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Effects of Ionic Strength on the Thermal Unfolding Process of Granulocyte-Colony Stimulating Factor

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This paper reports the effect of ionic strength on the process of thermal unfolding of recombinant methionyl human granulocyte-colony stimulating factor (rmethuG-CSF) at acid pH. We previously reported that the protein aggregates were formed at the highest temperature at pD 2.1 in the pD range of 5.5–2.1 and that the aggregation proceeded a little at pD 2.1 because of the strong repulsive interaction between the unordered structures that play the role of a precursor for the aggregation. In the present study temperature-dependent IR spectra and far-UV CD spectra were measured for rmethuG-CSF in aqueous solutions containing various concentrations of NaCl at acid pH. Second derivative and curve-fitting analysis were performed to examine the obtained IR spectra. The results revealed that the structure of rmethuG-CSF becomes less stable with increasing ionic strength at all pDs investigated (pD 2.1, 2.5, and 4.0). We have also demonstrated that, at pD 2.1, the temperature at which the protein aggregation starts becomes lower and that the amount of the aggregates becomes larger with the addition of NaCl. This is probably because the addition of NaCl masks the repulsive electrostatic interaction between the unordered structures.

Key words: FT-IR, G-CSF, ionic strength, secondary structure, thermal denaturation.

Abbreviations: rmethuG-CSF; recombinant methionyl human granulocyte-colony stimulating factor.

In many proteins, physical instability processes such as denaturation, adsorption to surfaces, aggregation, and precipitation may be accompanied by changes in secondary and higher-order structures, and may occur during the production, isolation, purification, delivery, and storage of protein pharmaceuticals (*1*). Protein aggregates in parenterally administered proteins can cause adverse patient reactions such as an immune response and anaphylactic shock (*1*, *2*). Furthermore, protein aggregation and subsequent precipitation are responsible for a number of diseases such as Alzheimer's disease and numerous neurodegenerative diseases (*3*). It is also important to understand the aggregation pathway to develop the optimum formulations in pharmaceutical science.

Recombinant methionyl human granulocyte-colony stimulating factor (rmethuG-CSF) is an approximately 19 kDa monomeric protein and a pharmaceutically relevant globular protein belonging to the four-helix bundle family of growth factors (*4*). Infrared (IR), circular dichroism (CD), and fluorescence studies on rmethuG-CSF have demonstrated that it undergoes structural changes induced by pH, heat, and denaturants such as guanidine hydrochloride, and that its secondary and tertiary structures are stable at low pH (*5–8*).

Many proteins become unfolded at acid pH through electrostatic repulsion by the net positive charge of polypeptide chains. The addition of anions leads to substantial refolding. This effect of anions is attributed to reduction of the charge repulsion on anion binding to the positively charged groups (*9–12*). Staphylococcal nuclease (SNase) at neutral pH shows greater resistance to urea and thermal denaturation with the addition of high concentrations of salts, for example, 0.4 M sodium sulfate. These salt-induced effects on the stability of SNase are attributed to the binding of counterions, which results in minimization of the intramolecular electrostatic repulsion (*12*). On the other hand, Narhi *et al.* reported that higher ionic strength results in decreased reversibility or increased aggregation on heat-induced denaturation of recombinant human megakaryocyte growth and development factor (rHuMGDF) (*13*). In addition, rHuMGDF precipitates in a phosphate buffer (pH 7.0) upon thermal unfolding, while the unfolding process is at least partially reversible in a 10 mM imidazole solution (pH 7.0), with no visible precipitate. Interactions of rHuMGDF with negatively charged groups such as the phosphate group would be more favorable than ones with positively charged groups such as imidazole, because rHuMGDF has a positive charge at neutral pH. It seems that the binding of these anionic groups results in neutralization of the charge, decreasing the inhibition of aggregation due to charge-charge repulsion.

The increase in thermal stability at acid pH relative to neutral pH is unique to rmethuG-CSF (*7*). In addition, rmethuG-CSF tends to aggregate irreversibly, particularly at neutral pH (*e.g.*, pH 7 phosphate-buffered saline and 37°C). We investigated the acid stability and process of thermal unfolding of rmethuG-CSF by means of IR spectroscopy (*8*). Temperature-dependent (25–80°C) IR spectra were measured for rmethuG-CSF in aqueous solutions over the pD range of 5.5–2.1, and it was suggested that

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in the thermal unfolding process the α-helix structure of rmethuG-CSF partially changes to an unordered structure and then the unordered structure forms aggregates. It was also indicated that the stability at pD 2.1 is slightly lower than that at pD 2.5, but that the aggregates are formed at higher temperature at pD 2.1 than at pD 2.5, and the aggregation proceeds a little at pD 2.1. We concluded that the above results are due to the repulsive interaction between the unordered structures being stronger at pD 2.1.

In relation to the above conclusion, in the present study, we have investigated the effect of ionic strength on the process of thermal unfolding of rmethuG-CSF solution by means of circular dichroism (CD) and infrared (IR) spectroscopy. Far-UV CD spectroscopy is useful for exploring the secondary structures of proteins. It is particularly sensitive as to the secondary structure of α-helix, but not to that of β-sheet. IR spectroscopy allows one to extract information about secondary structure such as α-helix, β-sheet, turn, and unordered structure from amide I bands (*14–17*). Some spectral analysis approaches involving second derivative, Fourier-self deconvolution, and curve fitting analysis of amide I bands have been used for quantitative estimation of secondary structure elements from IR spectra. Furthermore, IR spectroscopy can be used to estimate quantitatively the amount of aggregates as the formation of intermolecular β-structure.

In the present study, we have studied the thermal denaturation of rmethuG-CSF containing NaCl in the concentration range of 0–100 mM or Na_2SO_4 in that of 0–20 mM at acid pH with particular emphasis on the mechanism of protein aggregation in the presence of NaCl or Na_2SO_4. It was found in the present study that there is a marked difference in the aggregation of rmethuG-CSF at pD 2.1 between protein solutions with and without NaCl.

