NMR Spectroscopic Detection of Interactions between a HIV Protein Sequence and a Highly Anti-HIV Active Curdlan Sulfate

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Abstract: A new method is found for the detection of interactions between a highly anti-HIV (human immunodeficiency virus) active curdlan sulfate (CS) and a HIV peptide with the aid of NMR spectroscopy. To elucidate the action mechanism of the anti-HIV activity in curdlan sulfate, a partial peptide sequence in an envelope glycoprotein gp120 was synthesized. The sequence consists of a dimer (D518) of the sequence from no. 506 to 518, which is one of putative reaction sites for the sulfated polysaccharide. This was used to examine its interactions with CS. When CS and D518 were mixed in appropriate molar ratios, gel-like materials were formed. ^{1}H NMR spectra of the gel-like material revealed the formation of interpolymeric ionic interactions between a negatively charged CS and a positively charged D518. At the molar ratio [CS]/[D518] of 0.27, 100% gel formation was observed. Effects of molar ratio, pH, and temperature on the gel formation, that is, the degree of interaction, were also studied. In addition, although for a V3 region peptide sequence, i.e., nos. 309 to 331, the same method was applied, the mixing of CS with the V3 peptide did not provide gels, but yielded precipitates which afforded no structural information by solution NMR spectroscopy.

Introduction

A number of efforts have been made to find anti-human immunodeficiency virus (HIV) agents for the treatment against the acquired immunodeficiency syndrome (AIDS). Reverse transcriptase inhibitors and protease inhibitors improved remarkably for AIDS patients.[1,2] However, these drugs induce serious side effects and appearance of drug-resistant HIV strains in long-term clinical trials.[3,4] Recently, a new series of anti-HIV agents were reported such as conjugates of a reverse transcriptase inhibitor with a protease inhibitor[5] or with a sulfated polysaccharide,[6] and protease inhibitors designed by new concepts.[7,8]

During searching for new AIDS drugs, anti-HIV activity was clarified in sulfated polysaccharides in vitro via prevention from the binding of HIV virions to CD4$^+$ cells and the syncytium formation.[9] We successfully synthesized curdlan sulfate which showed a unique combination of biological activities, that is, a very high anti-HIV activity but low anticoagulant activity.[10−13] The curdlan sulfate also exhibited low toxicities in experiments in vivo using experimental animals.[10] Phase I/II clinical tests using curdlan sulfate as an AIDS drug were carried out twice under permission of United States FDA in 1992 to 1993 and 1995 to 1996.[14,15] A large dosage-related increase was observed in the number of CD4$^+$ lymphocytes in the peripheral blood with intravenous administration of 100, 200, and 300 mg of curdlan sulfate to individual carriers, though significant improvements were not observed in a short-term test of patients.

Inhibitory effects on viral replication and syncytium formation have been supposed for sulfated polysaccharides. These two are, therefore, mainly mediated by ionic interactions between

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anionic portions of sulfated polysaccharides and positively charged portions of envelope glycoprotein gp120 in HIV.[16,17] In the case of curdlan sulfate, one of the reaction sites in gp120 was revealed to be positively charged amino acids concentrated in the V3 region.[18,19] Other ionic interactions between natural biological polymers are well-known for an anticoagulant polysaccharide heparin and its counterpart antithrombin III.[20] In the case of HIV infection, negatively charged sulfated proteoglycans are also promising such as heparan sulfate which interacts with positively charged proteins related to HIV binding to regulate the virus entry into T cells.[21−23]

According to the investigation on the secondary structure and charge profile of HIV envelope glycoprotein gp120, it was revealed that the gp120 contains six α-helices in which two highly positively charged amino acid sequences exist in the V3 loop and in the sixth helical region. In particular, the latter portion of residues nos. 506 through 518 contained several basic amino acids (three lysines and four arginines).[24] In the previous paper, we revealed the existence of interactions between curdlan sulfate and polylysine as a model compound for the positively charged protein portion of gp120 from NMR spectroscopy.[25]

