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NMR Studies on Interactions between a Sulfated Polysaccharide Heparin and a Protein Model Compound

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Abstract : To elucidate the action mechanism of bioactive sulfated polysaccharides which exhibited anticoagulant and antiviral activities, interactions of an anticoagulant polysaccharide heparin (Hep) with polylysine (PL) which can be regarded as a model compound for an anticoagulant protein antithrombin-III, respectively, were examined by NMR spectroscopy. It was found that a gellike complex was formed by the ionic interaction of Hep with PL. Since in ¹H NMR spectra of the mixture of Hep and PL, absorptions due to the lysine side chain appeared as two species which were due to excess free polylysine and gellike complex, the proportion of the complex was determined. The proportion of the gellike complex was affected by $\bar{M}_w$ of heparin and polylysine, molar ratio, pH, concentration, temperature, and time. Maximal amount of the gellike complex was attained with Hep having 3×10^3 Da and PL having 10×10^3 Da.

1. Introduction

Heparin is a natural sulfated polysaccharide which plays an important role in blood anticoagulation, and has been long used as a blood anticlotting agent in surgeries and also in blood dialyzers[1,2]. Since heparin is composed of nearly alternative repeating sequences of D-glucosamine and D-glucuronic acid, partly L-iduronic acid, the hydroxyl and amino groups of which are sulfonated to provide sodium sulfate and sodium sulfamide groups, respectively, it is negatively charged in physiological conditions[3].

Various biological activities possessed by heparin are caused mainly by its specific interactions with proteins. Of these biomolecular interactions, anticoagulant activity of heparin has been investigated in detail. A negatively charged pentasaccharide portion of heparin is supposed to interact with a positively charged portion of antithrombin-III containing three positively charged lysine residues in spatial positions suitable for the interaction[4]. Several studies were performed for identification of lysine residues in

specific binding with anions of heparin using by chemical modification of lysine residues or by charged oligopeptides[5~7].

Recently, it was revealed that sulfated polysaccharides such as heparin[8], dextran sulfate[9], lentinan sulfate[10], pentosan sulfate[11], and curdlan sulfate[12,13] possess anti-HIV (human immunodeficiency virus) activity. The anti-HIV activity is caused by ionic interactions between several sulfate anions of the sulfated polysaccharides and basic amino acid residues of a virus glycoprotein gp120[14]. In the previous paper, we reported the formation of the gellike polyion complex by electrostatic interactions between anionic segments of curdlan sulfate and cationic segments of polylysine by NMR spectroscopy[15].

In this study, we wish to report interactions of heparin with a model compound polylysine in order to elucidate the mechanism of action for the sulfated polysaccharide in biological system. The present study is focused on ionic complexation of heparin containing sulfate and carboxyl anions with the positively charged protein. Formation of gellike materials caused by

interactions between heparin and polylysine was detected by ^1H and ^{13}C NMR spectroscopy. In addition, effects controlling the gel formation are examined in detail.

2. Experimental

2.1 Materials

Heparin sodium salt with $\overline{M}_w$ of 3×10^3 and 6×10^3 isolated from bovine intestinal mucosa and porcine intestinal mucosa, respectively, were purchased from Sigma. Heparin sodium salt isolated from porcine intestinal mucosa with $\overline{M}_w$ of 18×10^3 was obtained from Serbio, Co. Lysine hydrochloride was purchased from Junsei Chemical Co., Tokyo. Lysine dimer hydrochloride, oligolysine acetates from trimer to pentamer and poly-L-lysine hydrobromide ($\overline{M}_w$ $2.7 \times 10^3 \sim 346 \times 10^3$, which was measured by LALLS) were purchased from Sigma. All materials were used without further purification.

2.2 Preparations of polyion complexes

Samples for the NMR measurements were prepared as follows. An aliquot of a solution of lysine compounds of 2.8% (w/v) was added to a heparin (Hep) solution in different concentrations in NMR tube and then were shaken for 2 minutes. The solution was kept 37°C for 1 hr, unless otherwise mentioned. In the case of measuring the amount of gellike complexes, 1.0% (w/v) of polylysine solution was used. The pD of the samples was adjusted using NaOD or DCl. pD was measured with a TOA HM-30V pH meter with GS-5016 electrode by reading the pH of solutions in D_2O without correction.

