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Methanol-Induced Tertiary and Secondary Structure Changes of Granulocyte-Colony Stimulating Factor

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We have studied methanol-induced conformational changes in rmethuG-CSF at pH 2.5 by means of circular dichroism (CD), fluorescence and infrared (IR) spectroscopy, and 8-anilino-1-naphthalene sulfonic acid (ANS) binding. Methanol has little effect on the secondary and tertiary structures of rmethuG-CSF when its concentration is in the range of 0 to 20% (v/v). At 30% (v/v) methanol, rmethuG-CSF has ANS binding ability. In the methanol concentration range of 30 to 70% (v/v) the amount of α-helix decreases a little, and the tertiary structure decreases significantly. At methanol concentrations above 70% (v/v), a transition to a more helical state occurs, while there is little change in the tertiary structure, and no ANS binding ability. Thermal denaturation studies involving CD have demonstrated that as the methanol concentration increases the melting temperature and the cooperativity of transition decrease, and the transition covers a much wider range of temperature. It seems that the decreased cooperativity means an increase in the concentration of partially folded intermediate states during the unfolding of rmethuG-CSF.

Key words: G-CSF, conformational changes, intermediate state.

Abbreviations: rmethuG-CSF, recombinant methionyl human granulocyte-colony stimulating factor; ANS, 8-anilino-1-naphthalene sulfonic acid.

It is now well known that protein folding from an unfolded state into a native state takes place through intermediates (*1–5*). The molten globule state is an intermediate state formed under relatively mild denaturating conditions such as low pH, high ionic strength, or moderate concentrations of denaturants (*6–11*). This intermediate is compact with a significantly native-like secondary structure, but it has lost a rigid tertiary structure. It has been suggested that the molten globule state is a common folding intermediate state for small globular proteins. The lifetimes of intermediate states during a folding process are very short and their concentrations are very low under physiological conditions. Therefore, it is difficult to obtain information about the structures of intermediate states under equilibrium conditions due to the low population. Therefore, one must find the conditions that maintain the population of intermediate states for the long time period required to obtain structural information by means of spectroscopic techniques such as circular dichroism and fluorescence. In the present study to obtain information about the process of folding of rmethuG-CSF at low temperature, we investigated partially folded intermediates induced by methanol.

Recently, an organic solvent has often been used to generate partially folded intermediates (*12–21*). Alcohols stabilize the helical structure of a protein but destabilize its tertiary structure. Alcohols, especially trifluoroethanol and methanol, have been used to enhance the intrinsic secondary structure propensity of peptides that fold only weakly in water (*22–24*). It was demonstrated by recent studies that a decrease in the dielectric constant of an alcohol solution can induce the transformation of protein molecules into the denatured intermediate state that possesses all the properties of the molten globule state (*13*).

RmethuG-CSF is a monomeric pharmaceutically relevant globular protein (approximately 19 kDa) belonging to the four-helix bundle family of growth factors (*25*, *26*). It is a cytokine that is widely used to treat neutropenia and marketed by Kirin Brewery Co., Ltd. In the biotechnology and pharmaceutical industries, protein aggregation is often encountered, for example, during the production, isolation, purification, shipping, storage and administration of protein pharmaceuticals (*27*). 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 (*28*). It is important for pharmaceutics to understand the aggregation pathway for rmethuG-CSF. RmethuG-CSF tends to aggregate irreversibly at neutral pH (*29*). An equilibrium intermediate state is implicated in the mechanism of aggregation.

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, and that the secondary and tertiary structures of rmethuG-CSF are stable at low pH (*30–33*). We have investigated the acid stability and thermal unfolding process of rmethuG-CSF by means of IR spectroscopy (*33*).

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Temperature-dependent (25–80°C) IR spectra were measured for rmethuG-CSF in aqueous solution over the pD range of 5.5–2.1, and it was concluded that this protein is most stable at pH 2.5 as to the secondary structure. A stopped-flow kinetics study revealed that there are two intermediates in the process of folding of rmethuG-CSF; its refolding occurs in distinct stages with half of the helix being formed first, followed by the remaining half of the helix including the third helix, and finally the loop between the first and second helices folds (*34*).

In this paper we report CD, fluorescence and IR spectroscopy and ANS binding studies on the conformational changes in rmethuG-CSF in methanol/water mixtures. We have demonstrated the presence of an intermediate state of rmethuG-CSF induced by methanol and characterized its structure. It has been found that in an rmethuG-CSF solution at pH 2.5 containing 30% (v/v) methanol, the native-like secondary structure remains and the tertiary structure is partially lost. Hydrophobic clusters accessible to ANS are present, indicating that its structure differs from the native one.