In this study, we wish to report interactions between curdlan sulfate and a peptide sequence in gp120. The latter is regarded as a reaction site for anti-HIV active curdlan sulfate. It will be confirmed by ^{1}H and ^{13}C NMR spectroscopy. We synthesize a peptide sequence of nos. 506 to 518 at the carboxy terminus containing several basic amino acids as a possible candidate of potent reaction sites to curdlan sulfate. Since it can be expected for a medium molecular weight peptide to more easily form NMR-detectable gel-like materials, a 26 amino acid sequence corresponding to a dimer of the sequence nos. 506 through 518 is chosen as the HIV protein portion. We wish to analyze NMR signals of the mixture between the curdlan sulfate and the protein sequence for the quantitative study of polymeric interactions. In addition, interactions of the V3 region peptide sequence with curdlan sulfate are also examined.

Experimental Section

Materials. Sodium curdlan sulfate (abbreviated as curdlan sulfate or CS) containing 14.4% of sulfur with a weight average molecular weight of 79 000 was provided from Ajinomoto Co., Ltd.[11] The hydroxyl groups in curdlan sulfate are regioselectively substituted by an average of 1.5 sulfate groups per glucose residue. A dimer (denoted as D518) of the helical region consisting of amino acid residues of no. 506 (Thr) to no. 518 (Arg^+) at the carboxy terminus in gp120 was synthesized through the FastMoc method on an ABI 433 peptide synthesizer using Fmoc-amino acids. The peptide sequence composed of 26 amino acids was as follows: NH_2-(Thr)(Lys^+)(Ala)(Lys^+)(Lys^+)-(Arg^+)(Val)(Val)(Gln)(Arg^+)(Glu)(Lys^+)(Arg^+)(Thr)(Lys^+)(Ala)(Lys^+)-(Lys^+)(Arg^+)(Val)(Val)(Gln)(Arg^+)(Glu)(Lys^+)(Arg^+)-COOR. The sequence and residue number were obtained from the original gene analysis.[26] The peptide was purified by high-performance liquid chromatography. The molecular weight was determined by FAB mass spectrometry to be 3236.9. The purity of the peptide was in excess of 95%. A 25 amino acids sequence of the V3 region consisting of amino acid residues of nos. 309 (Asn) to 331 (Gly) in addition to lysines at both termini, that is NH_2-(Lys^+)(Asn)(Thr)(Arg^+)(Lys^+)(Ser)(ILeu)-(Arg^+)(ILeu)(Gln)(Arg^+)(Gly)(Pro)(Gly)(Arg^+)(Ala)(Phe)(Val)(Thr)-(ILeu)(Gly)(Lys^+)(ILeu)(Gly)(Lys^+)-COOR, was also synthesized.

Preparation of Samples for Detection of Polymeric Interactions. NMR samples were prepared in situ in a NMR tube for determining interactions between curdlan sulfate and polypeptide. For example, 0.25 mL of D_2O solution containing 5 mg (4.02×10^{-5} mol based on the average molecular weight of the component amino acids = 3236.9/26 = 124.50) of D518 and 0.25 mL of D_2O solution containing 3.43 mg (1.09×10^{-5} mol based on the glucose unit) of CS were put into the NMR tube, followed by mixing for 2 min. The NMR tube was kept at 37 °C for 1 h for the formed gel-like material to adhere to the wall of the NMR tube. The molar ratio [CS]/[D518] of this solution was 0.27. Since each residue of CS contained an average of 1.5 sulfate groups negatively charged and each molecule of D518 had 15 amino groups positively charged, the ion ratio [CS^-]/[$D518^+$] of the above sample was 0.7. In different molar ratios, the NMR solution contained a constant amount (5 mg) of D518 and different amounts of CS ranging from 1.51 to 14.65 mg. For ^{13}C NMR measurements, the amount of CS was changed in a similar manner, keeping a constant amount (14 mg) of D518.

