{-}\mathbf{d}\text{-}\mathbf{e}\text{-}\mathbf{f}\text{-}\mathbf{g})_n$, in which hydrophobic amino acid residues commonly occur in positions **a** and **d**, and positively and negatively charged amino acid residues regularly occur in positions **e** and **g** (Figure 1). The coiled-coil structure is assumed to be stabilized by both the hydrophobic and ion-pairing interactions presumed from the homology of the amino acid sequence. The former interaction is due to hydrophobic bonding between the side chains of Leu (positions **a** and **d**), and the latter is electrostatic bonding between positively charged amino acids, Lys and Arg, and negatively charged amino acids, Glu and Asp (positions **e** and **g**). The peaks corresponding to the side chain carbons of these amino acids were scarcely detected at pH 5.0 and 6.0 but abruptly appeared at pH 7.0 (Figures 2 and 3). The chemical shifts of all detected peaks indicated the random-coil values. Peak intensities increased remarkably in this pH region, which represented carbons at the end of the side chain of these amino acids (Figure 5). Especially, the end of the side chain of Glu, Asp, and Lys underwent the ion-pairing interaction which was not detected at pH 5.0 but increased rapidly at pH 7.0. The abrupt increase in the mobility of the side chain correlating with formation of the coiled-coil structure is due to its disruption by exposing the side chain to neutral or alkaline solution. The coiled-coil structure seems to be restricted at low pHs under 6.0 but to be disrupted predominantly into the random coil structure between pH 6.0 and 7.0. In the peptide backbone region of the ^{13}C NMR spectra (Figure 3), the carbonyl carbon of amino acids forming mainly the rod domain, such as Leu, Glu, Asp, and Lys, was detected above pH 6.0 to 7.0, while those forming the N- and C-terminal domains, such as Thr, Ser, Pro, and Gly, were detected even at pH 5.0. It is suggested that the backbone forming the coiled-coil structure is disrupted into the random structure, when the hydrophobic and ion pairing interactions between side chains are weakened. Such disruption of the coiled-coil structure at pH 7.0 was also confirmed by analysis of the aromatic region in the ^{13}C NMR spectra. The peak intensities in this region increased abruptly, when the coiled-coil structure changed to a random coil at high pHs. This result suggests that the rapid rotation of aromatic rings occurs by exposure of the side chain of Phe and Tyr to aqueous solution. The chemical shifts of C^{α} and C^{β} in the amino acid side chain provide the information concerning the change in crystalline form under various pHs, which is especially susceptible to the conformational change. The chemical shift values did not change over the whole pH range examined and indicated the random-coil structure, whereas the peak for α-helix structure was not observed at all. It is assumed from the results that the coiled-coil structure is preferentially disrupted between pH 6.0 and 7.0, producing the random-coil structure having high mobility. During the disruption process caused above pH 6.0, the single chain with a helical form cannot exist in SCMKA or exists only for a very short time, even if it does.

Some of the coiled-coil structure still remained even at pH 9.6 which is the pH of the strongest permanent-waving lotion. The peak intensities over the entire regions of the ^{13}C NMR spectra increased slightly from pH 7.0

to 9.6 but were smaller than those in 8 M urea solution. The low mobility component must remain in pH 9.6 solution because of a somewhat broad spectral baseline.

These NMR analyses not only reveal the disruption process but also support that the regularity of the amino acid sequence to maintain the coiled-coil structure is present in KIF protein. Although the heptad repeats of the amino acids, $(\mathbf{a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g})_n$, have been assumed by the homology of amino acid sequence,[27,28] they have not been experimentally proved until now. The solution ^{13}C NMR method was useful for this point because of the site specific information. The results obtained from the NMR analyses supported that the hydrophobic interaction between the Leu residues of SCMKA and the ion-pairing interaction formed by negatively charged amino acids (Glu, Asp) and positively charged amino acid residues (Lys, Arg) served to maintain the coiled-coil structure. Although no peak or only a broad peak of Ile C^{δ} (10.9 ppm) was observed at pH 5.0 and 6.0, this peak intensity increased abruptly at pH 7.0. This finding indicates that Ile is also correlated with the hydrophobic interaction similarly to Leu, which has not been suggested by the homology of the amino acids sequence.

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