METHODS

Materials—Recombinant methionyl human G-CSF was produced and purified by Kirin Brewery Co., Ltd. (Gunma, Japan) using overexpression in *Escherichia coli*. Protein concentrations were determined from the absorbance at 280 nm using an extinction coefficient of 0.86 for a 0.1 w/v % protein solution.

Circular Dichroism Spectroscopy—CD spectra were measured for rmethuG-CSF at the concentration of 0.5 mg/ml in a 20 mM sodium phosphate buffer using a Jasco J-820 spectropolarimeter and thermal control (Jasco CDF-426L). A cuvette cell with a path length of 1 mm was employed for the far-UV (250–200 nm) CD measurements. The CD measurements were performed as a function of ionic strength by incrementally adding aliquots of additives such as sodium chloride and sodium sulfate to rmethuG-CSF in a 20 mM sodium phosphate buffer solution at pH 2.1, 2.5 or 4.0.

The process of thermal denaturation of rmethuG-CSF was monitored as the CD signal at 222 nm. The sample temperature was increased at 1°C/min. The fraction of the folded protein was calculated by the method previously reported (*18, 19*), and plotted as a function of temperature to obtain thermal unfolding curves.

The degree of reversibility of secondary structure was determined as follows. Immediately after the temperature of a rmethuG-CSF solution reached 90°C, the solution was cooled down to 25°C and then incubated at 25°C for 15 min. After that, a CD spectrum was measured. The intensity of the signal at 222 nm regained during the 25°C incubation relative to that of the corresponding signal at the initial stage was used to determine the percentage of the reversibility.

Infrared Spectroscopy—A solution of rmethuG-CSF at the concentration of 10 mg/ml was changed from a HCl solution (pH 4.0) to 20 mM sodium phosphate buffer containing 50 mM NaCl at pD 2.1, 2.5, and 4.0. The pD value of a D_2O solution was measured with a standard pH electrode and the value was corrected according to pD = pH + 0.4 (*20*). IR spectra of rmethuG-CSF buffer solutions were measured with a Nicolet NEXUS 670 FT-IR spectropolarimeter equipped with a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector and a temperature controller with a water bath (Thermo Haake DC30). The IR cell used consisted of CaF_2 transmission windows and a 50 μm Teflon spacer. For each spectrum, 256 interferograms of 2 cm^{-1} spectral resolution were co-added. The sample chamber was continuously purged with N_2 gas to prevent atmospheric water vapor from interfering with the amide I region. The IR spectra were measured over the temperature range of 25 to 80 or 84°C. At each temperature a spectrum was obtained by equilibrating the sample for 5 min prior to the data collection, it taking approximately 5 min to collect the spectrum. The IR spectrum of atmospheric water vapor was subtracted from each spectrum. An IR spectrum of the 20 mM sodium phosphate buffer solution was measured at each temperature under identical conditions and subtracted from the corresponding spectrum of the protein solution. The spectral subtraction was performed using the OMNIC program (Thermo Nicolet). The spectra thus obtained were subjected to smoothing with a seven-point Savitsky-Golay function to reduce the noise. Fourier self-deconvolution was carried out to resolve overlapping IR bands using the same software as above with a full width at half-height of 25.6 cm^{-1} and a k of 3. The smoothing and the calculation of the second-derivative spectra and curve fitting were performed with software named SPINA 3.0 (Y. Katsumoto, Kwansei Gakuin University).

RESULTS

CD Study of the Effect of Ionic Strength on the Thermal Denaturation of rmethuG-CSF—Far-UV CD spectra of rmethuG-CSF solutions with different NaCl concentrations at pH 4.0, 2.5, and 2.0 were measured over the temperature range of 25 to 90°C. Figure 1A shows CD spectra of rmethuG-CSF solutions with no and 100 mM NaCl at 25°C and pH 4.0. In the same figure, CD spectra of rmethuG-CSF solutions containing 0, 10, 20, 50, and 100 mM NaCl at 25°C and pH 4.0 after heating of the solutions up to 90°C are also shown. The spectrum of rmethuG-CSF containing 100 mM NaCl at 25°C exhibits the maximum wavelengths at 208 and 222 nm, as in the case of the rmethuG-CSF solution without NaCl. This observation suggests that the secondary structure of rmethuG-CSF at 25°C and pH 4.0 is not significantly affected by the addition of NaCl.

Thermally-induced unfolding of rmethuG-CSF at pH 4.0 was monitored by plotting the intensity of the signal

