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An Infrared Spectroscopy Study of Acid Stability and Thermal Unfolding Process of Granulocyte-Colony Stimulating Factor

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Temperature-dependent (25–80°C) infrared (IR) spectra were obtained for recombinant methionyl human granulocyte-colony stimulating factor (rmethuG-CSF) in aqueous solutions over the pD range of 5.5–2.1 to investigate its thermal stability at various pDs. Second derivative, Fourier self-deconvolution, and curve-fitting analyses were performed to analyze the obtained spectra. These spectral analyses demonstrated that 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. The temperature-dependent IR spectra revealed that the structure of rmethuG-CSF is the most stable at pD 2.5 in the pD range of 5.5–2.1. It has been suggested that the unordered structure formed before the marked structural change in the whole molecule is a perturbed form of the native structure of rmethuG-CSF and plays a role as a precursor for the aggregation. This alteration to the perturbed form is likely to be the first secondary structure change that occurs along the aggregation pathway. Of particular note is that the stability at pD 2.1 is slightly lower than that at pD 2.5, but that aggregates are formed at higher temperature at pD 2.1 than at pD 2.5, probably because the repulsive interaction between the unordered structure is stronger at pD 2.1.

Key words: FT-IR, G-CSF, secondary structure, thermal denaturation, unfolding process.

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

Granulocyte-colony stimulating factor (G-CSF) is a cytokine that plays a critical role in maintaining the population of neutrophilic granulocytes in peripheral blood and is also responsible for granulocytosis during inflammation (*1*, *2*). Recombinant methionyl human G-CSF (rmethuG-CSF) overexpressed in *Escherichia coli* is composed of a polypeptide chain of 175 amino acids and has a molecular weight of approximately 19 kDa. RmethuG-CSF is a pharmaceutically relevant globular protein belonging to a group of growth factors that share the common four-helix bundle architecture (*3*). Infrared (IR), circular dichroism (CD), and fluorescence studies on rmethuG-CSF have demonstrated that rmethuG-CSF undergoes structural changes induced by pH, heat and denaturants such as guanidine hydrochloride (GdnHCl), and that the secondary and tertiary structures of rmethuG-CSF are stable at low pH (*4–6*). At pH 2, rmethuG-CSF exists in a compact state with a well-defined tertiary structure that unfolds in a cooperative fashion upon heating (*6*). On the other hand, rmethuG-CSF forms aggregates rapidly under physiological conditions (*e.g.*, pH 7 phosphate-buffered saline and 37°C). Krishnan *et al.* have demonstrated by means of transmission electron microscopy (TEM) that the structure on precipitates of rmethuG-CSF comprises amorphous aggregates (*7*). It has been suggested by recent studies that most of the aggregates are formed from a partially unfolded state of the molecules, which are in equilibrium with the native molecules (*8*, *9*). It has also been indicated that the aggregation proceeds through a transition state within the native state ensemble that is only slightly perturbed in structure relative to the most compact species (*10*, *11*).

Studies on protein aggregates are important in biotechnology as well as medicine. In biotechnology industries, protein aggregation is encountered, for example, during production, isolation, purification, shipping, storage and administration of protein pharmaceuticals (*12*). It is also known that protein aggregation and subsequent precipitation are responsible for a number of diseases such as Alzheimer's disease and numerous neurodegenerative diseases (*13*). To effectively inhibit the aggregation, we must understand the mechanism of protein stability and aggregation under varying solution conditions.

IR spectroscopy has been used for protein research for many years (*14–17*). It is powerful for qualitative and quantitative estimation of all the secondary structure elements of proteins such as α-helix, β-sheet, β-turn, and unordered structure. On the other hand, CD spectroscopy is sensitive to the secondary structure of α-helix, but not to that of β-sheet. One of the significant advantages of IR spectroscopy for studies on proteins is that spectra of pro-