METHODS

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

Circular Dichroism Spectroscopy—CD measurements were performed on a Jasco J-820 spectropolarimeter equipped with a thermal controller (Jasco CDF-426L). Far-UV CD spectra in the 200–250 nm region and near-UV CD spectra in the 250–340 nm region were measured at 25°C for rmethuG-CSF with protein concentrations of 0.125 to 0.5 mg/ml in 20 mM sodium phosphate buffer, pH 2.5, containing various concentrations of methanol. The cell path lengths used were 1 mm and 10 mm, respectively, for the far-UV and near-UV CD spectra measurements. Under each condition the buffer spectrum was subtracted from the corresponding protein spectrum.

Thermally-induced denaturation of the secondary structure was performed by monitoring the signal change at 222 nm with a protein concentration of 0.5 mg/ml. The sample temperature was increased at 1°C/min. The fraction of the folded protein was calculated by the method previously reported (*35*, *36*), and plotted against temperature to give unfolding curves. To estimate the reversibility a rmethuG-CSF solution was cooled down to 25°C immediately after the temperature of the solution had reached 90°C, and then the protein solution was incubated at 25°C for 15 min. Then, a CD spectrum was measured. The signal intensity at 222 nm regained during the 25°C incubation relative to that of the initial state was measured and then used to determine the degree of the reversibility.

Fluorescence Spectroscopy—Fluorescence spectra were recorded with a spectrofluorometer (HITACHI F-4500) for the concentration of 0.25 mg/ml at 25°C. They were measured for rmethuG-CSF in 20 mM sodium phosphate buffer, pH 2.5, containing various concentrations of methanol. The excitation wavelength was 278 nm and emission was recorded from 280 to 450 nm.

ANS Binding—A stock solution (2 mM) of ANS was prepared by dissolving ANS in water. The ANS stock solution was added to 0.1 mg/ml rmethuG-CSF solutions containing various concentrations of methanol in 20 mM sodium phosphate buffer, pH 2.5. The final ANS concentration was 50 μM. ANS binding to the protein was analyzed by measuring emission fluorescence spectra of ANS. The excitation wavelength was 380 nm, and the emission spectra were obtained in the range of 400 to 600 nm. The spectrum of a free ANS solution obtained under the same conditions was subtracted from the spectrum of each ANS-protein solution.

Infrared Spectroscopy—A rmethuG-CSF solution, 0.5 mg/ml, was changed from a HCl solution (pH 4.0) to 20 mM sodium phosphate buffer, pD 2.5, containing various concentrations of methanol (CH_3OD). IR spectra of the rmethuG-CSF solutions and buffers were obtained using a Thermo 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 100 μm Teflon spacer. For each spectrum, 256 scans were coadded at a spectral resolution of 2 cm^{-1} at 25°C. The sample chamber of the spectrometer was continuously purged with N_2 gas to prevent atmospheric water vapor from interfering with the amide I region. Spectral subtraction was performed using the 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 twelve-point Savitsky-Golay function to reduce the noise. The smoothing was performed with software named SPINA 3.0 (Y. Katsumoto, Kwansei Gakuin University).

RESULTS

CD Spectroscopy—Figure 1A shows far-UV CD spectra of rmethuG-CSF in the 20 mM sodium phosphate buffer with methanol concentrations of 0–90% (v/v) at pH 2.5 and 25°C. The far-UV CD spectrum in the absence of methanol shows minima at 208 and 222 nm. A change in the signal at 222 nm reflects a change in α-helix. Figure 1B plots a change in the ellipticity at 222 nm of rmethuG-CSF versus the methanol concentration at pH 2.5. It can be seen from Fig. 1B that methanol concentrations of 0 to 20% (v/v) have little effect on the secondary structure. It seems that the secondary structure of rmethuG-CSF is almost fully maintained in this concentration region. In the methanol concentration range of 30 to 60% (v/v), the signal at 222 nm decreases a little, indicating that the secondary structure varies slightly. The intensity of the far-UV CD spectra increases when the methanol concentration becomes higher than 70% (v/v), meaning that the secondary structure increases. The methanol-induced transition changes the ratio of the ellipticity at 208 nm and that at 222 nm ($[\theta]_{208}/[\theta]_{222}$). It is 0.97 and 1.15, respectively, for the solutions without methanol and with 80% (v/v) methanol.

