Carbamoylated Poly-L-Lysine and its Helix-Coil Transition

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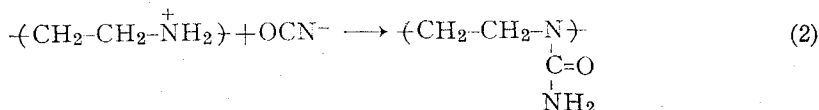
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The carbamoylation reaction of poly-L-lysine(PLL) and its influence on the helix-coil transition of the product polymer were studied. The rate constant decreased with increasing concentrations of PLL and cyanate ions. At lower concentrations, the rate constants were in agreement with theoretical values obtained by the Brönsted theory and Manning's theory on polyelectrolytes. The free energy, enthalpy and entropy of activation of the reaction at 40°C were 18.4 Kcal/mol, 0.2 Kcal/mol and −58 e.u., respectively. From the ORD measurements the carbamoylation raised the helix content (f_H) at lower pH and lowered f_H at higher pH. This tendency was accounted for in terms of two factors: The charge neutralization by the carbamoylation, which may facilitate the helix formation at lower pH and the steric hindrance effect of introduced carbamoyl groups which destroys partly the orderliness of the molecule. The viscosity-pH profile was in accord with the interpretation. Finally, the denaturation of proteins was suggested to be at least partly due to carbamoylation of amino groups.
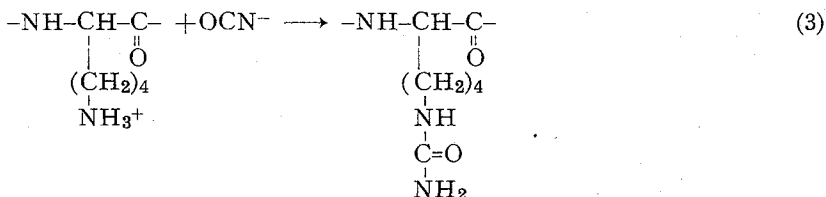
INTRODUCTION

The reaction between ammonium ions and cyanate ions

$$NH_4^+ + OCN^- \rightarrow (NH_2)_2CO \tag{1}$$

was found out by Wöhler in 1828 and the catalytic influence of polyelectrolytes thereon was thoroughly investigated.[1] A similar reaction of polyethylenimine hydrochloride (PEI·HCl) with cyanate ions, namely carbamoylation reaction, was recently studied.[2] In this present paper, we study the carbamoylation reaction in aqueous media of poly-L-lysine(PLL)

$$-(CH_2-CH_2-\overset{+}{N}H_2)- + OCN^- \longrightarrow -(CH_2-CH_2-N)- \tag{2}$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad C=O$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad NH_2$$

which contains primary amino groups. It is believed that the reaction proceeds as follows. We discuss the rate of the carbamoylation of PLL and we study how the newly

$$-NH-CH-C- \ +OCN^- \longrightarrow \ -NH-CH-C- \tag{3}$$
$$\qquad\quad | \quad \| \qquad\qquad\qquad\qquad\qquad\quad | \quad \|$$
$$\qquad\quad | \quad O \qquad\qquad\qquad\qquad\qquad\quad | \quad O$$
$$\qquad\quad (CH_2)_4 \qquad\qquad\qquad\qquad\quad (CH_2)_4$$
$$\qquad\quad NH_3^+ \qquad\qquad\qquad\qquad\qquad NH$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad C=O$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad NH_2$$

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introduced carbamoyl groups affect the stability of the helical structure of PLL.

EXPERIMENTAL

Materials

Silver cyanate was prepared from urea (for biochemical study, Merck) and silver nitrate (GR grade, Merck) by the method of Warner and Warrick.[3] Two samples of the hydrobromide of PLL were obtained from Sigma and Institute of Protein Research, Osaka University. The molecular weights were 79,000 and 100,000, respectively. The former was used for kinetic study and the latter for viscosity and ORD measurements. PLL(HBr) was dialysed first against 0.01 N HCl to replace Br^- by Cl^- and then against pure water. The substitution was necessary because of large molar absorption of Br^- in ultra-violet regions. The concentration of the hydrochloride of PLL was determined by conductometric titration. The pH of PLL solutions was adjusted by sodium hydroxide or hydrochloride. The water used was purified by ion-exchange resins.