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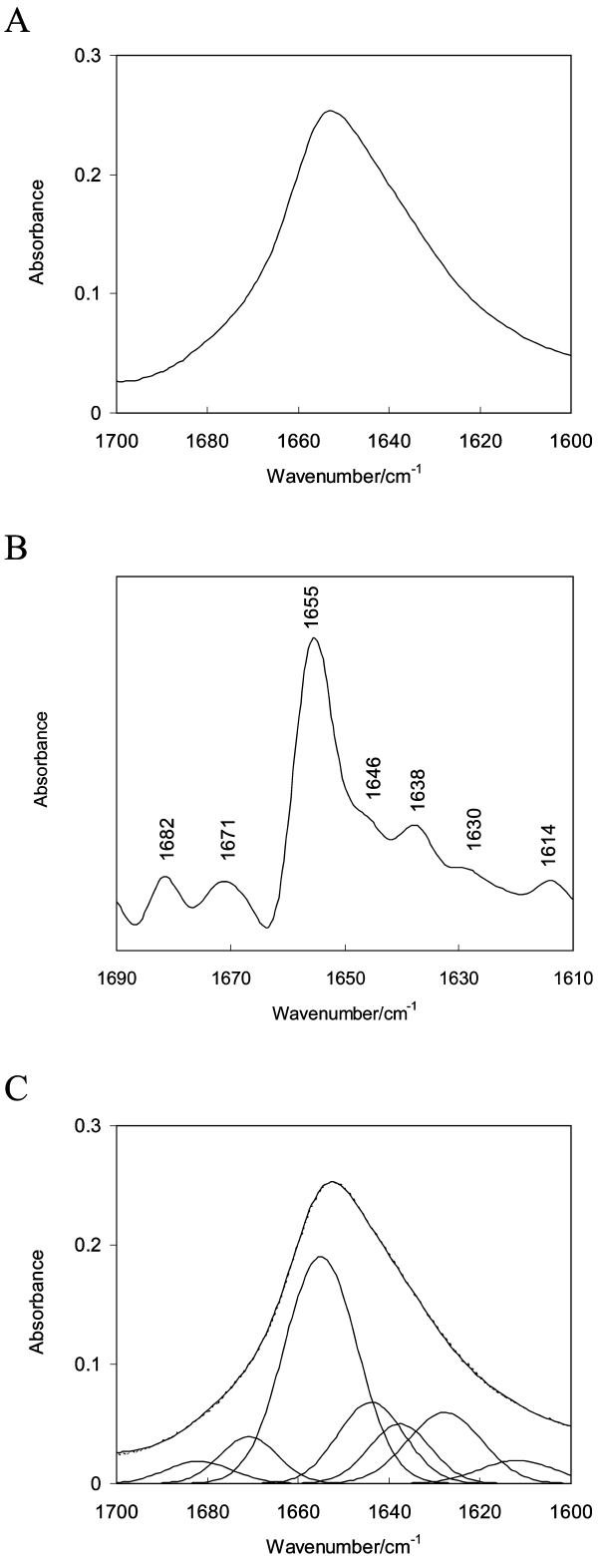

Fig. 1. **IR spectrum, Fourier self-deconvolved spectrum, and result of curve-fitting of the IR spectrum of rmethuG-CSF.** (A) An IR spectrum of rmethuG-CSF in the amide I′ region at 25°C in 20 mM Na phosphate buffer at pD 4.0. (B) A Fourier self-deconvolved spectrum of the original spectrum shown in (A). (C) The result of curve-fitting of the Fourier self-deconvolved spectrum shown in (B). A solid line represents the original spectrum while a dashed line represents the curve-fitted spectrum.

teins with a high signal-to-noise ratio can be obtained easily under various conditions irrespective of the size of the proteins. Amide I and II bands have been used to investigate the secondary structure of proteins. Although many attempts have been made to assign the components of the amide I and II bands to secondary structures such as α-helix and β-sheet, it is still a matter of controversy (*14–17*). Therefore, many spectral analysis methods such as second derivative, Fourier self-deconvolution, and difference spectra have been employed to enhance the spectral resolution in the amide I and II regions and to assign amide I and II component bands to given conformations.