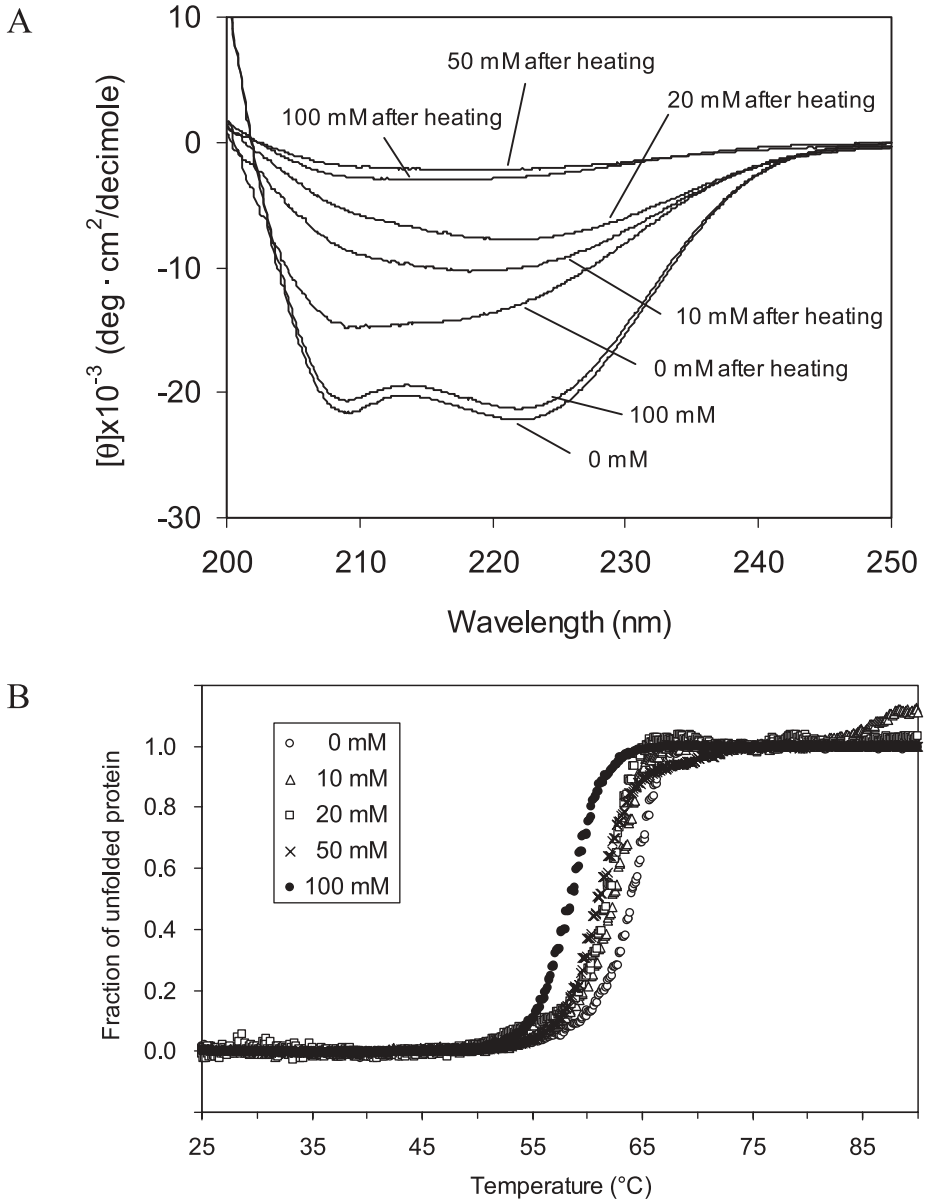

Fig. 1. **CD spectra before and after heating, and thermal unfolding curves for rmethuG-CSF solutions containing 0–100 mM NaCl at pH 4.0.** (A) CD spectra of rmethuG-CSF solutions at pH 4.0 (25°C) in the presence of 0 and 100 mM NaCl, and CD spectra of rmethuG-CSF solutions cooled down to 25°C from 90°C in the presence of NaCl of 0 to 100 mM at pH 4.0. (B) Thermal unfolding curves of rmethuG-CSF solutions in the presence of NaCl of 0 to 100 mM at pH 4.0.

at 222 nm versus temperature, as shown in Fig. 1B. The Tm obtained for the rmethuG-CSF buffer solutions with different concentrations of NaCl are summarized in Table 1. Figure 1B and Table 1 reveal that Tm decreased with the increase in the NaCl concentration. This means that the secondary structure of rmethuG-CSF is destabilized with an increase in ionic strength.

The CD spectra of a rmethuG-CSF solution at pH 4.0 cooled down to 25°C from 90°C depended on the NaCl concentration (Fig. 1A). As the NaCl concentration increased, the CD intensity decreased. When the NaCl concentration was 50 or 100 mM, a precipitate appeared. The reversibility of the secondary structure during the unfolding and folding processes decreased with the increase in the NaCl concentration (Table 2). These results suggest that the denaturation induced by the addition of NaCl was accompanied by a decrease in the reversibility of the secondary structure and by the formation of protein aggregates.

The thermal stability of the secondary structure of rmethuG-CSF in solutions with various concentrations of NaCl was also investigated at pH 2.5 and 2.0 using far-UV CD. The Tm and reversibility determined from these CD measurements are listed in Tables 1 and 2, respectively. It can be seen in Tables 1 and 2 that an increase in the NaCl concentration makes the secondary structure of the protein less stable at all pHs examined in this work. In contrast to the case of pH 4.0, there was no precipitation during the heat-induced denaturation in the presence of 100 mM NaCl at pH 2.5 and 2.0.

Table 1. **pH dependence of T_m (°C) of thermal denaturation of rmethuG-CSF solutions in the presence of 0–100 mM NaCl investigated by means of far-UV CD.**

pH	NaCl (mM)				
	0	10	20	50	100
pH 2.0	62.3	61.8	61.2	58.0	54.2
pH 2.5	65.1	64.6	62.8	60.1	56.7
pH 4.0	64.0	62.3	61.7	60.9	58.6

Table 2. **pH dependence of reversibility (%) of thermal denaturation of rmethuG-CSF solutions in the presence of 0–100 mM NaCl investigated by means of far-UV CD.**

pH	NaCl (mM)				
	0	10	20	50	100
pH 2.0	89	91	87	62	61
pH 2.5	85	83	65	61	61
pH 4.0	59	47	35	10	13

Table 3. **T_m (°C) and reversibility (%) of thermal denaturation of rmethuG-CSF solutions in the presence of 0-20 mM Na_2SO_4 at pH 2.0 investigated by means of far-UV CD.**

Parameter	Na_2SO_4 (mM)					
	0	1	3	5	10	20
T_m (°C)	62.3	62.5	60.8	59.6	57.3	55.5
Reversibility (%)	89	91	65	62	28	9

In this study, we heated rmethuG-CSF solutions at the heating rate of 1°C/min. We have demonstrated that the Tm of remthuG-CSF is easily affected by the measurement conditions such as the protein concentration and heating rate, as its reversibility is low, as reported in our previous paper (*21*). The reversibility of the secondary structure upon thermal denaturation decreases with an increase in the NaCl concentration. Therefore, it seems that the Tm of a rmethuG-CSF solution with NaCl is influenced by changes in the protein concentration and heating rate.