2.3 Measurements

400 MHz ^1H and 100 MHz ^{13}C NMR spectra were recorded on a JEOL Lambda-400 spectrometer. 4,4-Dimethyl-4-silapentane-1-sulfonate (DSS) and methanol solutions were used as the internal standard for ^1H and ^{13}C NMR measurements, respectively. Tetramethylsilane (TMS) was used as reference zero.

3. Results and discussion

3.1 NMR Spectra of Polyion Complexes Composed of Heparin and Polylysine

When heparin (Hep) solution was mixed with polylysine hydrobromide (PL·HBr) solution, gellike materials and powdery precipitates were formed.

Similarly to the case of a curdlan sulfate and polylysine mixture[15], the gellike material was produced by mixing Hep with lysine compounds ranging from a tetramer to polylysine having a molecular weight of a few ten thousands. On the other hand, clear solutions were obtained by mixing Hep with low molecular weight lysine compounds less than a trimer. Since this phenomenon is assumed to be caused by interactions between Hep with polylysines, NMR spectroscopy was used to elucidate structure and behaviors of the interaction.

Fig. 1 400 MHz ^1H NMR Spectra of mixtures of heparin (Hep) with poly-L-lysine·HBr (PL·HBr) in different molar ratios [Hep]/[PL·HBr]. (A) heparin, (B) PL·HBr and mixtures in the molar ratio of (C): 0.3, (D): 0.6, (E): 1.0, (F): 2.5 and (G): 5.0. The concentration of PL·HBr is 2.8% (w/v).

^1H NMR spectra of mixtures of heparin with $\overline{M}_w$ of 3×10^3 and polylysine hydrobromide with $\overline{M}_w$ of 8×10^3 were measured changing molar ratios. As shown in Figure 1, it was revealed that the ^1H NMR spectra were

influenced to a great extent by the molar ratio which was based on the respective monomeric units of heparin and polylysine.

In the molar ratio of Hep to PL·HBr of 0.3, individual absorptions due to polylysine protons split into two peaks. In comparison with spectrum of the starting PL·HBr, a major peak at 3.04 ppm is assignable to ϵ proton (designated as ϵ_{HBr}) of the PL·HBr, and the other small peak at 2.81 ppm (designated as ϵ_g) is due to a

Table 1 ^1H and ^{13}C NMR Chemical shifts of polylysine and forming polyion complex with heparin

	chemical shift (ppm) ^a			
	H α	H β and H δ	H γ	H ϵ
polylysine ^b	4.33	1.73	1.48	3.04
polylysine-heparin	4.08	1.50	1.25	2.81
polyion complex ^c				

	chemical shift (ppm) ^d					
	C=O	C α	C β	C γ	C δ	C ϵ
polylysine	173.75	53.54	30.74	22.32	26.49	39.36
polylysine-heparin	— ^e	— ^e	— ^e	22.05	26.24	39.26
polyion complex						

^aRecorded in D₂O, using DSS as a standard (0.015 ppm). ^bPolylysine hydrobromide with $\bar{M}_w$ of 8×10^3 . ^cPrepared by mixing heparin with $\bar{M}_w$ of 3×10^3 and polylysine with $\bar{M}_w$ of 8×10^3 in the molar ratio of 0.6. ^dRecorded in D₂O, using methanol as a standard (49 ppm). ^eNot appeared as peaks.

new gellike material formed by interactions between heparin and polylysine. In this case, a gellike complex was attached homogeneously on the wall of NMR tube. Other protons belonging to the lysine side chain appeared approximately 0.2 ppm upfield from the corresponding proton peaks of the PL·HBr. For polylysine included in each species, assignments of the ^1H and ^{13}C NMR absorptions are shown in Table 1.

As the molar ratio increased to 0.6, intensity of the peak increased to a great extent, indicating that the gellike material was produced in the maximum ratio of 93%. The peak intensity, i.e., the proportion, of the gellike material decreased with the molar ratio higher than 0.6, as seen in Figure 1D through 1G. In the molar ratio of 5.0, the ϵ_g peak disappeared completely. It is assumed that excess heparin might interrupt the formation of crosslinked complex because of its strong anionic charges to remain a part of free polylysine.