Figure 2 depicts near-UV CD spectra of rmethuG-CSF in the presence of 0, 30, and 70% (v/v) methanol at pH 2.5

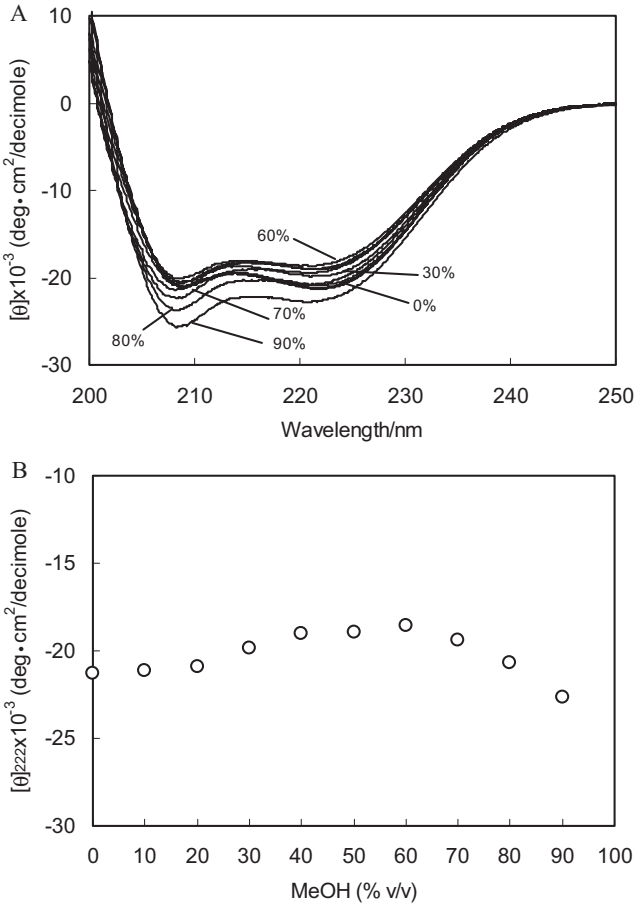

Fig. 1. **Far-UV CD spectra and a change in ellipticity of rmethuG-CSF at pH 2.5 as a function of methanol concentration.** (A) Far-UV CD spectra of rmethuG-CSF in 20 mM sodium phosphate buffer with methanol concentrations of 0–90% (v/v) at pH 2.5 (25°C). (B) A change in ellipticity of rmethuG-CSF in the 20 mM sodium phosphate buffer with methanol concentrations of 0–90% (v/v) at 222 nm. The protein concentration was 0.5 mg/ml.

and 25°C. The ellipticity of rmethuG-CSF in this region is very weak. The near-UV CD spectrum of rmethuG-CSF in the absence of methanol exhibits a broad maximum around 260 nm, and minima at 283 and 291 nm, indicating that the aromatic residues are in a specific tertiary structure. It should be noted that the intensity around 260 nm decreases and that at 291 nm increases in the near UV-CD spectrum of rmethuG-CSF with the methanol concentration of 30% (v/v). This suggests that the tertiary structure of rmethuG-CSF in the 20 mM sodium phosphate buffer with 30% (v/v) methanol differs from that of the native structure. The 70% (v/v) methanol protein solution no longer exhibits signal intensity at 283 and 291 nm, and the intensity around 260 nm decreases further, indicating that the tertiary structure of rmethuG-CSF is lost in the 70% (v/v) methanol solution.

Fluorescence Spectroscopy—RmethuG-CSF contains two tryptophan and three tyrosine residues, and therefore, one can study conformational changes of this protein by means of fluorescence spectroscopy. Figure 3A shows fluorescence spectra of rmethuG-CSF in the 20 mM sodium phosphate buffer with methanol concentrations of 0–90%

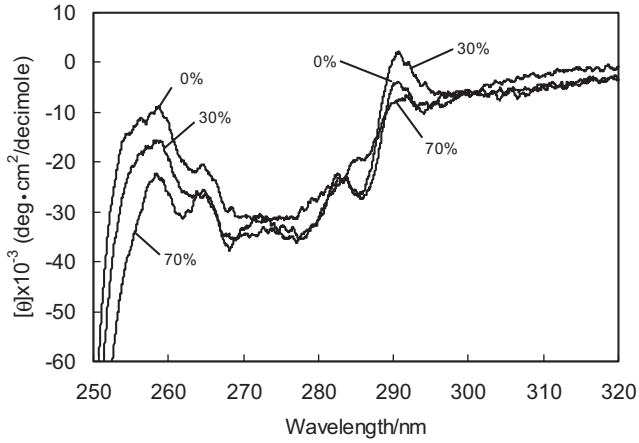

Fig. 2. **Near-UV CD spectra of rmethuG-CSF in 20 mM sodium phosphate buffer at pH 2.5 as a function of methanol concentration.** Near-UV CD spectra of rmethuG-CSF in 20 mM sodium phosphate buffer with methanol concentrations of 0, 30, and 70% (v/v) at pH 2.5 (25°C). The protein concentration was 0.5 mg/ml.