The purpose of the present study is to explore the pH dependence of the thermal stability of rmethuG-CSF and the mechanism of the stability by use of IR spectroscopy. The increase in the stability at acid pH relative to neutral pH is unique to rmethuG-CSF. In addition, rmethuG-CSF is prone to aggregate irreversibility at neutral pH. It is important for pharmaceutics to understand more deeply the protein stability and aggregation pathway of rmethuG-CSF. Thermal stability studies on rmethuG-CSF solutions have already been carried out by Kolvenbach *et al.* by means of IR, CD, and fluorescence spectroscopy (*6*), but spectra were only obtained at three pHs, 2, 4 and 7, and the qualitative estimation of secondary structural changes and interpretation of the unfolding mechanism have not been performed in detail. In the present study, IR spectra were obtained for rmethuG-CSF in aqueous solutions over the temperature range of 25 to 80°C at pD 2.1, 2.5, 3.0, 3.5, 4.0, 5.0, and 5.5. The spectra obtained were analyzed by means of second derivative, Fourier self-deconvolution, and curve-fitting analyses to investigate the thermal stability of rmethuG-CSF at various pDs and to explore the unfolding mechanism. One of the novel points of this papar is discussion of a transition state species of rmethuG-CSF during thermal denaturation. It was found in the present study that rmethuG-CSF is the most stable at pD 2.5 in terms of that the stability of α-helix is the highest at this pD, but aggregation takes place more easily at pD 2.5 than pD 2.1.

METHODS

Materials—Recombinant methionyl human G-CSF overexpressed in *Escherichia coli* was produced and purified at Kirin Brewery Co., Ltd. (Gunma, Japan). The concentration of rmethuG-CSF was determined spectrophotometrically using an extinction coefficient of 0.86 at 280 nm for a 0.1 w/v % protein solution.

Infrared Spectroscopy—Experimentally it is much simpler to obtain protein spectra in a D_2O solution than in a H_2O solution. An IR band due to the D_2O bending mode appears at a lower wavenumber (~1,230 cm^{-1}) than the corresponding band for H_2O (~1,650 cm^{-1}), creating a region of relatively low absorbance between 1,400 and 1,800 cm^{-1}, an ideal window for observing weak IR bands of proteins. Much longer pathlengths of 40–80 μm may be used for a D_2O solution, and hence much lower sample concentrations are required to obtain high-quality spectra.

A rmethuG-CSF solution of 10 mg/ml was changed from a HCl solution (pH 4.0) into 20 mM Na phosphate

Table 1. **The relative integrated intensities of amide I' bands of rmethuG-CSF at 25°C and 80°C at pD 4.0.**

Wavenumber/cm^{-1}	25°C	80°C
1682	4.2	6.7
1670	7.7	8.6
1654–1658	47.0	28.7
1644–1646	15.5	13.2
1638–1640	10.5	9.9
1630	15.1	13.6
1618	N.D.[a]	19.2

[a]N.D.: Not detected.

Table 2. **Wavenumbers (cm^{-1}) and assignments of IR bands in the amide I′ region of rmethuG-CSF.**

Wavenumber/cm^{-1}	Assignment
1685	intermolecular β-sheet (aggregation)
1682	extended strands
1670	reverse turn
1654–1658	α-helix
1644–1646	unordered structure
1638–1640	β-strand (3_{10}-helix)
1630	extended strands
1618	intermolecular β-sheet (aggregation)
1614	amino acid residues

buffer at pD 2.1, 2.5, 3.0, 3.5, 4.0, 5.0 and 5.5. IR spectra of the rmethuG-CSF solutions and buffers were obtained using a Nicolet NEXUS 670 FT-IR spectrophotometer equipped with a liquid nitrogen cooled mercury cadmium telluride (MCT) detector and a temperature controller with a water bus (Thermo Haake DC30). The samples were sandwiched between CaF_2 windows with a 50 μm Teflon spacer. For each spectrum, 256 scans were coadded at a spectral resolution of 2 cm^{-1}. The sample chamber of the spectrometer was continuously purged with N_2 gas to prevent atmospheric water vapor from interfering with the amide I and II regions. Spectra were obtained over the temperature range of 25 to 80°C. The equilibration time for each temperature was 5 min. Spectral subtraction was performed using OMNIC program (Thermo Nicolet). The IR spectrum of atmospheric water vapor was subtracted from each spectrum, and then the spectrum of a buffer solution obtained under the same conditions was subtracted from the spectrum of a protein solution. The spectra thus obtained were subjected to smoothing with a seven-point Savitsky-Golay function to reduce the noise, and Fourier self-deconvolution was carried out using the same software with a full width at a half-height of 25.5 cm^{-1} and a k of 3. The smoothing and the calculation of second-derivative spectra and curve fitting were performed with software named SPINA 3.0 (Y. Katsumoto, Kwansei Gakuin University).