It is supposed that hydrophobic gellike complex having ultrahigh-molecular weight formed by macromolecular ionic interactions between segments of anion groups of heparin and segments of ammonium groups of PL·HBr through intermolecular cross-linkings. Absorptions due to heparin did not appear in the spectra for the mixtures containing the gellike polyion complex as a major component, because backbone of the heparin might become so immobile as not to appear as the NMR signal.

For anti-HIV active curdlan sulfate previously reported[15], the *in vivo* action mechanism has not been made clear. On the other hand, relationship between the structure of heparin and its interaction with an anticoagulant protein, i.e., antithrombin-III, has been elucidated[3,4,16,17]. A negatively charged pentasaccharide portion of heparin ionically interact with helical 8 amino acids portion of antithrombin-III in which 282, 286 and 289 lysine residues are positively charged. Therefore, the interaction between heparin and polylysine can be regarded as an appropriate model system to detect interactions of heparin with biological proteins by NMR spectroscopy.

Fig. 2 100 MHz ^{13}C NMR Spectra of poly-L-lysine·HBr (PL·HBr) and mixtures of Hep and PL·HBr in different molar ratios [Hep]/[PL·HBr] in the polylysine C α to C ϵ region. (A) PL·HBr and in the molar ratio of (B): 0.3, (C): 0.6, (D): 1.0, (E): 2.5 and (F): 5.0. The concentration of PL·HBr is 2.8% (w/v).

Concerning with morphology of individual components in the gellike polyion complex, an information was acquired from ^{13}C NMR spectra shown in Figure 2. In Figure 2 indicating the polylysine absorption region, absorptions due to the gellike complex appeared. For example, ϵ_g and δ_g peaks due to polylysine included in the gel were observed at 39.26 and 26.24 ppm, respectively, being 0.1 to 0.25 ppm upfield from the corresponding PL·HBr peaks. However, the peak separations were smaller than those in the ^1H NMR spectra.

Fig. 3 100 MHz ^{13}C NMR Spectra of heparin (Hep) and mixtures of Hep with PL·HBr in different molar ratios $[\text{Hep}] / [\text{PL}\cdot\text{HBr}]$ in the absorption range due to heparin component. (A) heparin and in the molar ratio of (B): 0.6, (C): 1.0 and (D): 5.0.

On the other hand, for ^{13}C NMR absorptions due to heparin component, there was a large difference in line width between heparin included in the gel and free heparin (Figure 3). In the molar ratio of 0.6, the heparin absorption completely disappeared as broad baseline. This phenomenon suggests that local motion of the heparin backbone in the gel was so restricted as not to give NMR absorptions.

Similar ionic interactions between a heparin and a basic fibroblast growth factor have been examined[18]. The X-ray crystallographic analysis revealed that heparin binds to the basic fibroblast growth factor by interactions of negatively charged sulfate groups with a positively charged region of the protein containing lysine and arginine residues.

3.2 Effects of molecular weights of heparin and polylysine

Since the interaction between heparin and polylysine is a macromolecular-macromolecular interaction which is usually affected by their molecular weights, its dependence of the molecular weight of heparin was examined by fixing the molecular weight of polylysine at $\bar{M}_w$ of 8×10^3 .

The proportion of PL contained in gellike materials depended to a large extent on the molecular weight of heparin as well as the molar ratio. Proportions of the gellike complexes were determined by measuring intensities of ϵ_g and ϵ_{HBr} proton absorptions corresponding to the gel and the free polylysine, respectively, by means of ^1H NMR spectroscopy. As

Fig. 4 Dependence of the proportion of the gellike complex on molecular weight of heparin. The concentration of PL·HBr ($\bar{M}_w = 8 \times 10^3$) is 1.0% (w/v). Kept at 37°C for 1 hr. ●: Hep with $\bar{M}_w$ of 3×10^3 . ▲: Hep with $\bar{M}_w$ of 6×10^3 . ■: Hep with $\bar{M}_w$ of 18×10^3 . No precipitates were observed.

shown in Figure 4, when polylysine was mixed with heparin having $\bar{M}_w$ of 3×10^3 , 6×10^3 , or 18×10^3 , the maximum proportion of the formed gellike complex was 93%, 83%, or 41%, respectively. This phenomenon indicates that lower molecular weight heparins had a tendency to more easily produce the complex. It is assumed that high molecular weight heparins can not penetrate into cross-linked gels after the gels were formed in an early stage of the reaction.