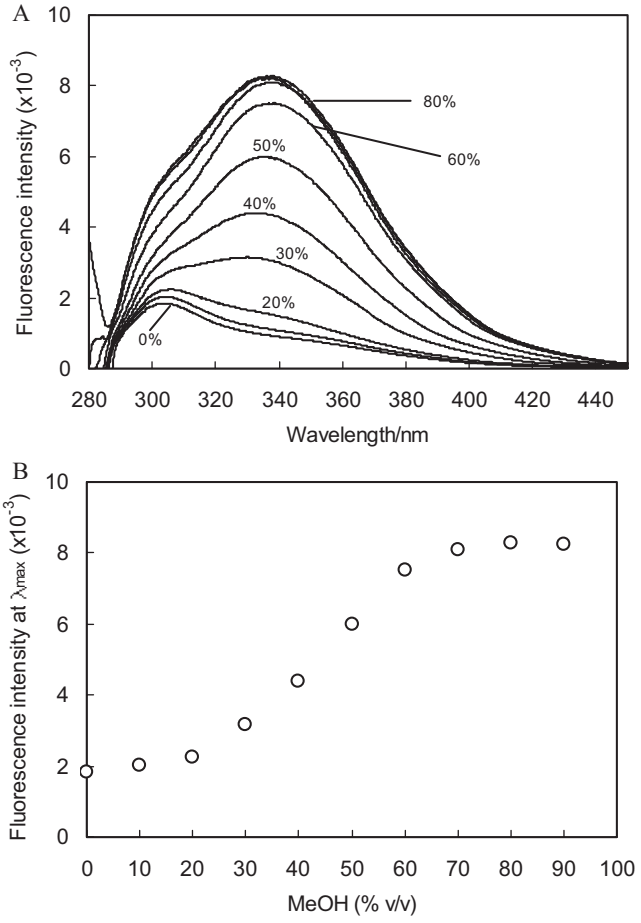

Fig. 3. **Fluorescence spectra and intensity of fluorescence of rmethuG-CSF in 20 mM sodium phosphate buffer at pH 2.5 as a function of methanol concentration.** (A) Fluorescence spectra of rmethuG-CSF in 20 mM sodium phosphate buffer with different concentrations of methanol at pH 2.5 (25°C). (B) Dependence of fluorescence intensity on the methanol concentration. The protein concentration was 0.25 mg/ml.

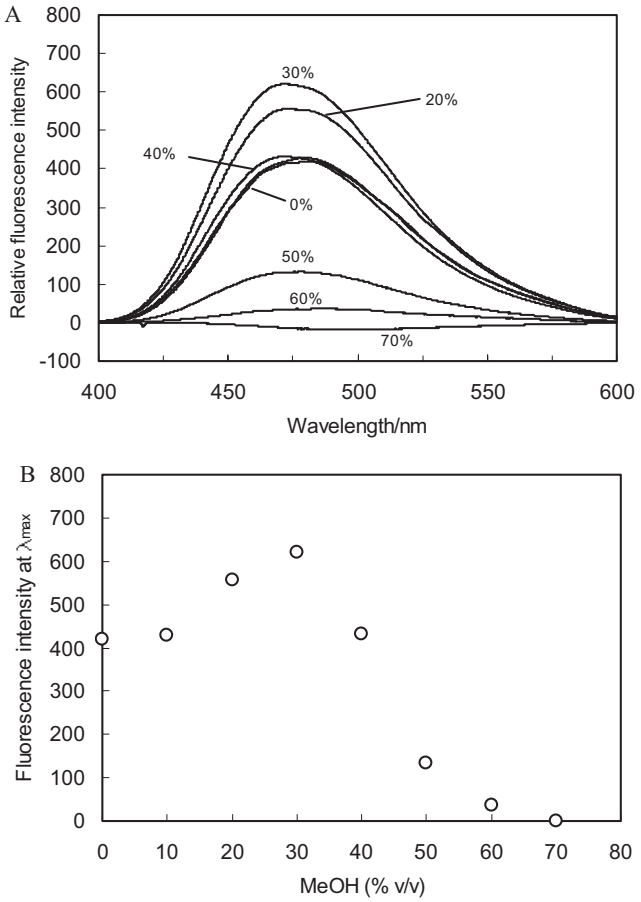

Fig. 4. **Results of ANS binding experiments.** (A) Emission spectra of ANS-rmethuG-CSF solutions as a function of methanol concentration at pH 2.5. (B) Dependence of fluorescence intensity at λ_{max} on the methanol concentration. The protein concentration was 0.1 mg/ml. The final concentration of ANS was 50 μM.