Kinetic Measurements

Aqueous solutions of PLL(HCl) were mixed under violent stirring with equivalent amounts of AgOCN. The mixture was ice-cooled and precipitates of AgCl were filtered off. Because of its low solubility, the filtrate can be claimed to contain only cyanate ions and PLL cations. The carbamoylation reaction was then followed by the electric conductivity of the reaction mixtures using a Jones-Ballinger type conductivity cell (cell constant$=4.97$ cm^{-1}). The conductivity was measured by a Wayne-Kerr autobalance precision bridge (B-331) at a frequency of 1592 H$_z$. The capacitance correction was automatically carried out and the precision of the conductivity is believed to be $\pm0.001\%$. The pH of the solution was between 6 and 8.

As the reaction proceeded, reactant ions were transformed to neutral product, causing gradual decrease in the conductivity. The second-order rate constant (k_2) was calculated by a rate equation

$$k_2 = (\mathcal{K}_0^* - \mathcal{K}^*)/\mathcal{K}^* m_2 t, \tag{4}$$

where t is the time, $\mathcal{K}_0^*$ and $\mathcal{K}^*$ are the specific conductivities at $t=0$ and $t=t$ and m_2 is the initial concentration of the reactants. The solvent correction to the conductivity was of course effected. The k_2 value was evaluated below 1% conversion.

Optical Rotary Dispersion(ORD)

A spectrophotometer, ORD/UV 5 of JASCO, was used in a wave-length range between 200 and 500 nm, with a 1 cm cell. The concentration was approximately 4×10^{-3} residue mole/1. The helix content f_H was obtained either by

$$f_H = \{[m']_{233}(obs) - [m']_{233}(coil)\} / \{[m']_{233}(helix) - [m']_{233}(coil)\} \tag{5}$$

or by

$$f_H = \{b_0(obs) - b_0(coil)\} / \{b_0(helix) - b_0(coil)\} \tag{6}$$

where $[m']$ is the specific rotation, b_0 is a constant of the Moffitt-Yang equation. It was

N. Ise, T. Okubo, T. Sekiguchi, and K. Mita

assumed that $[m']_{233}$ (helix)$=-12700$, $[m']_{233}$ (coil)$=-2200$, b_0 (helix)$=-630$ and b_0 (coil)$=0$.

PLL samples of various degrees of carbamoylation were prepared as follows. To the hydrochloride of PLL, AgOCN was added in amounts necessary according to the desired degree of carbamoylation and the mixture was kept at 30°C for 24 hrs, and AgCl was precipitated by 15 min. centrifugation at 12,000 RPM. The degree of carbamoylation was determined by conductometric titration.

Viscosity.

A dilution-type viscometer was used. The flow time of water was 120 sec at 30°C. The PLL concentration was approximately 0.125 g/dl and the concentration of added salt (NaCl) was 0.45 M.

RESULTS AND DISCUSSION

Kinetic Data

Figure 1 shows the dependence of the second-order rate constant (k_2) on the reactant
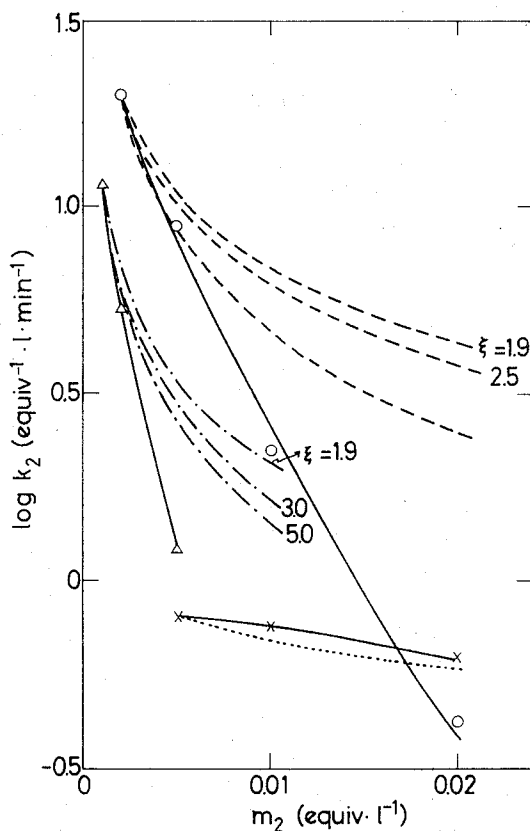