RESULTS

Temperature-Dependent IR Spectra of rmethuG-CSF at pD 4.0—Figure 1A shows an IR spectrum in the 1,700–1,600 cm^{-1} region of a rmethuG-CSF solution at pD 4.0. The spectrum in Fig. 1A is characterized by a dominant band at 1,654 cm^{-1} due to the amide I′ vibration of rmethuG-CSF. This wavenumber is indicative of α-helix. The number and wavenumbers of component bands in the amide I′ region were determined by Fourier self-deconvolution (Fig. 1B). The deconvoluted spectrum exhibits seven component bands in the 1,700–1,600 cm^{-1} region. The relative integrated intensity of each band was determined by curve-fitting of the original spectrum (Fig. 1C and Table 1). The assignment of each band to a secondary structure element was made based on previous reports on IR spectra of proteins (*14–20*). The wavenumbers and assignments of bands observed in the Fourier self-deconvolued spectrum are summarized in Table 2. The peak at 1,614 cm^{-1} is not due to an amide I′ component but to amino acid residues. The relative integrated intensity of each band in the amide I′ region is very similar to that in the IR spectrum of rmethuG-CSF reported by Arakawa *et al.* (*21*). Arakawa *et al.* obtained the IR spectrum under the conditions with 0.32 mM DCl (pD 3.5) as the buffer and a protein concentration of 1.7 mM (*21*).

We have studied the thermal stability of a rmethuG-CSF solution at pD 4.0. Figure 2A depicts IR spectra for the amide I′ region of rmethuG-CSF obtained over the temperature range of 25–80°C. Figure 2B presents the second derivatives of the spectra shown in Fig. 2A. Figure 2C shows difference spectra calculated by subtracting the spectrum obtained at 25°C from all the spectra in Fig. 2A. It can be seen from Fig. 2, A, B and C, that the intensity of the band at 1,654 cm^{-1} decreases as the temperature increases, new bands appearing instead near 1,685 and 1,618 cm^{-1}. The second derivative spectra and difference spectra demonstrate that the new amide I′ bands at 1,685 and 1,618 cm^{-1} emerge at 52°C. The appearance of the new bands indicates that intermolecular antiparallel β-sheet is formed through aggregation (*22*). The aggregates are produced as the consequence of thermal denaturation of rmethuG-CSF. Apart from the appearance of the 1,685 and 1,618 cm^{-1} bands, the broad maximum of the spectra shifts from 1,654 to 1,646 cm^{-1}, indicating that the secondary structure of rmethuG-CSF changes mainly from α-helix, the major component, to unordered structure upon heating.

Figure 3 shows the original and curve-fitted spectra of rmethuG-CSF at 80°C (pD 4.0). The relative integrated intensities of the amide I′ component bands at 80°C are also listed in Table 1. The relative amounts of α-helix, intermolecular antiparallel β-sheet, and unordered structure are approximately 50%, 0%, and 15% at 25°C, and 30%, 20%, and 15% at 80°C, respectively. Comparison of the curve fitting in Fig. 3 with that in Fig. 1C reveals that α-helix (1,654 cm^{-1}) decreases and intermolecular antiparallel β-sheet (1,685 and 1,618 cm^{-1}) increases. The relative amounts of minor components such as extended strands, reverse turn, and 3_{10}-helix change a little with temperature.

Figure 4 plots the percentages of secondary structure elements calculated from the curve-fitted spectra as a function of temperature. The plots show that α-helix gradually decreases from 40 to 50°C, and then decreases rapidly above 50°C. On the other hand, an increase of the unordered structure starts from 40°C, and aggregation (intermolecular antiparallel β-sheet) increases from 50°C. The unordered structure gradually decreases from