(v/v) at pH 2.5 and 25°C. Figure 3B illustrates the methanol concentration dependence of the fluorescence intensity at the fluorescence maximum. Narhi *et al.* assigned a peak at 303 nm to tyrosine residues and a peak at 345 nm to tryptophan residues (*30*). It is known that although a free tryptophan shows fluorescence maximum at around 350 nm, the embedding of a tryptophan in the nonpolar core of a globular protein results in a characteristic blue shift of its fluorescence maximum. The fluorescence spectrum of rmethuG-CSF in the absence of methanol is characterized by a peak at 304 nm and a shoulder in the region of 340–350 nm. There is little change in the intensity, spectral shape, and wavelength of the maximum emission in the methanol concentration range of 0 to 20% (v/v). Therefore, it seems that tryptophans are buried in the hydrophobic core, and consequently the native tertiary structure of rmethuG-CSF is maintained. With 30 to 70% (v/v) methanol there is a significant increase in the fluorescence intensity and a shift in the wavelength of maximum emission. This indicates that tryptophans are exposed to the solvent, leading to the unfolding of the tertiary structure of rmethuG-CSF. Above 70% (v/v) methanol, the intensity and wavelength of maximum emission become constant.

ANS Binding—The fluorescent dye, ANS, binds to a hydrophobic cluster of proteins provided that they are accessible to the dye. The binding of ANS to proteins is frequently utilized to demonstrate changes in the accessibility of hydrophobic clusters (*37*). It is known that the binding induces an increase in the fluorescence intensity of ANS and a blue shift of the fluorescence maximum (*38*, *39*). Figure 4A shows the emission spectra of mixtures of rmethuG-CSF and ANS as a function of the methanol concentration. Figure 4B plots the fluorescence intensity at λ_{max} versus the methanol concentration. The fluorescence intensity increases as the methanol concentration increases from 0 to 30% (v/v) (Fig. 4B). Above 30% (v/v) methanol, the fluorescence intensity decreases significantly. ANS does not bind to the protein in the solutions containing methanol at more than 70% (v/v). The intensity maximum shifts from 484 nm to 471 nm as the methanol concentration increases in the range of 0 to 30% (v/v). There is a red shift of the maximum for the solutions with methanol concentrations of more than 30% (v/v). This finding shows that the changes in the fluorescence intensity and wavelength maximum reflect the conformational transition in the presence of methanol. The rmethuG-CSF solution with 30% (v/v) methanol is characterized by the presence of hydrophobic clusters that are accessible to ANS. The solution with 70% (v/v) methanol loses the ability to bind ANS, which means the collapse of hydrophobic clusters.

Thermal Denaturation—Thermal denaturation of rmethuG-CSF with different concentrations of methanol was monitored as the CD spectra change at 222 nm. Figure 5A shows the thermally-induced denaturation curves of this protein for 0 to 60% (v/v) methanol. Figure 5B plots the melting temperature as a function of the methanol concentration. The observations in Fig. 5 reveal that with an increase in the methanol concentration the transition moves to a lower temperature. The melting temperatures without and with 20% (v/v) methanol at pH 2.5 are 66.0 and 56.4°C, respectively. Of particular note is that the cooperativity of the transition decreases. The transition covers a much wider range of temperature. At above 30% (v/v) methanol, there is no observation of a transition point. It seems that the decreased cooperativity means populated intermediate states upon thermal unfolding. Figures 1A, 3A, and 5A demonstrate that rmethuG-CSF retains its native structure at 25°C with up to 20% (v/v) methanol, but heating of the protein with 5, 10, and 20% (v/v) methanol has a significant influence on the melting of the native structure. The thermal-induced transition of rmethuG-CSF was found to be reversible in the temperature range of 25 to 90°C, when the scan rate was 1°C/min.

pH Dependence of Methanol-Induced Far-UV CD Changes—We also measured far-UV CD spectra changes of rmethuG-CSF in the presence of 0–90% (v/v) methanol at pH 4.0 as well as at pH 2.5, at 25°C (Fig. 6). The far-UV CD spectrum of the rmethuG-CSF solution without methanol shows minima at 208 and 222 nm, as in the case of pH 2.5. It is clear on comparison of Fig. 6 with Fig. 1A that there is a difference between the spectra of the 50–70% (v/v) methanol solutions at pH 4.0 and pH 2.5. The far-UV CD spectra of rmethuG-CSF solutions with 50–60% (v/v) methanol do not show minima at 208 and 222 nm,