Fig. 1. The observed rate constants of carbamoylation reactions of polyethylenimine, poly-L-lysine and ethylenediamine in water at 30°C. The theoretical values are given by the dashed and broken curves. The dashed curve without ξ value was obtained for $\xi=\infty$.

○: PEI   △: PLL,   ×: ED

concentration(m_2). The k_2 value decreased with increasing concentration in accordance with the primary salt effect[4] for reactions between oppositely charged ionic species. For comparison, the k_2 values for polyethylenimine(PEI) and ethylenediamine(ED)[5] were shown. The k_2 for PEI is larger than that for PLL. This is probably due to the higher spacial charge density of PEI, which is resulted from the electric charges being localized on the main chain. It is, furthermore, seen that the k_2 values for the two polymers decrease much more sharply with increasing cnocentration than that for ED. This can be easily understood if one accepts the Brönsted relation,[6] $k_2/k_{20}=f_A \cdot f_B/f_X$ (k_{20}: the second-order rate constant at zero ionic strength, f: the activity coefficient, A, B: reactants, X: critical complex) and if f_A or f_B decreases drastically when A or B is a macromolecular species. Such a concentration dependence has been definitely observed.[7]

By using the Brönsted equation,[6] the k_2/k_{20} value can be theoretically evaluated if the activity coefficients (f) of the reactants can be estimated. As a theory for polyelectrolyte solutions, we use here Manning's limiting law[8] to estimate the f's. It is assumed here that we have z-valent polycations and monovalent gegenions in the reaction system. According to Manning's theory, the mean activity coefficient of the polyelectrolyte $f_\pm$ is given by

$$\log f_\pm = A - z(1-\xi^{-1/2})\log c/(z+1) \tag{7}$$

where c is the concentration (eq/1) and ξ is defined by

$$\xi = e^2/DkTb \tag{8}$$

where e is the proton charge, D the dielectric constant, k the Boltzmann constant, T the temperature, and b the distance between charges on infinitely long line. (Thus $b=L/P$ where L is the contour length of the polyelectrolyte chain, and P is the number of monovalent charged groups on the chain.). By using the Brönsted equation and Eq. (7), we have

$$\log k_2/k_2{}^* = [(z+1)/z]\log f_\pm/f_\pm{}^* = -(1-\xi^{-1/2})\log c/c^* \tag{9}$$

where the asterisk denotes the reference. It is assumed in the calculation that f_X is unity since the critical complex was formed from monovalent cation and monovalent anion so that it has no electric charge. Thus the kinetic constant k_2 can be calculated if ξ and c are given. The k_2/k_{20} values thus obtained are shown by dotted and broken curves in Fig. 1. From the definition, ξ should have the same value (1.9) for PEI and PLL. In Fig. 1, however, ξ was assumed to be an adjustable parameter. The agreement is gratifying at low concentrations and at larger ξ, especially so for PLL.

The disagreement at higher concentration seems reasonable, because the theory is a "limiting" law which is expected to be valid at extremely low concentration. However, the large values assigned to ξ may be viewed with caution. For PLL, which has fairly long side chains, the theory or its model for polyelectrolytes might be inapplicable. For PEI, it may be true that the theory should not be applied, because this polyelectrolyte has a branched structure.

The agreement between the theory and experiment is noteworthy. It implies that the observed concentration dependence of the rate constant (or deceleration by increasing ionic concentration) is nothing else than the primary salt effect, which has been well-established: No ad hoc assumption is needed.