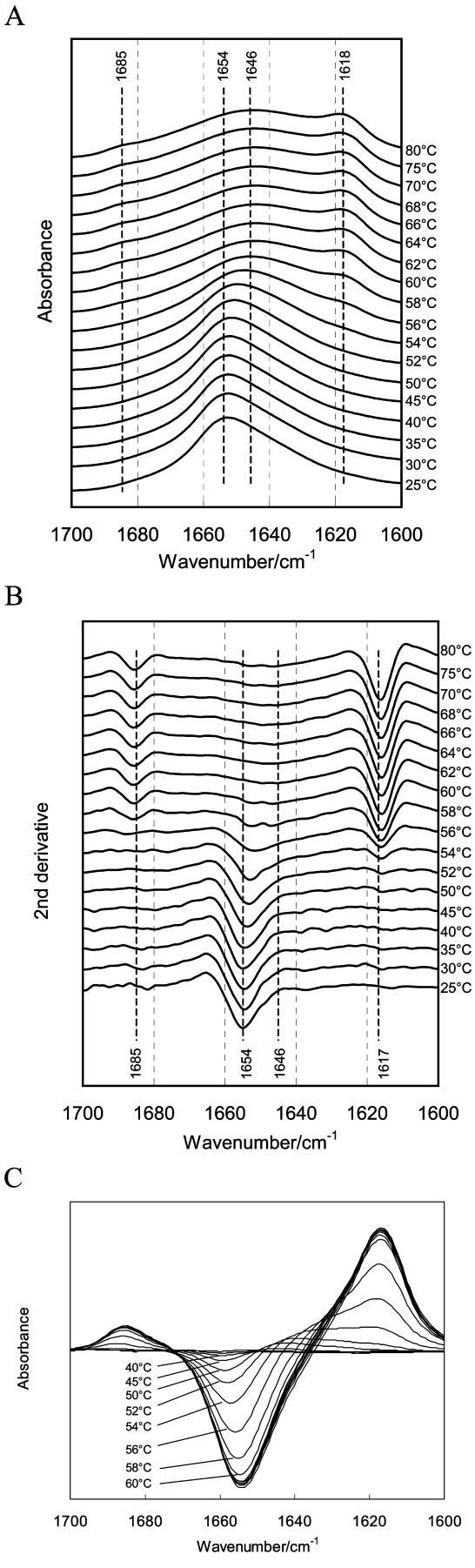

Fig. 2. **IR spectra, second-derivative spectra, and difference spectra of rmethuG-CSF as a function of temperature.** (A) IR spectra of rmethuG-CSF in the amide I′ region as a function of temperature from 25 to 80°C. (B) Second-derivative spectra in the amide I′ region from 25 to 80°C at pD 4.0. (C) The difference spectra calculated by subtraction from spectra obtained at 25°C.

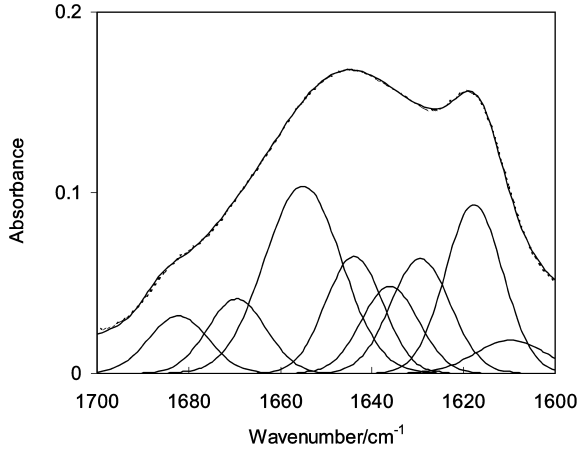

Fig. 3. **The curve-fitting spectrum of rmethuG-CSF at 80°C.** A solid line represents the original spectrum. A dashed line represents the curve-fitted spectrum.

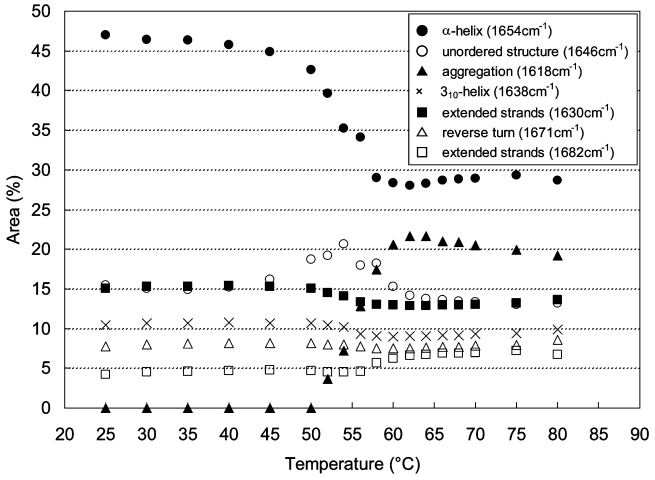

Fig. 4. **The area percentage of each amide I′ band of rmethuG-CSF at pD 4.0 as a function of temperature.** Temperature-dependent variations in the percentages of secondary structure elements calculated from the curve-fitted spectra at pD 4.0.