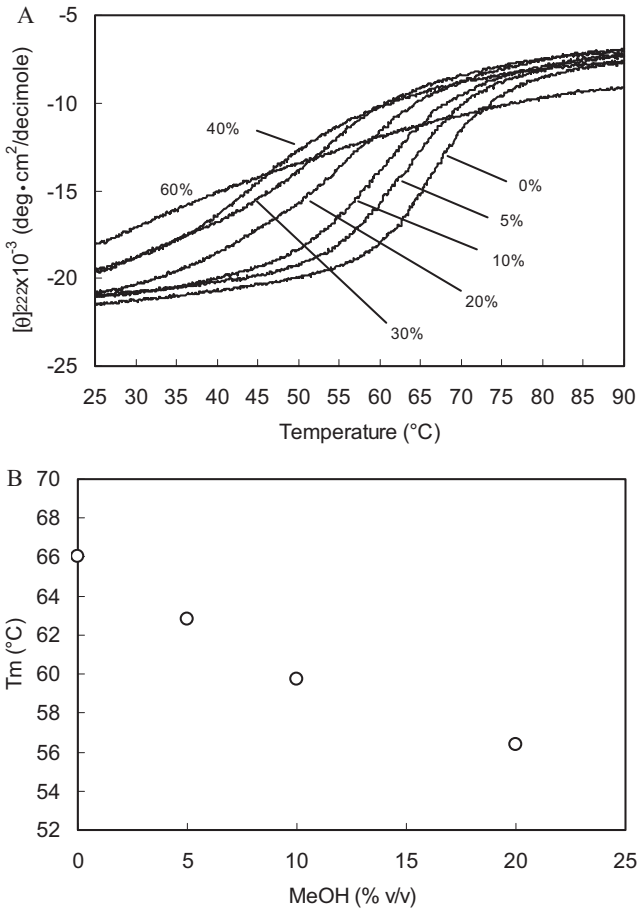

Fig. 5. **Thermal denaturation of rmethuG-CSF in 20 mM sodium phosphate buffer with different methanol concentrations at pH 2.5.** (A) Thermal unfolding curves of rmethuG-CSF as a function of methanol concentration, monitored as the ellipticity change at 222 nm. (B) Dependence of the melting temperature (T_m) on the methanol concentration.

indicative of α-helix, which means that the protein no longer maintains the secondary structure in this methanol concentration range.

We have investigated the acid stability and thermal unfolding process of rmethuG-CSF by means of IR spectroscopy (*33*). It was indicated that the thermal stability of rmethuG-CSF at pD 2.5 was higher than that at pD 4.0. Aggregates are formed at higher temperature at pD 2.5 compared to at pD 4.0, because of the decrease in the repulsive interaction between unordered structures of rmethuG-CSF molecules at pD 4.0. Taking this result into account, it is very likely that the secondary structure is maintained after disruption of the tertiary structure through an increase in methanol at pH 2.5, but the secondary structure is not maintained at pH 4.0 because of the decrease in the repulsive interaction between rmethuG-CSF molecules that is caused by the loss of the tertiary structure.

Detection of Aggregates by IR Spectroscopy—As mentioned above, it is likely that the secondary structure of rmethuG-CSF in solutions with 50–70% (v/v) methanol at pH 4.0 is not maintained. Figure 7A shows IR spectra for the amide I′ region (1,700–1,600 cm^{-1} region) of

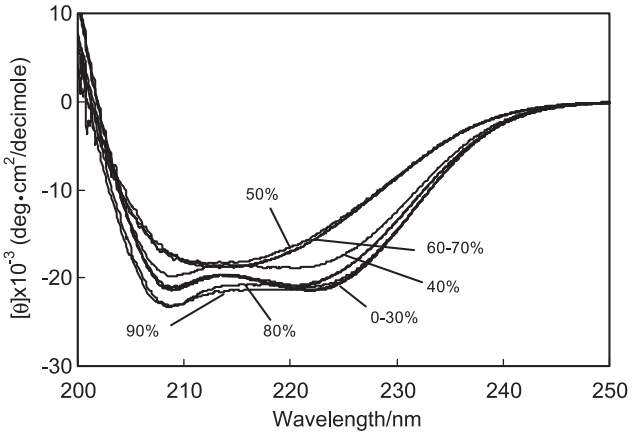

Fig. 6. **Far-UV CD spectra of rmethuG-CSF in 20 mM sodium phosphate buffer at pH 4.0 as a function of methanol concentration.** Far-UV CD spectra of rmethuG-CSF in 20 mM sodium phosphate buffer with methanol concentrations of 0–90% (v/v) at pH 4.0 (25°C).

rmethuG-CSF solutions containing 0 and 60% (v/v) methanol at pD 4.0 (25°C). It should be noted that the intensities of the bands at 1688 and 1618 cm^{-1} are increased in the spectrum of the 60% (v/v) methanol protein solution. The appearance of these bands indicates that intermolecular antiparallel β-sheet is formed on protein aggregation (*40*). IR spectroscopy can be used to determine the amount of aggregates as formation of intermolecular β-sheet. Figure 7B plots the intensity at 1,618 cm^{-1} at pD 4.0 and 2.5 versus the methanol concentration. The peak intensity increases as the methanol concentration increases from 20 to 60% (v/v) at pD 4.0. On the other hand, there is little aggregation below 40% (v/v) methanol at pD 2.5. This protein undergoes aggregation a little at pD 2.5 with 60% (v/v) methanol. However, the amount of aggregates with 60% (v/v) methanol at pD 2.5 is a much lower than that at pD 4.0. Above 60% (v/v) methanol, the intensity decreases. This finding indicates that the amount of aggregates decreases with high methanol concentrations. The signal intensity for an 80% (v/v) methanol solution is almost the same as that without methanol, indicating that the rmethuG-CSF solution with 80% (v/v) methanol exhibits little aggregation.