Measurements. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a JEOL Lambda-500 spectrometer. 4,4-Dimethyl-4-silapentane-1-sulfonate (DSS) was used as the internal standard for ^{1}H and ^{13}C NMR measurements. TMS was used as reference ($d = 0$). For the assignment of proton absorptions of D518, 2-dimensional NMR measurements such as COSY and PTOCSY[27] were carried out. Assignments of carbon absoprtions of D518 were performed by use of 2-dimensional NMR measurements such as PHSQC, HMQC, and HMBC.[27]

Results and Discussion

^{1}H NMR Spectra of Mixtures of D518 with Curdlan Sulfate. Each peak of the ^{1}H NMR spectra of a HIV peptide sequence D518 were assigned with the aid of 2D PTOCSY and COSY NMR spectra. Measurements were done in D_2O solution. All proton absorptions of D518 appeared in the range of 0.9 to 4.5 ppm. The δ-proton of arginine (δ_R), ϵ-proton of lysine (ϵ_K), and γ-proton of valine (γ_V) appeared at 3.22, 3.02, and 0.94 ppm, respectively, without overlapping other absorptions.

When a curdlan sulfate solution was mixed with a D518 solution in an appropriate molar ratio in a NMR tube, adhesion of gel-like materials occurred on the wall of the tube. Figure 1 shows ^{1}H NMR spectra of the D518 and curdlan sulfate mixtures in different molar ratios. As seen in spectra C through E, absorptions due to D518 in the range of 0.8 to 3.3 ppm shifted considerably depending on molar ratio. In spectrum C of the molar ratio [CS]/[D518] of 0.12, new peaks appeared at 3.03, 2.83, and 0.75 ppm due to δ_{R-G}, ϵ_{K-G}, and γ_{V-G} absorptions

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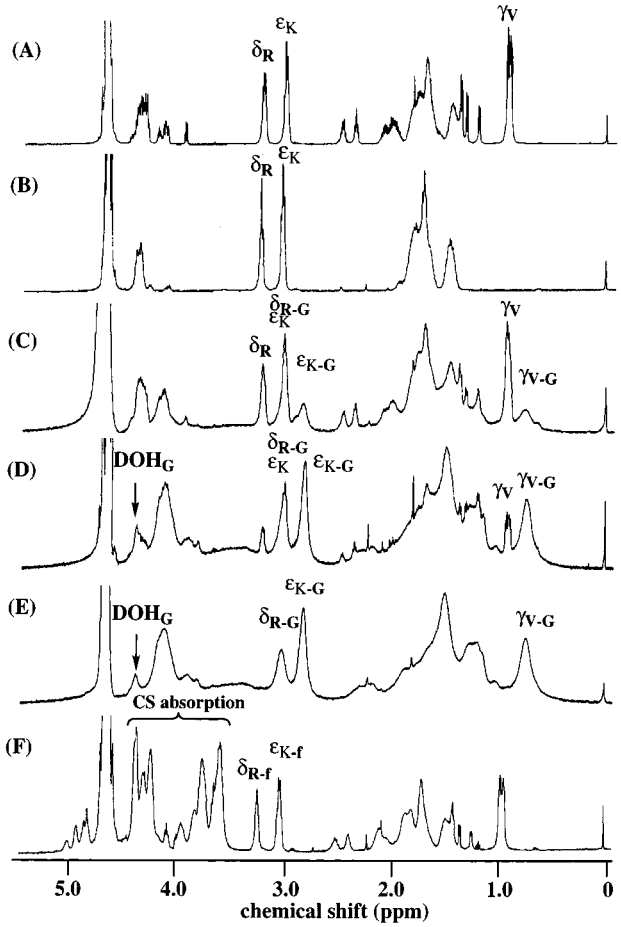

Figure 1. 500 MHz ^{1}H NMR spectra of D518 and mixtures of curdlan sulfate (CS) with D518 in different molar ratios: D518 (A) and a mixture of polylysine (PL) with polyarginine (PA) in the molar ratio of PL to PA of 4:3 (B) and in the molar ratio ([CS]/[D518]) of 0.12 (C), 0.23 (D), 0.27 (E), and 1.16 (F).

corresponding to those in the gel state, respectively, being 0.19 ppm upfield shifted from the corresponding original peaks. Other absorptions due to amino acids in the range of 1.2−2.5 ppm exhibited the existence of peaks accompanied by the formation of gel-like materials, although those were not clearly separated.