54°C. It is very likely that the unordered structure is converted to aggregates. Therefore, it seems that α-helix changes into the unordered structure in the range of 40–50°C and above approximately 50°C the unordered structure is converted to intermolecular antiparallel β-sheet.

pD Dependence of Thermal Stability—To investigate the pD dependence of thermal stability, temperature-dependent IR spectra of rmethuG-CSF solutions were also obtained at pD 2.1, 2.5, 3.0, 3.5, 5.0, and 5.5. Figure 5 compares temperature-dependent variations in the percentages of the three major secondary structure elements (α-helix, unordered structure, and intermolecular antiparrallel β-sheet) among pD 2.1, 2.5, 3.0, 4.0, and 5.0.

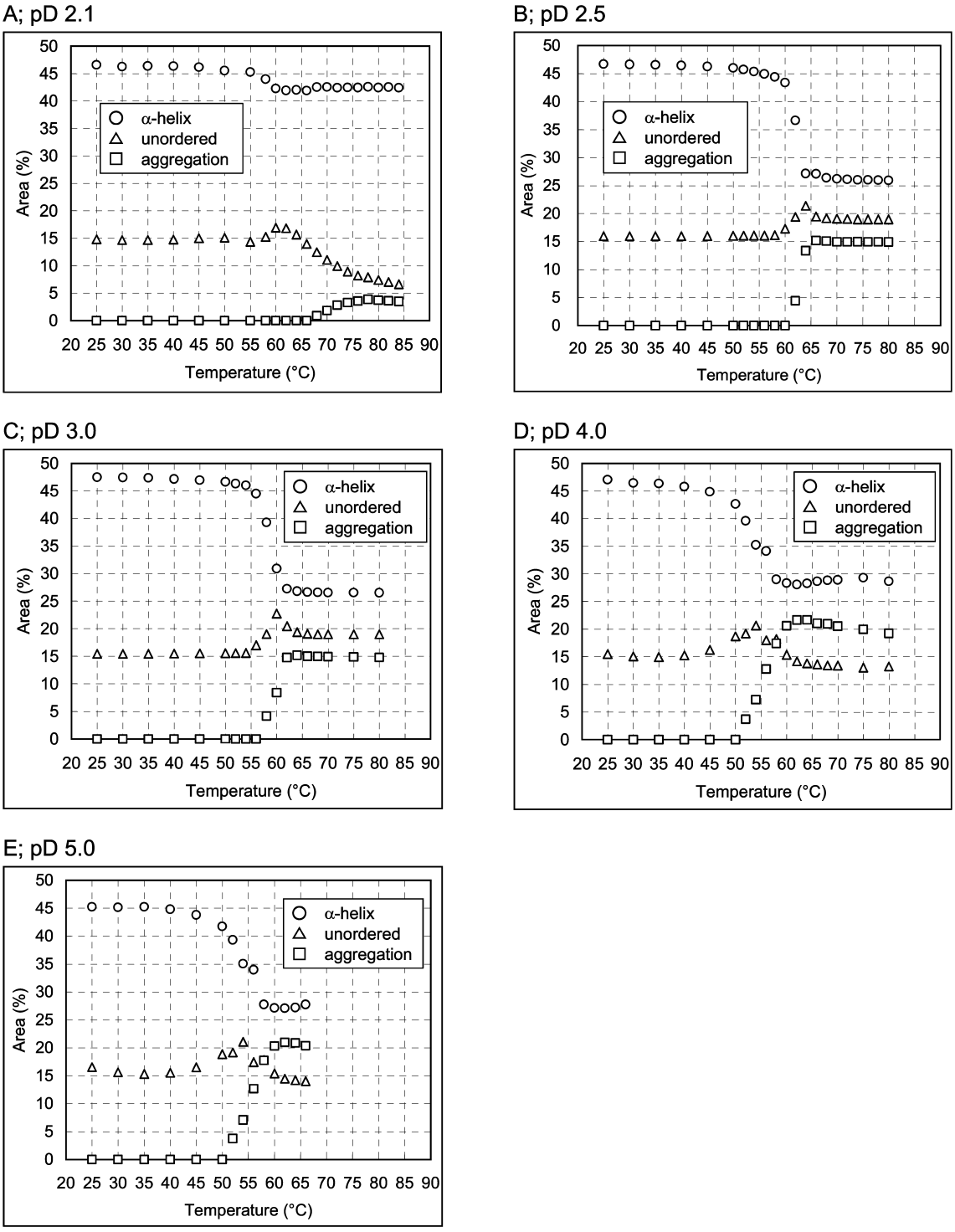

Fig. 5. **The result of curve-fitted analysis of rmethuG-CSF at various pDs as a function of temperature.** Temperature-dependent variations in the percentages of α-helix, unordered structure, and aggregation calculated from the curve-fitted spectra at (A) pD 2.1, (B) pD 2.5, (C) pD 3.0, (D) pD 4.0, and (E) pD 5.0.

The process of thermal unfolding of rmethuG-CSF at pD 5.0 is very similar to that at pD 4.0, as demonstrated in Fig. 5. A significant change of α-helix occurs from around 45°C, and aggregation starts from 50°C (Fig. 5E). However, we could not obtain IR spectra above 66°C at pD 5.0, because a precipitate appeared. As the pD of the rmethuG-CSF solution approaches the isoelectric point, pH 6.1, rmethuG-CSF tends to precipitate. We also observed the precipitation of rmethuG-CSF upon heating at pD 5.5 (data not shown).

It can be seen from Fig. 5 that the thermal stability of rmethuG-CSF increases significantly upon going from pD 4.0 to pD 3.0. At pD 3.0, rmethuG-CSF starts to lose the α-helix structure at 54°C, and aggregation becomes