N. Ise, T. Okubo, T. Sekiguchi, and K. Mita

Thermodynamic Quantities

Table I shows the free energy (ΔG^{*}), the enthalpy (ΔH^{*}) and the entropy (ΔS^{*}) of activation of the carbamoylation reaction of PLL and PEI. It is to be mentioned that PLL gives lower ΔH^{*} and ΔS^{*} than PEI. Though the reason for this is not clear, we note that ΔH^{*} for PLL is nearly zero; the reaction is entropy-controlled.

ORD Measurements

Figure 2 shows $[m']_{233}$ values. We note that $[m']_{233}$ obtained for untreated PLL agreed with literature values.[9,10] At 15°C, it is known that PLL assumes α-helical structure at pH=12 and random-coil conformation at pH=7.[11] At lower pH, $[m']$ decreased with increasing degree of carbamoylation whereas it increased at higher pH.

Table I. Thermodynamic Quantities of the Carbamoylation Reaction of PEI and PLL in Water at 40°C.

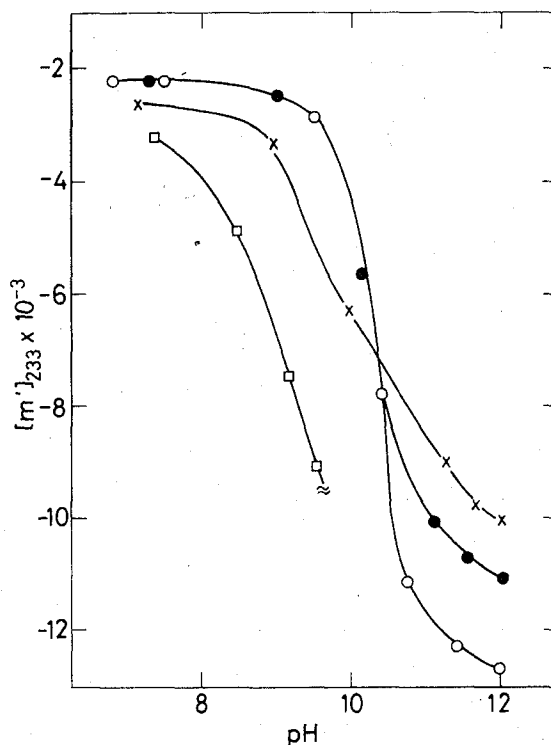|	ΔG^{*} (Kcal mole^{-1})	ΔH^{*} (Kcal mole^{-1})	ΔS^{*} (e.u.)
PEI	17.9	2.5	−48
PLL	18.4	0.2	−58

[PEI·OCN]=0.005 equiv·l^{-1}
[PLL·OCN]=0.002 equiv·l^{-1}

Fig. 2. The rotary power of carbamoylated poly-L-lysine at 233 nm and 15°C. The degree of carbamoylation; ○:0%, ●:8.8%, ×:53.0%, □:85.0%

Figure 3 shows the Moffitt-Yang parameter b_0 at various pH and degrees of carbamoylation. For untreated PLL at pH$=12$, b_0 was -630, indicating that the PLL chains are fully helical. At pH 7, b_0 was zero. It is seen that the carbamoylation reaction destroyed the helical structure at higher pH and promoted the ordered structure at lower pH. This would be due to destruction of the orderliness by bulky carbamoyl groups on one hand and due to partial elimination of electrostatic repulsive interaction as a result of the carbamoylation. The fact that the untreated PLL and the 8%-carbamoylated PLL gave the same curve at lower pH indicates that the positive charges were not eliminated so much as to stabilize the helical structure. Furthermore, the observed shift of the transition point toward lower pH by increasing degree of carbamoylation can be accounted for in terms of the partial elimination of the electric charges.

It should be mentioned here of the precipitation phenomenon of 85%-carbamoylated PLL at higher pH. Above pH 9.5, the ORD measurements of this material could not be carried out because the solution became turbid. This seems to be due to the β structure, which could be more easily formed at higher pH, temperature and concentration. According to Fasman,[12] the β structure of PLL is stabilized by hydrogen bonds between carbonyl and amide groups of peptide bonds of neighboring molecules and by hydrophobic interaction between lysine groups. Carbamoylation of PLL neutralizes the positive charges of lysine groups so that the hydrophobic interactions are enhanced. Thus, the β structure can be formed with greater ease for carbamoylated PLL than for untreated PLL. Furthermore, at high degrees of carbamoylation, hydrogen bonds between newly introduced carbamoyl groups would be a stabilizing factor of the β structure.