We also investigated the thermal stability of rmethuG-CSF solutions containing 1, 3, 5, 10, and 20 mM Na_2SO_4 at pH 2.0 by means of far-UV CD. As in the case of NaCl, the T_m and reversibility of the secondary structure of rmethuG-CSF decrease as the concentration of Na_2SO_4 increases (Table 3). A precipitate appeared in the solution containing Na_2SO_4 at above 10 mM. The degree of the decrease in the reversibility was greater for Na_2SO_4 than for NaCl at the same concentration. The reversibility of a rmethuG-CSF solution at pH 2.0 with 10 mM NaCl and that with 10 mM Na_2SO_4 was 91% and 28%, respectively. In addition, the thermal stability of rmethuG-CSF solutions containing Na_2SO_4 is lower than that of ones containing NaCl with the same concentration of sodium ions (*e.g.*, 10 mM Na_2SO_4 solution *vs.* 20 mM NaCl solution) because of the higher ionic strength of Na_2SO_4.

IR Study of the Effect of Ionic Strength on the Process of Thermal Unfolding of rmethuG-CSF at pD 4.0—Figure 2, A, B, and C, shows an IR spectrum in the amide I′ region (1,700–1,600 cm^{-1} region) of a rmethuG-CSF solution containing 50 mM NaCl at pD 4.0 (25°C), its Fourier-self deconvolved spectrum, and the result of curve-fitting of the original spectrum, respectively. The IR spectrum, its

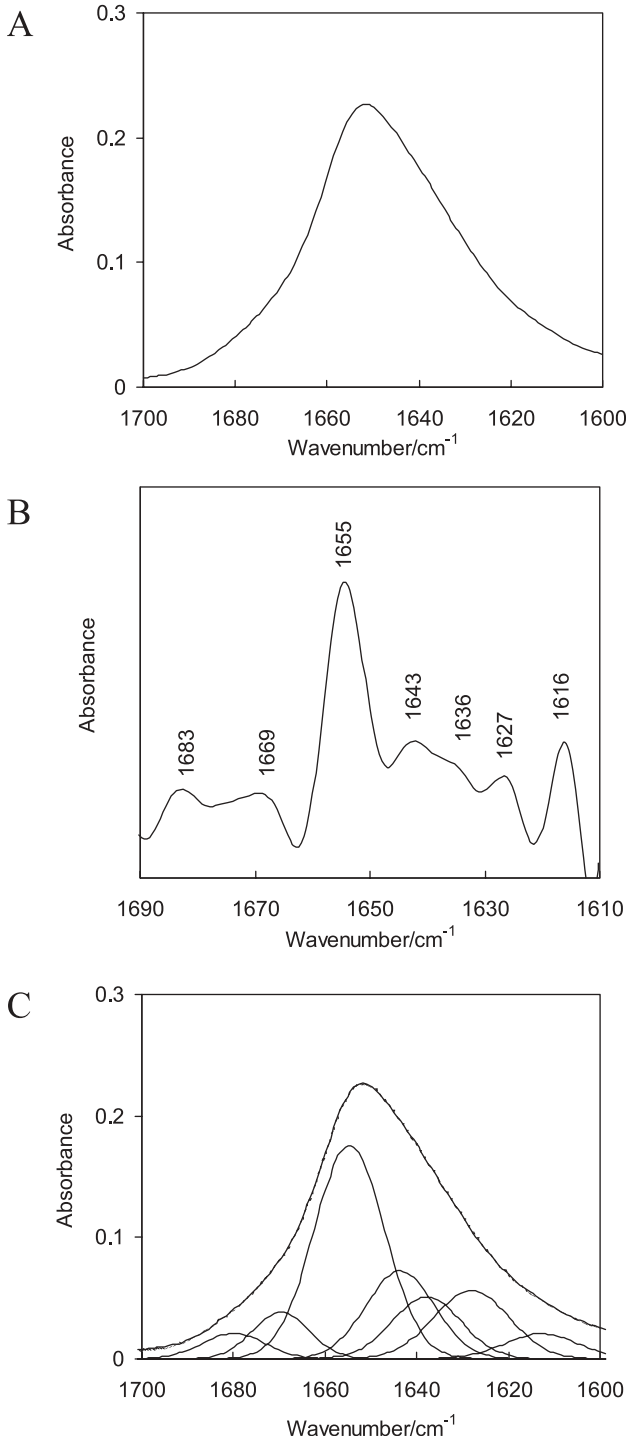

Fig. 2. **An IR spectrum, its Fourier-self deconvolved spectrum, and the result of curve-fitting of the original spectrum of a rmethuG-CSF solution containing 50 mM NaCl.** (A) An IR spectrum in the amide I′ region of rmethuG-CSF at pD 4.0 (25°C) containing 50 mM NaCl. (B) A Fourier-self deconvolved spectrum of the original spectrum shown in (A). (C) The result of curve-fitting of the original spectrum shown in (A).

Fourier-self deconvolved spectrum, and the result of curve-fitting of the spectrum in Fig. 2, A, B, and C, are almost identical with those of a rmethuG-CSF solution without NaCl at pD 4.0 (25°C) that we previously reported (*8*).

Table 4. **Wavenumbers (cm^{-1}) and assignments of IR bands in the amide I′ region of rmethuG-CSF. The percentages of intensities of amide I′ bands of a rmethuG-CSF solution at pD 4.0 containing 50 mM NaCl at 25°C.**

Wavenumber/cm^{-1}	Assignment	25°C
1685	intermolecular β-sheet (aggregation)	N.D.[a]
1682–1683	extended strands	4.6
1669–1671	reverse turn	7.6
1654–1658	α-helix	43.3
1643–1647	unordered structure	17.3
1636–1638	β-strand (3_{10}-helix)	12.3
1627–1630	extended strands	14.9
1616–1618	intermolecular β-sheet (aggregation)	N.D.[a]

[a]N.D.: Not detected.