significant at 58°C. A further increase in the thermal stability is observed at pD 2.5. The percentage of α-helix shows a marked change from 60°C, and aggregation is recognized at 62°C. The results at pD 2.1 are quite interesting in that the stability of rmethuG-CSF at pD 2.1 is lower than that at pD 2.5 (α-helix starts changing at 55°C at pD 2.1 but at 60°C at pD 2.5), but aggregates are formed at higher temperature at pD 2.1 (68°C) than at pD 2.5 (60°C) (Fig. 5, A and B). Another notable point at pD 2.1 is that the α-helix content does not decrease greatly even at high temperatures and the aggregation proceeds only a little at pD 2.1. Based on all the above results as to the pD dependence of thermal stability, we conclude that rmethuG-CSF is the most stable at pD 2.5 in the pD range of 5.5 to 2.1. When pD increases, the thermal stability of rmethuG-CSF decreases, and it is more liable to undergo aggregation.

DISCUSSION

In the present study, we have employed the second derivative, difference spectral, Fourier self-deconvolution and curve-fitting methods to analyze temperature-dependent IR spectra of rmethuG-CSF. The combined use of these methods has allowed us to explore the mechanism of thermal unfolding of rmethuG-CSF and the pD-dependence of its thermal stability. IR spectroscopy can detect protein aggregation as the formation of intermolecular β-structure, and one can also quantify the aggregates by use of curve-fitting. The characteristic bands for the intermolecular β-structure appear at 1,685 and 1,618 cm^{-1}, and increase with temperature. This aggregation pathway is irreversible (data not shown). The curve-fitting analysis in the present study revealed that α-helix changes to the unordered structure in the range of 40–50°C, and above approximately 50°C it changes to both the unordered structure and intermolecular β-structure (aggregation) at pD 4.0. Of note is that the unordered structure is also converted to the intermolecular β-structure (aggregation).