Comparing with peptide sequences dissolved in the solution, those in the gel are assumed to be surrounded by electron-donating sulfated polysaccharide, causing the upfield shift of ^{1}H NMR absorptions.

Proportions of the absoprtions due to the gel-like complex were determined by means of the relative intensities of the ϵ proton of lysine, and of the δ proton of arginine. Results are summarized in Table 1. For the molar ratio of 0.12, the proportion of the gel-like complex was 40%. The proportion increased with increasing molar ratio up to 0.27 (calculated ion ratio of 0.7), reaching to 100%. Beyond the molar ratio of 0.27, the proportion of gel-like material decreased. In the molar ratio of 0.72, the gel-like material completely disappeared.

It has been reported that in the interaction between heparin and antithrombin III, three anions consisting of two sulfamides and a sulfate in heparin react with three amino groups (=three ammonium cations in an aqueous solution) of the lysine side chain in antithrombin III, which causes a conformational change in the complexed antithrombin III.[20,28] Considering that reaction sites for polymers to form gelled materials are a few percent of

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Table 1. Effects of the Molar Ratio [CS]/[D518] on the Proportion of Gel-like Complexes

	curdlan sulfate (CS)		D518			
no.	wt mg	mol[a] $\times 10^{-5}$	wt mg	mol[b] $\times 10^{-5}$	molar ratio [CS]/[D518]	proportion of gel-like complex,[c] %
1	1.51	0.48	5.0	4.02	0.12	40
2	2.94	0.93	5.0	4.02	0.23	86
3	3.43	1.09	5.0	4.02	0.27	100
4	4.55	1.44	5.0	4.02	0.36	82
5	6.97	2.21	5.0	4.02	0.55	32
6	9.08	3.12	5.0	4.02	0.72	0
7	14.65	4.65	5.0	4.02	1.16	0

[a] Based on the molecular weight of the glucose residue having 1.5 sulfate groups. [b] Based on average residue molecular weight (124.50), i.e., the molecular weight of D518 (3236.9) divided by the number of amino acids (26). [c] Determined by ^{1}H NMR spectroscopy in terms of the absorptions due to the ϵ proton of lysine and the δ proton of arginine.

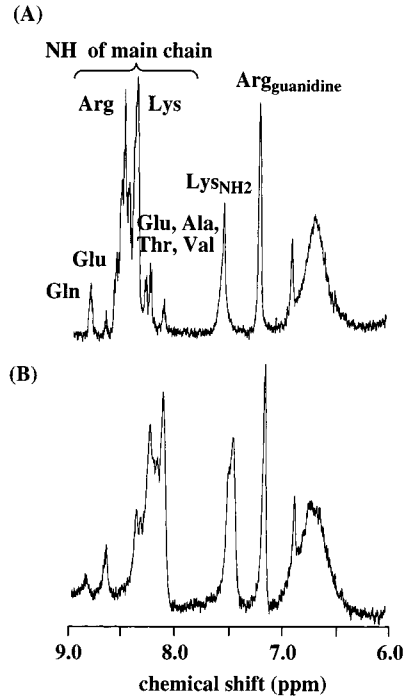

Figure 2. 500 MHz ^{1}H NMR spectra of D518 and mixtures of D518 with curdlan sulfate (CS) in the molar ratios of 1.16 in the N−H absorption region of main chains and side chains of D518: D518 (A) and D518 in the molar ratio of 1.1 (B). 10% D_2O was used as NMR solvent.

the total functional groups in the polymers, interactions are expected to be induce between the negatively charged curdlan sulfate and the positively charged peptide D518. A few percent of the total charged segments participated and the remaining molecules or segments were confined in the cross-linked network, providing NMR signals. Accordingly, the formation of the gel by the reaction between CS and D518 revealed that the peptide sequence including D518 in gp120 is one of the reaction sites for curdlan sulfate to manifest its anti-HIV activity.