Figure 4 gives the helix fraction f_H at pH$=11.5$. It should be noted that, in Eq. (5) PLL and carbamoylated PLL were assumed to have the same $[m']_{233}$ value when the
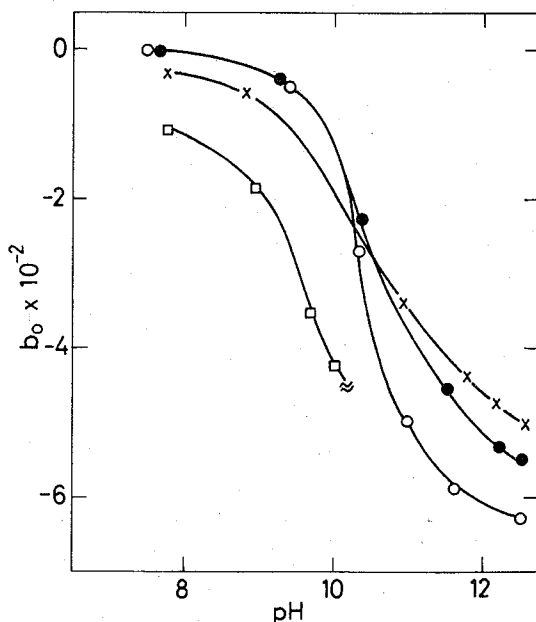

Fig. 3. The b_0 values of carbamoylated poly-L-lysine at 15°C. The degree of carbamoylation; ◯: 0%, ●: 8.8%, ×: 53.0%, ☐: 85.0%

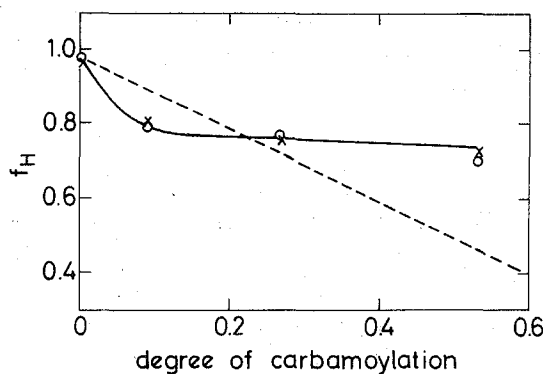N. Ise, T. Okubo, T. Sekiguchi, and K. Mita

Fig. 4. The helix content f_H as a function of the degree of carbamoylation
pH $=11.5$, $15°C$.
◯: from $[m']_{233}$,   ×: :from b_0

chains are fully helical or fully coiled. This would be acceptable since $[m']_{233}$ (helix) is -12700, -15300, and -15500, for PLL, poly-L-ornithine, and poly-L-diaminobutyric acid, respectively and $[m']_{233}$ (coil) is -2200, -2060, and -2270.[13] The value of $[m']_{233}$ (obs) was read from the curves given in Fig. 2. The b_0 (obs) value in Eq. (6) was from Fig. 3. Figure 4 shows that the f_H value decreased sharply at first with increasing degree of carbamoylation and then gradually. The dotted line in Fig. 4 shows a hypothetical proportionality between the degree of carbamoylation and f_H. The fact that the observed f_H value is larger than the hypothetical one indicates the important contribution of the charge elimination.

Figure 5 shows the viscosity of PLL and carbamoylated PLL as a function of pH. The viscosity behavior of PLL was in agreement with that by other researchers.[14] PLL has a relatively high viscosity at lower pH because of elongation of the chain due to electrostatic repulsive forces by charged lysine groups. At the transition point, the viscosity is low because the molecules are folded up with the coiled parts as joints. At higher pH, the viscosity increases because the fraction of the helical structure, which is rigid, becomes larger.