It was recently suggested that protein aggregates are formed through the interaction between partially folded intermediates containing significant native-like structure (*22*). As for rmethuG-CSF, equilibrium intermediates have previously been identified by use of CD and fluorescence spectroscopy (*4*). Nahri *et al.* (*4*) revealed that the GdnHCl-induced denaturation of rmethuG-CSF is a complex event involving a transition from the native structure to a partially denatured structure, equilibrium intermediates, at low GdnHCl concentrations (1 or 2 M), followed by a major conformational change involving the loss of the secondary and tertiary structures at concentrations of above 2 M. It is considered that the above-mentioned unordered structure is a perturbed form of the native structure of rmethuG-CSF and a precursor for aggregation. The appearance of the perturbed structure seems to be the first secondary structure change that occurs along the aggregation pathway. As mentioned above, the process of unfolding of rmethuG-CSF is similar in the pD range of 2.1–5.5, and the unordered structure is produced before the marked structural change. Therefore, it is very likely that this species induces the aggregation irrespective of pD. It is likely that both the unordered structure discussed in the present paper and the partially denatured structure reported by Nahri *et al.* are so-called partially unfolded intermediates (*4*). However, they may be somewhat different from each other because the unordered structure appears irrespective of pH and its structure does not change with pH, while the appearance of the partially denatured structure depends on pH (*4*). We monitored the change in the amount of the unordered structure and its conversion to aggregates quantitatively, but Nahri *et al*. examined the partially denatured structure only qualitatively.

Recently, based on the results of folding kinetics studies, Brems demonstrated that the third helix of rmethuG-CSF is a part of the latest of the helical segments to fold (*23*). Taking account of our results, the first event in the unfolding process may be the step in which the unordered structure is formed after the third helix of rmethuG-CSF collapses. Next, the unordered structures interact each other and the aggregates are formed.

The results in Fig. 5 demonstrate that the secondary structure of rmethuG-CSF is the most stable at pD 2.5. This conclusion is similar but somewhat different from to that of Kolvenbach *et al.* (*6*). They concluded that rmethuG-CSF is the most stable at pH 2, but they obtained IR spectra of rmethuG-CSF only at pH 2, 4, and 7 (*6*). As pD decreases, the thermal stability of the secondary structure, especially that of α-helix, increases and aggregates are formed at higher temperature. At pD 5.0 and 5.5, the precipitation of rmethuG-CSF was observed upon heating around 60–70°C. In the pD range of 5.0–5.5, it seems that the precipitation occurs through the collision between the aggregates produced through thermal denaturation, because the electrostatic repulsion between the protein molecules decreases due to the decreasing net charge of rmethuG-CSF. Recently, Chi *et al.* (*24*) proposed that the stability of rmethuG-CSF should be discussed by taking two steps, conformational stability and colloidal stability, into account. The stability of the conformation (the first step) is concerned with the process of modification from the native state to the expanded transition state, and colloidal stability (the second step) means the process of assembly of the expanded transition states. Based on colloidal stability, Chi *et al.* (*24*) suggested that the increase in the repulsive interactions between protein molecules is effective in reducing the aggregation in rmethuG-CSF solutions at low pH. Therefore, the decrease in the repulsive interaction between denaturatued rmethuG-CSF molecules seems to be the cause of the precipitation at pD 5.0 and 5.5.

The thermal stability of rmethuG-CSF at low pD is somewhat complicated. As for the temperature when the α-helix structure starts changing, the structure of rmethuG-CSF is the most stable at pD 2.5 because at pD 2.5 the conformational stability is very high. However, if one considers the temperature when the aggregation occurs, the structure of rmethuG-CSF is more stable at pD 2.1 than at pD 2.5. At pD 2.1, the electrostatic repulsive interactions become very strong, interfering with the formation of aggregates. In this way the two factors, the conformational stability of the protein molecule and the strength of the repulsive interactions between the protein molecules control the thermal stability of rmethuG-CSF.

CONCLUSION

The present study has provided new insights into the pD-dependence of the thermal stability of rmethuG-CSF through the use of IR spectroscopy. It has been found that the thermal stability is determined by two factors. One is the conformation stability of the protein molecules and the other is the electronic repulsive interaction between the protein molecules. Due to the balance of the two factors, rmethuG-CSF is the most stable at pD 2.5 but the aggregation occurs at lower temperature at pD 2.5 than at pD 2.1. The present study has also revealed that the thermal denaturation process starts with the conversion of α-helix to an unordered structure and then aggregates are formed from the unordered structures. A similar process has been proposed by other research groups, but for the first time we have monitored the process quantitatively and have shown that it occurs irrespective of pH.

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