To examine the secondary structure of the peptide sequence D518 existing in the excess CS and D518 mixture after decomposition of the gel, ^{1}H NMR spectra of the NH and NH_2 regions were measured in 10% D_2O solutions. As shown in Figure 2, all absorptions due to NH and NH_2 protons shifted upfield, and especially, NH absorptions due to the main-chain amide bonds exhibited an upfield shift by 0.25−0.28 ppm. As reported elsewhere,[25] in a mixture consisting of an excess

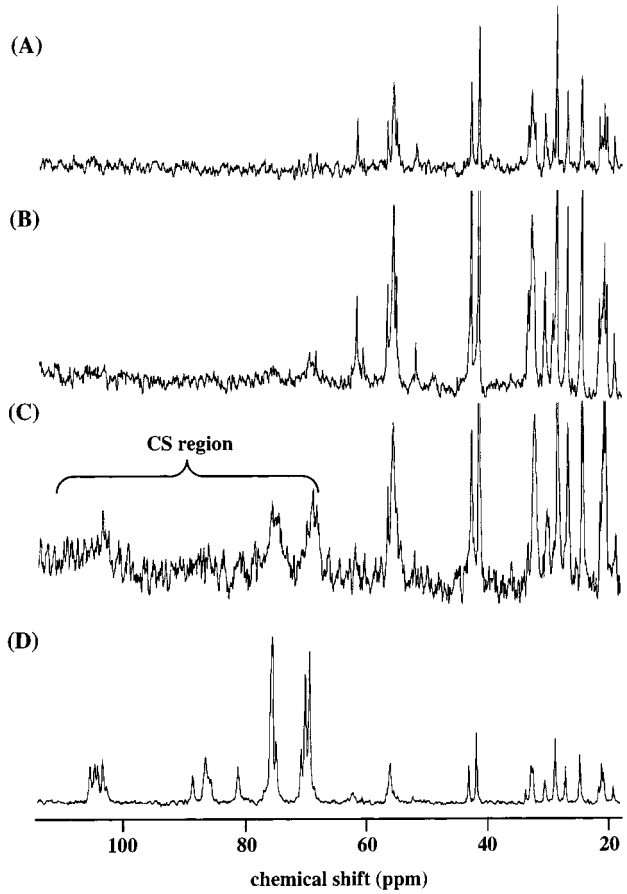

Figure 3. 125 MHz ^{13}C NMR spectra of D518 and mixtures of D518 with CS in different molar ratios: D518 (A) and in the molar ratio of 0.12 (B), 0.27 (C), and 1.16 (D).

curdlan sulfate and polylysine the secondary structure of polylysine changed from α-helix to random coil. It implies that a change in the secondary structure of D518 also took place in the excess CS and D518 mixture. Structural perturbation in helical domains of a protein has been demonstrated in an ionic interaction between heparin and platelet factor-4.[29]

^{13}C NMR Spectra of Mixtures of D518 with Curdlan Sulfate. By the formation of the gel-like material, ^{13}C NMR absorptions from curdlan sulfate appeared in the region between 65 and 110 ppm as broad peaks, part of which were hidden in the baseline, as shown in Figure 3B,C. The broadening is attributed to restriction of the local motion of curdlan sulfate molecules or segments in the polymer complex forming the gel. When curdlan sulfate existed in a noncomplexed state such as in the [CS]/[D518] of 1.16, the CS absorptions were observed as sharp peaks (Figure 3D). In the CS absorption region, it is impossible to distinguish signals due to the gel-like material from those due to the noncomplexed.