Clearly, the viscosity of carbamoylated PLL is lower than that of PLL. Furthermore, the drop of the viscosity at the transition region became smaller with increasing degree of carbamoylation; at 70% carbamoylation, the viscosity increased monotonously with increasing pH. The transition point shifted toward lower pH. These changes can be accounted for in terms of the electrostatic interactions being weakened as a result of neutralization by carbamoylation. The rather small drop from lower pH at higher degree of carbamoylation appears to be brought about by the simultaneous influence of the neutralization and enhanced helical conformation. The shift of the minimum point toward lower pH seems to be due to weakened effect of protonation caused by the neutralization.

Figure 6 shows the effect of urea on the conformation of PLL. Evidently, the helix content was decreased by 10% by addition of a large amount of urea (urea: PLL$=200$: 1). As is well recognized,[15,16] proteins can be denatured by 8 M urea. Usually, the denaturation is attributed to destruction of the hydrogen bonds or the hydrophobic interactions by urea.[17] We point out, here, that the carbamoylation by cyanate ions may cause the denaturation. As a matter of fact, urea is in a dissociation equilibrium with ammonium
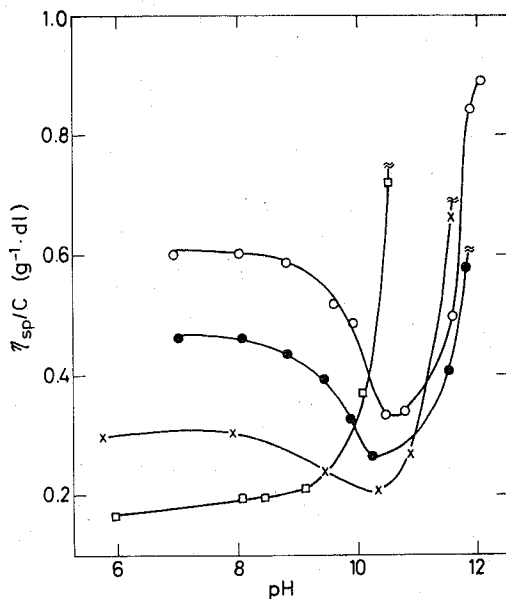
Fig. 5. The reduced viscosity of carbamoylated poly-L-lysine at 30°C.
The concentration=0.125 g/dl, [NaCl]=0.45 M.
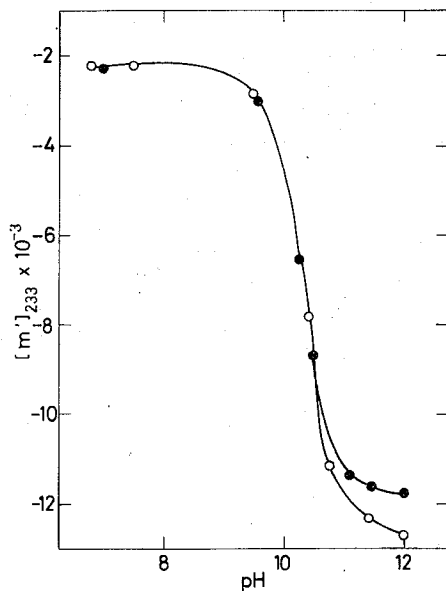The degree of carbamoylation; ○: 0%, ●: 26.5%, ×: 53.0%,
□: 70.0%

Fig. 6. The rotary power of poly-L-lysine in the presence and absence of
urea, at 233 nm and 15°C.
○: PLL   ●: PLL+urea

ions and cyanate ions; 8 M urea corresponds to 0.02 M cyanate ions,[18] which is not a
negligible amount at all. The decrease in the helical content (demonstrated in Fig. 6)
correspond to the fact demonstrated in Fig. 2 that the helical content of carbamoylated

PLL was smaller than that of PLL. Our inference is supported by the observation[19] that poly-L-glutamic acid, which lacks amino groups, cannot be denatured by urea and also by a report that lysine groups of proteins were carbamoylated in the presence of a large excess of urea.[20]

ACKNOWLEDGMENTS

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