Next, polymeric interactions of heparin with the molecular weight of 3×10^3 were examined by changing the molecular weight of polylysine from 530 to 25×10^3 . As the molecular weight of PL·HBr increased up to 10×10^3 , the proportion of the formed gellike material

Fig. 5 Dependence of the proportion of the gellike complex on molecular weight of PL·HBr in molar ratio of 0.6. The concentration of PL·HBr is 1.0% (w/v).

increased to reach 94%, with remaining 6% as a free polylysine (Figure 5). Precipitation occurred with use of high molecular weight PL·HBr. Since very high molecular weight polylysines not only have many interacting points consisting of segments but also show low solubilities than polylysine with $\bar{M}_w$ of 10×10^3 , it is assumed that resulting polymer complexes precipitated due to formation of hydrophobic materials having very high molecular weights. The formation of precipitates caused by the interaction of heparin or a

sulfated polysaccharide dextran sulfate with plasma proteins has been known as a reason for toxicity [19].

3.3 Effects of pH

For a mixture with molar ratio of 0.6 consisting of heparin with $\bar{M}_w$ of 3×10^3 and polylysine with $\bar{M}_w$ of

Fig. 6 Dependence of the proportion of the gellike complex on pH in a mixture of heparin ($\bar{M}_w = 3 \times 10^3$) and polylysine·HBr ($\bar{M}_w = 8 \times 10^3$) in the molar ratio of 0.6. The concentration of PL·HBr is 1.0% (w/v). No precipitates were observed.

8×10^3 , effects of pH on the formation of gellike materials was examined. As shown in Figure 6, proportion of the gel remarkably depended on pH of the mixture. In pH range of 3.8 to 8.3, the proportion of gel was high in approximately 90%, indicating that there were strong intermolecular ionic interactions. On the other hand, both below pH 2.5 and above pH 9, the proportion of gel was low. In addition, under strong alkaline conditions above pH 10, almost no gel formation took place. Since in the strong alkaline condition, the amino group in the side chain of polylysine must not exist as the ammonium group necessary for the ionic interaction.

3.4 Effects of concentration and molar ratio

The proportion of gels was determined by changing the molar ratio of Hep to PL·HBr and the concentration of polylysine. The proportion was plotted against the concentration of added polylysine

Fig. 7 Dependence of the proportion of the gellike complex on concentration and molar ratio. Molar ratio of ■: 0.3, ●: 0.6, ▲: 1.0, ◆: 2.5 and ▼: 5.0. No precipitates were observed.

in Figure 7. It was demonstrated that the proportion of gels increased with increasing concentration. In addition, the proportion depended to a large extent on the molar ratio. In the molar ratio of 0.6, the proportion was more than 90%, when the concentration was above 0.2 w/v%. On the other hand, in higher or lower molar ratios than 0.6, the proportion was low even in high sample concentrations. Therefore, it was revealed that both excess polymers seem to interfere with formation of the gellike material.

Furthermore, when salts with strong ionic strengths such as NaClO_4 and KSCN were added to the aqueous solution of heparin and $\text{PL} \cdot \text{HBr}$, ionic interactions were suppressed to a large extent. These salts are known not only to have high solubilities in water but also to affect the conformation of polylysine and other polyamino acids [20,21]. Thus, it is suggested that the gellike complex is formed by releasing their counterions as NaBr out of the gel. Such salt effects have been demonstrated in the binding of heparin to antithrombin-III and basic fibroblast growth factor (bFGF) [22,23].

The present NMR study provides a detailed evidence of strong ionic interactions between heparin and polylysine the amino functional group of which was

known to act as a cation in the biological system. These results afford a useful information on the biomolecular adhesion properties of sulfated polysaccharides to proteins.

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