On the other hand, in the D518 region at 41−44 ppm, new absorptions attributable to gel-like materials were clearly observed, as shown in Figure 4. For the original D518 (Figure 4A), the δ carbon of arginine and the ϵ carbon of lysine appear at 43.20 and 41.84 ppm, respectively, while in the 100% gel-like complex (Figure 4C) these peaks shifted downfield by approximately 0.05 through 0.08 ppm. In the excess CS state (Figure 4D), there were two species, i.e., one existing in the free state represented by δ_{R-F} and ϵ_{K-F}, and the other existing in the cross-linked network designated as δ_{R-G} and ϵ_{K-G}.

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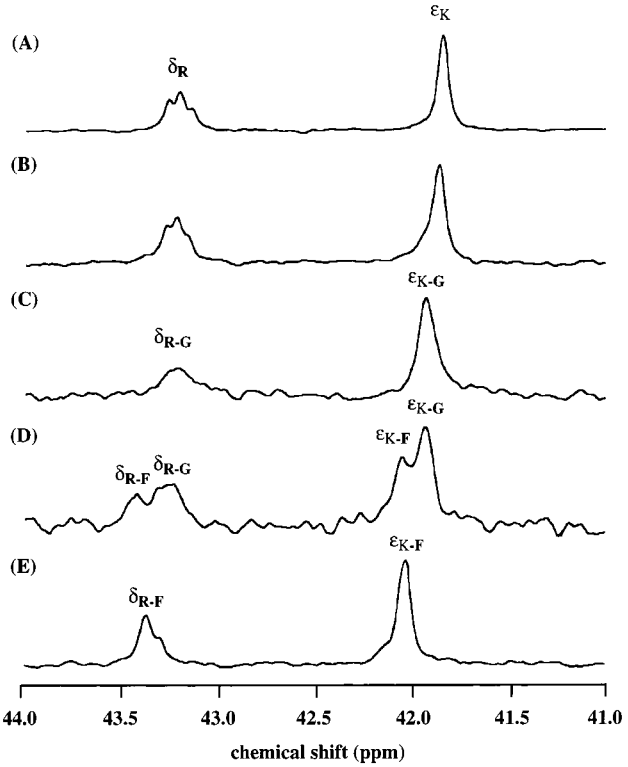

Figure 4. 125 MHz ^{13}C NMR spectra of D518 and mixtures of D518 with CS in different molar ratios in the absorption region of the δ carbon of the arginine and the ϵ carbon of the lysine side chain: D518 (A) and in the molar ratio of 0.12 (B), 0.27 (C), 0.39 (D), and 1.16 (E).

Finally, for a largely excess CS state (Figure 4E), the absorptions corresponding to the free state were exclusively observed. The downfield shift of the δ_{R-F} and the ϵ_{K-F} peaks suggests that the electron-withdrawing effect in the δ-carbon of arginine and the ϵ-carbon of lysine in D518 was caused by nonbonding interactions with the surrounding curdlan sulfate.

Effects of pH on the Formation of the Gel-like Polyion Complexes. To examine the pH range in which curdlan sulfate can exhibit the ability to form the gel-like material by mixing with D518, i.e., the ability to manifest anti-HIV activity, ^{1}H NMR spectra were measured on the CS and D518 mixture of the molar ratio of 0.27 by changing the pH in the range of 0.8 to 11.1 (Figure 5). The pH of the original solution containing the mixture of the molar ratio of 0.27 was 4.0.

Since two gel peaks consisting of δ_{R-G} at 3.03 ppm and ϵ_{K-G} at 2.83 ppm exclusively appeared in the pH range of 0.8 to 5.6 (Figure 5A,B), it was revelaed that 100% gel was formed in acidic to weakly acidic conditions. On the other hand, at a slightly basic condition of pH 7.2, small peaks due to free species appeared in addition to large ones due to the gel-like material. In a strong basic condition of pH 11.1, the gel peaks completely disappeared. In Figure 6, the proportion of the gel-like material, which was determined from the ^{1}H NMR absorptions due to the δ proton of arginine and the ϵ proton of lysine, was plotted against pH values. This figure indicates that since the guadinium group of arginine and the ammonium group of lysine were sensitive to pH and deprotonated by the action of hydroxyl groups, the ionic interactions between CS and D518 did not occur in the strong basic solution.