DISCUSSION

RmethuG-CSF has never been shown to undergo a methanol-induced transition. For the first time, we have studied methanol-induced conformational changes in rmethuG-CSF at pH 2.5 by means of CD, fluorescence, IR and ANS binding experiments. Table 1 summarizes the results of CD, florescence, and IR spectroscopy and ANS binding experiments. According to the results of the CD and fluorescence measurements, methanol has little effect on the secondary and tertiary structures when the methanol concentration is in the range of 0 to 20% (v/v). At 30% (v/v) methanol, the tertiary structure surrounding the Trp moiety is partially destroyed. RmethuG-CSF contains two Trp residues. One Trp at residue 118 is located within the third helix, while the other Trp at residue 58 is in the long loop between the first and

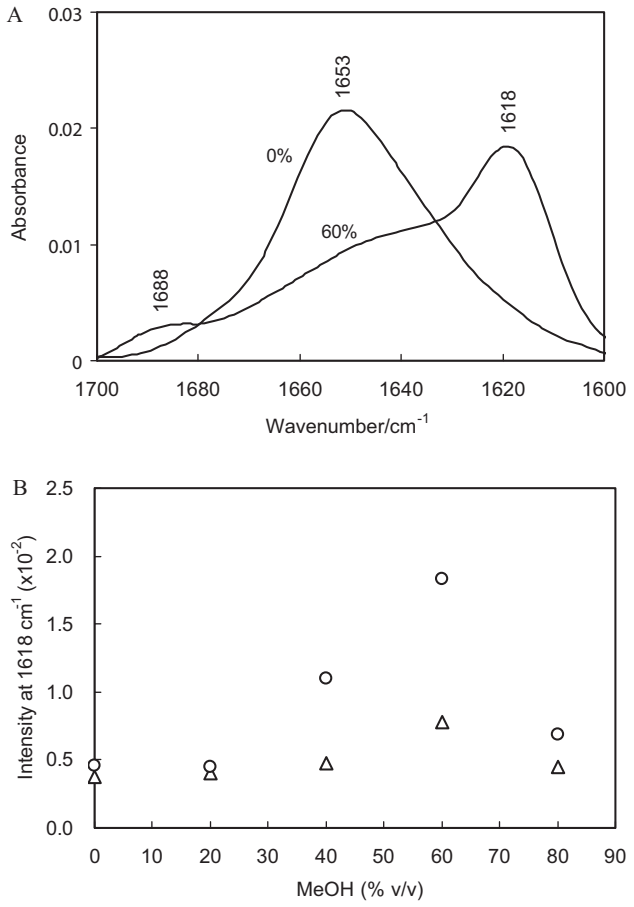

Fig. 7. **IR spectra and a change in peak intensity at 1618 cm^{-1} of rmethuG-CSF in 20 mM sodium phosphate buffer as a function of methanol concentration.** (A) IR spectra for the amide I′ region of rmethuG-CSF in 20 mM sodium phosphate buffer with methanol concentrations of 0 and 60% (v/v) at pD 4.0 (25°C). (B) A change in peak intensity at 1,618 cm^{-1} in the 20 mM sodium phosphate buffer with methanol concentrations of 0–80% (v/v) at pD 4.0 and 2.5 (25°C). Circles, pD 4.0; triangles, pD 2.5.

second helices. It seems that the conformational change of the Trp58 moiety takes place, followed by that of the Trp118 moiety, because Trp58 is more solvent-exposed than Trp118, and the conformational change of this long loop is the latest event observed in the folding of rmethuG-CSF (*25*, *34*, *41*). The state of rmethuG-CSF with 30% (v/v) methanol is characterized by the presence of hydrophobic clusters that are accessible to ANS. Therefore, we conclude from the ANS experiment, and the near-UV CD and fluorescence measurements that the structure of the long loop between the first and second helices collapses, and the tertiary structure is partially lost for the 30% (v/v) methanol solution. The secondary structure is mostly maintained at this methanol concentration. The conformations surrounding Trp58 and Trp118 are lost with 70% (v/v) methanol. This state has no ANS binding ability, indicative of the loss of the hydrophobic core. When the methanol concentration becomes more than 70% (v/v), a transition to a more helical state occurs through an increase in the secondary structure, while there is no change in the tertiary structure.