However, as will be shown later, there are clear differences in the temperature-dependent variations in the 1,700–1,600 cm^{-1} region between the rmethuG-CSF solution in the presence of 50 mM NaCl and that in the absence of NaCl.

The deconvoluted spectrum in Fig. 2B shows that there are seven component bands in the 1,700–1,600 cm^{-1} region. The contribution of each band to the total amide I′ contour can be determined by the curve fitting procedure for the original spectrum (Fig. 2C and Table 4). The assignment of each band to a secondary structure element was carried out based on comparison with the assignments of previously reported IR spectra of proteins (*14–17*, *22–24*). Table 4 summarizes the assignments of amide I′ bands in Fig. 2C. The area percentage of an individual amide I′ band of the IR spectrum of the rmethuG-CSF solution with 50 mM NaCl is very similar to that of the solution without NaCl (*8*).

Figure 3, A and B, compares temperature-dependent (25–80°C) IR spectra in the amide I′ region of the rmethuG-CSF solution containing 50 mM NaCl with those of the solution without it. Figure 4, A and B, presents the second derivatives of the spectra shown in Fig. 3, A and B, respectively. It should be noted in Fig. 3, A and B, that the intensity of the band at 1,654 cm^{-1} decrease with temperature, and instead new bands appear near 1,685 and 1,617 cm^{-1}. These spectral changes are more clearly recognizable in the second derivative spectra in Fig. 4, A and B. The appearance of the new bands indicates that intermolecular antiparallel β-sheet is formed through protein aggregation (*25*). The aggregation occurs as the consequence of thermal denaturation of rmethuG-CSF. Of particular note in the second derivative spectra is that the aggregation starts at 50 and 44°C in the solution without NaCl and that with NaCl, respectively (Fig. 4, A and B). Therefore, the results in the second derivative spectra indicate that the structure of rmethuG-CSF becomes less stable with an increase in the ionic strength. Apart from the appearance of the 1,685 and 1,617 cm^{-1} bands, the broad maximum of the spectra shifts from 1,654 to 1,647 cm^{-1}, indicating that the secondary structure of rmethuG-CSF changes mainly from α-helix, the major secondary structure component, to unordered structures upon heating.

Figure 5, A and B, shows plot of temperature-dependent variations in the percentages of the major secondary structure elements (α-helix, unordered structure, intermolecular antiparallel β-sheet, and 3_{10}-helix) of rmethuG-CSF in solutions at pD 4.0 containing no and 50 mM NaCl calculated from the curve-fitted spectra. Comparison of the results in Fig. 5, A and B, reveals the

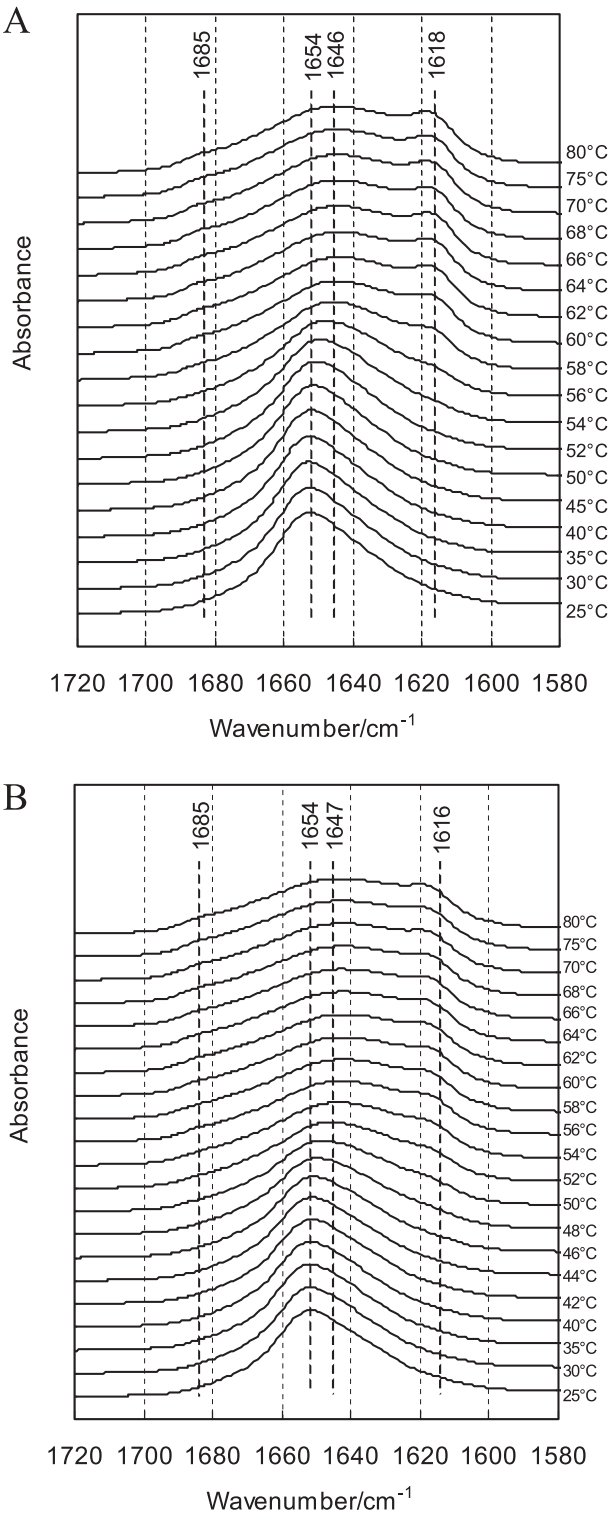

Fig. 3. **Temperature-dependent IR spectra in the amide I′ region of rmethuG-CSF solutions.** IR spectra of rmethuG-CSF solutions in the absence (A) and presence of NaCl (50 mM) (B) were measured over the temperature range of 25 to 80°C at pD 4.0.