Previously, we reported the gelation induced by interactions between chondroitin-6-sulfate and polylysine hydrobromide; the proportion of gel-like material was low at about 25% in an acidic pH of 2.0.[30] This phenomenon implies a lower degree of

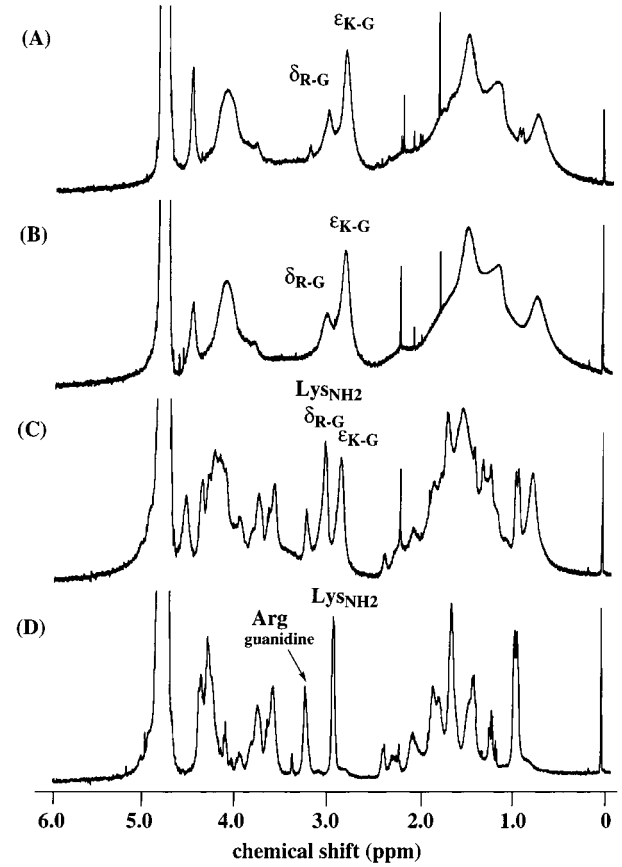

Figure 5. Effects of pH on formation of the gel-like materials by ionic interactions between D518 and CS in the molar ratio of 0.27: pH of 0.8 (A), 5.6 (B), 7. (C), and 11.1 (D).

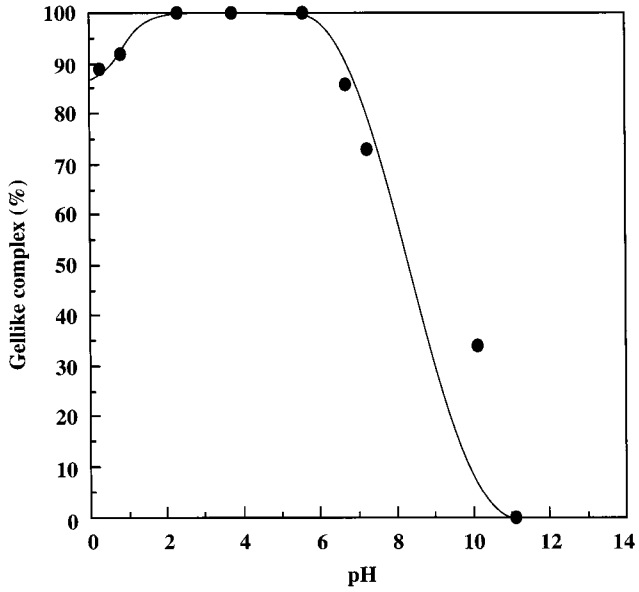

Figure 6. Dependence of the proportion of the gel-like materials on pH in a mixture of CS with D518 in the molar ratio of 0.27. The concentration of D518 is 1.0% (w/v). No precipitates were formed.

dissociation for the carboxylate group in chondroitin-6-sulfate which contains both carboxylate and sulfate anions.