Table 1. **Methanol-induced conformational changes in rmethuG-CSF observed in CD, florescence, and IR spectroscopy and ANS binding experiments.**

Methanol concentration	Conformational changes
0–20% (v/v)	Little effect on the secondary and tertiary structures
30% (v/v)	The native-like secondary structure remains, the tertiary structure is partially lost, hydrophobic clusters accessible to ANS are present, and there is no clear transition point on thermal denaturation
30–70% (v/v)	The secondary structure decreases a little and there is a significant change in the tertiary structure, and aggregates are formed
Above 70% (v/v)	Transition to a more helical state and collapse of the hydrophobic clusters

There have been many reports on protein conformational changes induced by high alcohol concentrations (*15*, *17*, *42*, *43*). According to these reports, in general, proteins in solutions with high alcohol concentrations show more pronounced far-UV CD spectra compared with that of the native structure, suggesting a substantial increase in their helicity. At high methanol concentrations, hydrogen bonds between a protein and a solvent become weaker, resulting in the formation of stronger hydrogen bonds in the protein main chain. Therefore, it seems that the proteins have a more pronounced helical structure.

We compared the change in secondary structure induced by methanol between pD 2.5 and pD 4.0. IR spectra demonstrated that the amount of aggregates formed as a result of conformational changes of rmethuG-CSF at pD 4.0 is much higher than that at pD 2.5. In our pervious 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 the aggregates are formed through an intermolecular interaction between the unordered structures (*33*). We have also shown that the addition of a salt such as NaCl masks the intermolecular repulsive electrostatic interaction between the unordered structures, resulting in an increase in the aggregates (*44*). RmethuG-CSF solutions are positively charged at acid pD (the isoelectric point is pH 6.1). The electrostatic repulsive interactions at pD 2.5 become stronger than those at pD 4.0, interfering with the formation of aggregates. When the methanol concentration becomes more than 60% (v/v), the amount of aggregates significantly decreases. There is little aggregation in 80% (v/v) methanol solutions at pD 4.0 and 2.5. As mentioned above, a transition to a more helical state takes place at high alcohol concentrations through formation of intramolecular hydrogen bonds in the protein main chain. Therefore, it seems that the formation of a more helical state through an intramolecular interaction is preferable for the formation of aggregates through an intermolecular interaction in rmethuG-CSF with 80% (v/v) methanol at both pDs.

The present study has shown that the tertiary and secondary structure changes do not occur simultaneously in the methanol-induced transition for rmethuG-CSF, which

suggests the existence of at least one intermediate state. It is likely that an intermediate state is formed in a solution containing 30% (v/v) methanol.

Recently, based on the results of folding kinetics studies on rmethuG-CSF, Brems (*34*) concluded that there are two intermediates in the folding mechanism: $U \leftrightarrow I_1 \leftrightarrow I_2 \leftrightarrow N$; where U, I_1, I_2, and N represent the unfolded protein, intermediate state 1, intermediate state 2, and the native state. The I_2 state shows mostly native-like fluorescence for the Trp 118 moiety and mostly denatured-like fluorescence for the Trp 58 moiety, and it has native-like helicity. He also concluded that the conformation change of the long loop between the first and second helices where Trp58 is located is the latest event observed in the folding of rmethuG-CSF (*34*). The secondary structure is maintained but the tertiary structure surrounding Trp 58 is lost for a 30% (v/v) methanol solution, and therefore it seems that the conformation of rmethuG-CSF with 30% (v/v) methanol is similar to that of the I_2 intermediate state.

We have also investigated the effect of methanol on the thermal denaturation of rmethuG-CSF. It is important to note that the decreased cooperativity is evidence of the stabilization of partially intermediate states induced by methanol. One can measure the level of the cooperativity as the temperature ratio for Fd = 0.1 and Fd = 0.9 (Fd is the fraction of the unfolded state in the process of thermal unfolding) (*20*). The breadths of the transitions extend from about 15°C for 0% (v/v) methanol to about 30°C for 20% (v/v) methanol. This ratio change increases as the methanol concentration increases from 0 to 20% (v/v). It is known that the cooperativity in a conformational change from a molten globule state to an unfolded state decreases upon thermal unfolding in some proteins (*45*, *46*). We have found that there is no clear transition point when the methanol concentration is more than 30% (v/v).

CONCLUSION

The present study has provided new insights into the methanol-induced conformational changes of rmethuG-CSF through the use of CD, fluorescence and IR spectroscopy, and ANS binding experiments. We have demonstrated that the conformational transitions induced by methanol can be divided into several groups depending on the methanol concentration range. In the 20 mM sodium phosphate buffer, pH 2.5, containing 30% (v/v) methanol, the native-like secondary structure remains, the tertiary structure is partially lost, hydrophobic clusters accessible for ANS are present, and there is no a clear transition point on thermal denaturation. It seems that the conformation surrounding Trp58 collapses and that surrounding Trp118 is maintained in a 30% (v/v) methanol solution. The structure of rmethuG-CSF in the 20 mM sodium phosphate buffer, pH 2.5, with the methanol concentration of 30% (v/v) is an intermediate state that differs from the native structure.

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