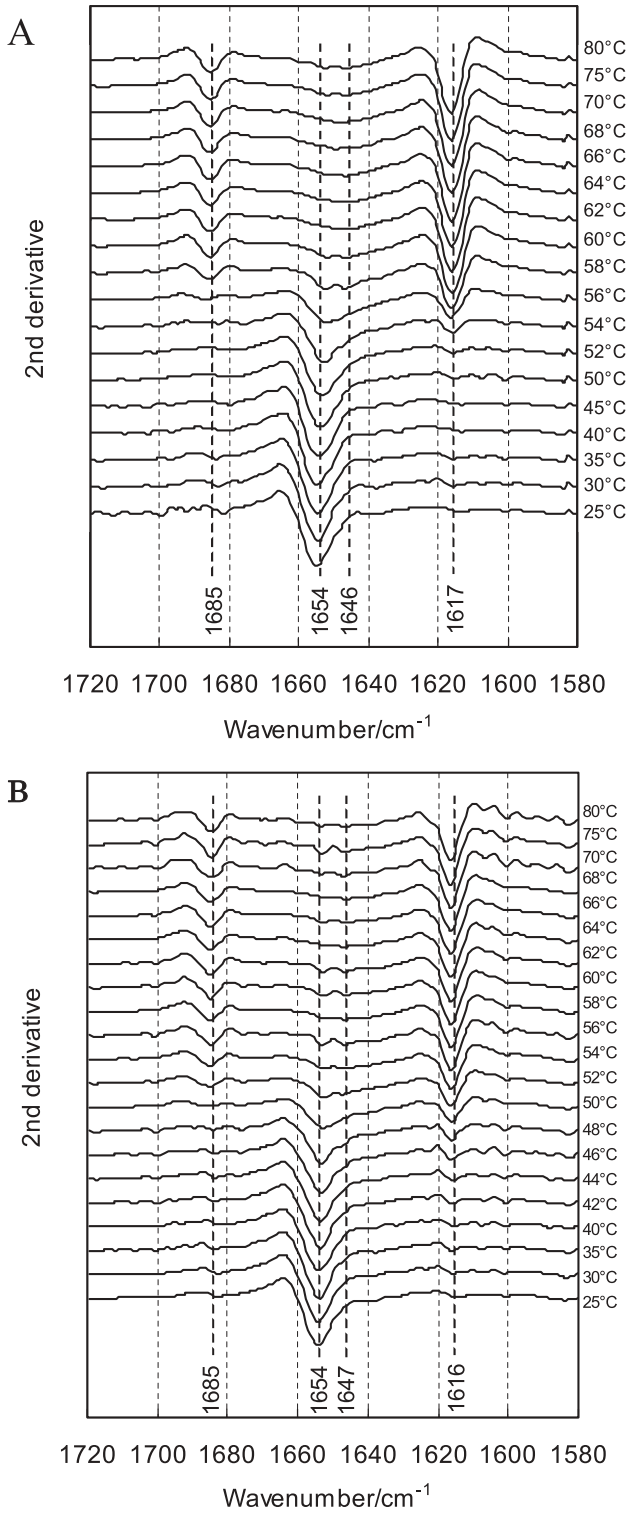

Fig. 4. **Temperature-dependent second-derivative IR spectra in the amide I′ region of rmethuG-CSF solutions.** (A and B) Second-derivative spectra of the IR spectra shown in Fig. 3, A and B, respectively.

following three major points: (i) The aggregation starts at a lower temperature in the rmethuG-CSF solution in the presence of 50 mM NaCl than in the solution without NaCl. (ii) The slope of the formation curve for aggregates is apparently lower for the rmethuG-CSF solution in the presence of 50 mM NaCl. (iii) The amount of the aggregates is smaller for the rmethuG-CSF solution in the presence of 50 mM NaCl.

pD Dependence of the Effect of Ionic Strength on the Thermal Unfolding Process—To investigate the pD dependence of the effect of ionic strength on the thermal stability of rmethuG-CSF, temperature-dependent IR spectra of rmethuG-CSF solutions containing 50 mM NaCl were also measured at pD 2.1 and 2.5. Figure 5, C and D, compares the temperature-dependent changes in the percentages of α-helix, unordered structure, aggregates, and 3_{10}-helix between the rmethuG-CSF solutions with and without 50 mM NaCl at pD 2.5. Figure 5, E and F, compares the corresponding results at pD 2.1. The following three points should be noted in Fig. 5, C, D, E, and F: (i) As in the case of pD 4.0, at pD 2.5 and pD 2.1 the aggregation begins at a lower temperature in the solutions containing 50 mM NaCl than in those without NaCl. In the case of pD 2.1, it occurs at 45°C in the solution containing 50 mM NaCl (Fig. 5F), which is 22°C lower than the temperature (67°C) at which the aggregation starts in the solution without NaCl (Fig. 5E). The temperature at which aggregates of rmethuG-CSF solutions are formed decreases clearly with the addition of NaCl at all the pDs investigated. (ii) In the case of the solution in the absence of NaCl, there are marked differences in the temperature-dependent changes in the percentages of secondary structure elements between pD 2.5 and 2.1 (Fig. 5, C and E), but in the case of that containing 50 mM NaCl, the differences are much smaller (Fig. 5, D and F). (iii) The aggregation proceeds much more in the solution containing 50 mM NaCl at pD 2.1 compared with that in the absence of NaCl (Fig. 5, E and F).