Temperature Dependence on the Gel Formation. Since the gel formation is assumed to be caused by ionic interactions between negatively charged segments and positively charged

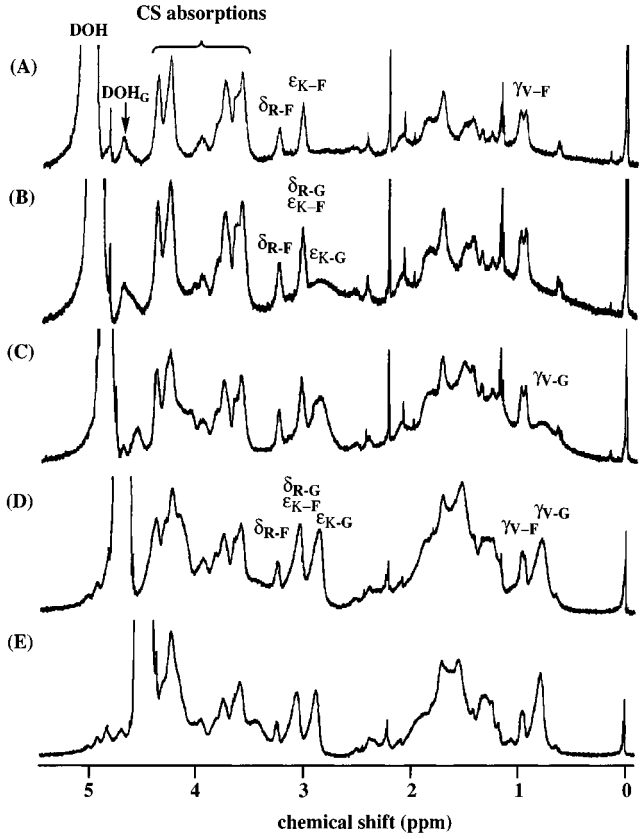

Figure 7. Effects of temperature on the molecular motion of gel-like materials formed by interactions between D518 and CS in the molar ratio of 0.35: measuring temperature of 5 (A), 10 (B), 20 (C), 37 (D), and 60 °C (E).

segments, the conformation and molecular motion should be temperature dependent. ^{1}H NMR spectra were, therefore, measured changing the temperature in the range of 5 to 60 °C for a CS and D518 mixture consisting of the molar ratio of 0.35.

As shown in Figure 7, the gel formation was to a large extent influenced by the temperature. At 5 °C, absorptions due to the arginine δ and the lysine ϵ protons ranging from 2.5 to 3.5 ppm clearly exhibited that all species existed in a free state. At 10 °C, there were a broad ϵ_{K-G} peak in addition to sharp δ_{R-F} and δ_{R-G} peaks. As the temperature was increased to 20 °C, the broadness of the ϵ_{K-G} peak decreased to a considerable extent. The broad peak due to the ϵ proton suggests that at low temperatures ionic interactions occurred for the most part with lysine residues and that local motions of the lysine residue confined in the gel were restricted to a larger extent than those of the arginine. This is due to stronger interactions of the former with negatively charged curdlan sulfate. The proportion of the gel formed at 20 °C was 74%. At higher temperatures than 37 °C, interactions leading to the gel were caused with both arginine and lysine residues and the proportion of the gel was constant in 82−84%.

Using the V3 region peptide sequence consisting of nos. 309 to 331 in the envelope glycoprotein gp120, the mixing of the sequence with curdlan sulfate failed to provide gel-like materials under various conditions. Instead, precipitation of the mixture occurred. It has been revealed that the interaction of curdlan sulfate with this sequence takes place in the competitive adhesion reaction to CD4 cells by using an antibody to the V3 sequence and curdlan sulfate.[18,19] Therefore, it was concluded that although interactions between the two compounds took

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place, the detection method that used the gelation could not be applied to such a peptide sequence containing low proportions of basic amino acids, that is, 8 basic amino acids of 25 amino acids.

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Supporting Information Available: 2D PTCSY and COSY NMR spectra of D518 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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