DISCUSSION

In this study, we have explored the effect of ionic strength on the process of thermal unfolding of rmethuG-CSF using CD and IR spectroscopy. It was found in the CD study that the Tm and degree of reversibility of the secondary structure decrease at all the pHs investigated with an increase in the concentration of NaCl. At pH 4.0, precipitation occurs in the protein solutions containing NaCl at concentrations of more than 50 mM, which means that the level of the aggregation is beyond the solubility limit. This finding is similar to that in a thermal stability study on rHuMGDF, which belongs to the four-helical cytokine family like rmethuG-CSF (*13*). Narhi *et al.* investigated the effect of the NaCl concentration on the reversibility of the thermal denaturation of rHuMGDF. This study demonstrated that higher ionic strength results in decreased reversibility or increased aggregation of rHuMGDF, and in a lower melting temperature. The addition of Na_2SO_4 to a rmethuG-CSF solution destabilizes the protein more than that of NaCl due to the higher ionic strength.

CD spectroscopy cannot be used to estimate protein aggregation, but IR spectroscopy can detect it as the formation of intermolecular β-structure, and also can be used to quantify the aggregates by means of the curve-fitting procedure. We have employed temperature-dependent IR spectra to monitor the heat-induced denaturation of

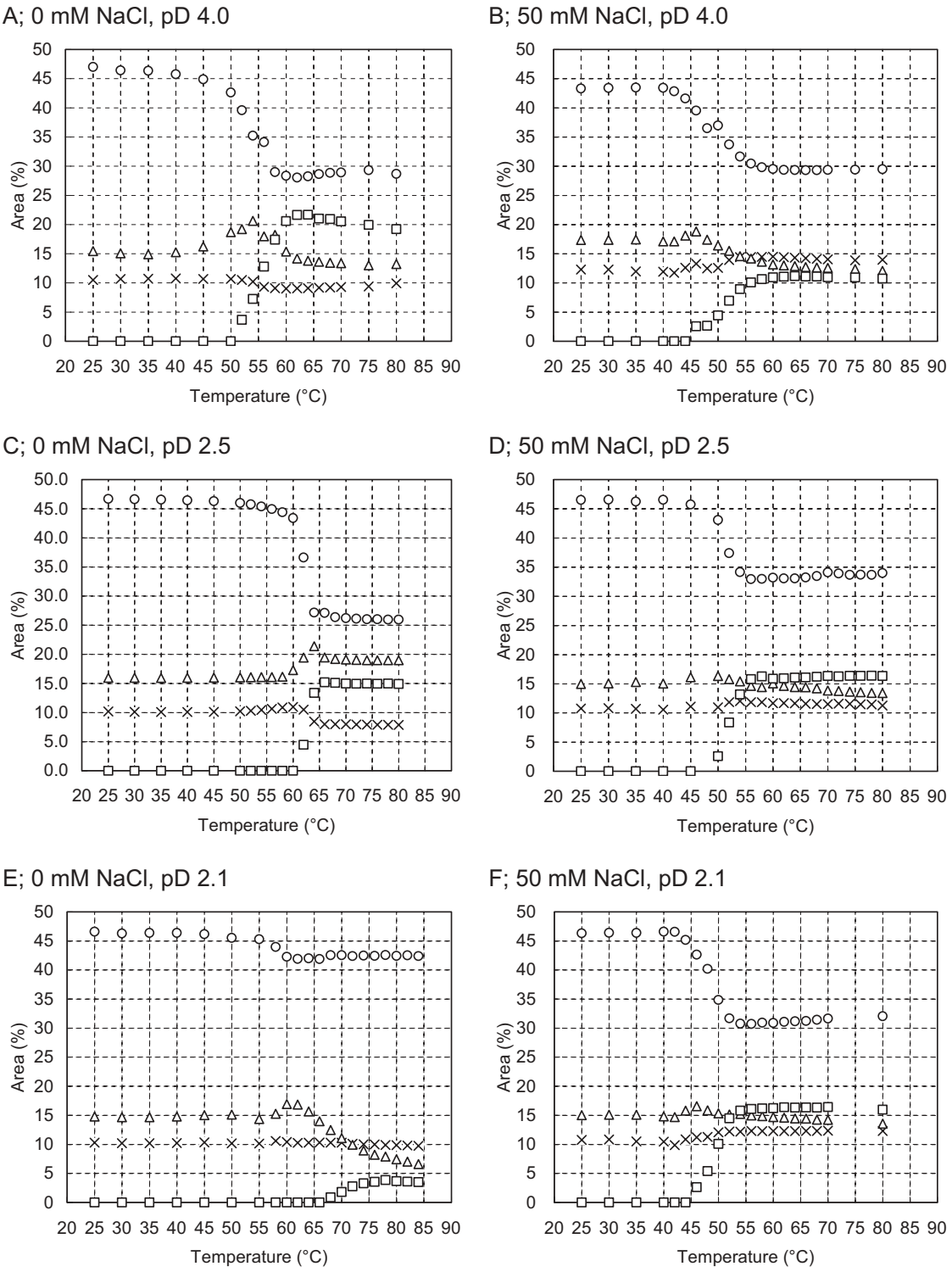

Fig. 5. **The results of curve-fitting of temperature-dependent IR spectra of rmethuG-CSF solutions at pD 4.0, 2.5, and 2.1 in the absence and presence of NaCl (50 mM).** Temperature-dependent variations in the percentages of α-helix (circles), unordered structure (triangles), 3_{10}-helix (crosses), and aggregates (squares) determined from the curve-fitted spectra. (A) 0 mM and (B) 50 mM NaCl at pD 4.0; (C) 0 mM and (D) 50 mM NaCl at pD 2.5; (E) 0 mM and (F) 50 mM NaCl at pD 2.1.

rmethuG-CSF, especially the formation of aggregates, in more detail.

As seen on comparison of CD and IR spectra, the Tm of rmethuG-CSF obtained from the CD spectra is higher than the thermal stability of secondary structure, α-helix, obtained from the IR spectra. For instance, the Tm of the rmethuG-CSF solution with 50 mM NaCl at pH 2.5 obtained from CD and IR spectra are 60.1 and 51°C,

respectability (Table 1 and Fig. 5D). In this study, the CD and IR spectra were measured with protein concentrations of 0.5 and 10 mg/ml, and heating rates of 1°C/min and approximately 0.3°C/min, respectability. We have demonstrated that the T_m of rmethuG-CSF is easily affected by the measurement conditions such as the protein concentration and the heating rate, because the process of thermal denaturation of rmethuG-CSF is irreversible (*21*). As the protein concentration becomes higher and the heating rate becomes slower, T_m decreases. Therefore, it seems that the T_m determined from the CD spectra does not agree with that determined from the IR spectra.

In our previous study, it was revealed by temperature-dependent IR spectra of rmethuG-CSF that the process of thermal denaturation of rmethuG-CSF starts with the conversion of α-helix to an unordered structure, and then aggregates are formed by the unordered structure (*8*). The unordered structure is a perturbed form of the native structure and is produced before the marked structural changes of rmethuG-CSF. The unordered structures interact with each other and form aggregates. The formation of the unordered structure seems to be the first secondary structural change that occurs in the aggregation pathway. This event occurs irrespective of pH. Furthermore, as pD increases, the thermal stability of the secondary structure, especially that of α-helix, decreases and aggregates are prone to be formed at lower temperature. The thermal stability of rmethuG-CSF at low pD is somewhat complicated. The thermal stability of the secondary structure of rmethuG-CSF at pD 2.1 is lower than that at pD 2.5. At pD 2.1 the α-helix begins to change at 55°C, which happens at 60°C at pD 2.5. However, aggregates of this protein are formed from 60°C and 66°C at pD 2.5 and pD 2.1, respectively. The aggregation takes place at a higher temperature at pD 2.1 than at pD 2.5. Another notable point at pD 2.1 is that the α-helix content does not decrease greatly even at high temperatures and that the aggregation proceeds a little at pD 2.1. We inferred that this happens because the repulsive interaction between the unordered structures of rmethuG-CSF is stronger at pD 2.1.

The present study increases our understanding of the mechanism of aggregation of a rmethuG-CSF solution at acid pH. The temperature-dependent IR spectra at pD 4.0 demonstrated that the aggregation started at a lower temperature in the solution containing 50 mM NaCl than in that without NaCl. As mentioned above, the unordered structure is the precursor for aggregation in the process of unfolding of rmethuG-CSF irrespective of the presence or absence of NaCl. Therefore, it is concluded that the aggregation is liable to occur in the presence of 50 mM NaCl at pD 4.0 because of masking of the repulsive interaction between the unordered structures. The aggregation proceeds at a lower rate and the amount of the aggregates is also lower for a rmethuG-CSF solution with 50 mM NaCl (Fig. 5B). This finding seems to be responsible for the formation of a precipitate. The precipitation occurs in protein solutions containing 50 mM NaCl upon heating. The precipitate is probably produced immediately after the soluble aggregates are formed from the unordered structure in the presence of 50 mM NaCl at pD 4.0 because of reduction of the repulsive interaction. It seems that the soluble aggregates can be detected while the precipitate cannot be detected on IR spectroscopy. Therefore, the percentage of aggregates for all the secondary structure elements is apparently smaller in a rmethuG-CSF solution containing 50 mM NaCl than in one without NaCl upon heating (at 80°C). In addition, the aggregation apparently proceeds at a lower rate for a rmethuG-CSF solution with 50 mM NaCl.

We investigated the effects of ionic strength on the structure of rmethuG-CSF at different pDs (pD 4.0, 2.5, and 2.1). In this study, it was found that the addition of 50 mM NaCl decreases the temperature at which aggregates are formed at pD 2.5 and 2.1. In addition, there is no significant difference in the amount of aggregates of rmethuG-CSF in a solution with 50 mM NaCl between pD 2.5 and 2.1 upon heating. It may be concluded that after conversion of α-helix to the unordered structure at pD 2.1, aggregates are immediately formed by the unordered structures. The equilibrium between α-helix and unordered structure shifts to the unordered structure with the formation of aggregates from the unordered structure, and hence the degree of the decrease in α-helix is greater in a solution with 50 mM NaCl than in one without NaCl at pD 2.1. RmethuG-CSF solutions have a positive charge at acid pD (the isoelectric point is pH 6.1). The effect of salts is attributed to a reduction of the charge repulsion through masking of the positively charged groups by the salts, thereby minimizing the repulsive interaction. Recently, Chi *et al.* (*26*) proposed that rmethuG-CSF aggregation is controlled by both conformational stability and colloidal stability. Conformational stability is concerned with the modification change from the native state to an expanded transition state, and colloidal stability means the assembly step of the expanded transition states. Based on the conformational stability and colloidal stability, Chi *et al.* (*26*) suggested that the addition of 150 mM NaCl masks repulsive electrostatic interactions, reducing the maximum interaction energy barrier of two protein molecules sufficiently, so that aggregation occurs. In the present study, the results obtained from the IR and CD spectra were similar to those of Chi *et al.*, but we have first monitored the process of thermal unfolding of rmethuG-CSF in high ionic solutions quantitatively.

CONCLUSION

This paper has reported the effect of ionic strength on the process of thermal unfolding of rmethuG-CSF at acid pH investigated by means of far-UV CD and IR spectroscopy. The Tm and reversibility on the heat-induced denaturation decreased as the ionic strength increased at all the pHs investigated. The temperature-dependent IR measurements have provided information about secondary structure changes and the formation of aggregates quantitatively. It has been found that the temperature at which protein aggregates are formed at pD 2.1 becomes lower and the amount of aggregates becomes greater with the addition of NaCl. Moreover, the present study has also revealed that after the conversion of α-helix to unordered structure at pD 2.1, aggregates are immediately formed by the unordered structures due to masking of the repulsive interaction between the unordered structures by the salts.

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