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Effects of Cyclodextrin on Thermal Stability of Cytochrome *c*

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To clarify the effects of cavity size and substituent of cyclodextrin (CD) on the stability and the conformation of cytochrome *c*, thermal denaturations of cytochrome *c* were measured in several CD solutions at various concentrations by differential scanning calorimetry and circular dichroism. The secondary structure and the tertiary structure of cytochrome *c* were unfolded cooperatively in the thermal denaturation. The midpoint temperature was decreasing by addition of CD indicating that cytochrome *c* was destabilized by CD. The destabilization effect of CD was dependent on the interior diameter of CD, β-CD > α-CD > γ-CD, and also the substituent, mono-acetyl > methyl > 2-hydroxyl propyl. The bound number and binding constant of methyl-β-CD to the unfolded state of cytochrome *c* was estimated to be 5.0 ± 1.0 and 10.3 ± 2.9 M^{-1}, respectively, indicating the stronger interaction with protein and the limited binding site on protein as compared with usual denaturants. These results indicated that CD destabilizes the folded state of cytochrome *c* by stabilizing the unfolded state due to inclusion of hydrophobic part of unfolded state into interior of CDs. CD will be used as a new arbitrary denaturant for protein with the combination of arbitrary cavity size and substitution of CD.

Keywords: Cyclodextrin, cytochrome *c*, stability, DSC, circular dichroism

1. Introduction

The protein structure plays an important role for an own function and physical properties as a stability and flexibility. It is very important to know the mechanism of protein folding for the protein engineering and drug development [1-5]. The protein structure is maintained by a significant small stability as a compensated result of hydrophobic interaction, hydrogen bond, electrostatic interaction, structural entropy, and other enthalpic and entropic factors. When the globular protein is folded state in water, the hydrophobic amino acids of the protein preferentially gather protein inside to avoid contact with the water and the hydrophilic amino acids are preferentially exposed to the water. However, when the folded structure is disordered by external perturbation like as temperature, pressure, pH, denaturant, and salt [6-8], the hydrophobic amino acids will be exposed to the solvent. The conformational stability of protein is expressed as the difference of Gibbs energy between the folded and unfolded states of the protein. To clarify the mechanism of protein structure, therefore, it is very important to know the conformational and thermodynamic properties of the two states.

Cyclodextrin (CD) is a family of cyclic oligosaccharides composed of several glucoses. The shape of CD is toroid and the interior of CD is hydrophobic compared to the exterior and environment. The typical CD is α-CD, β-CD, and γ-CD that consist of six, seven, and eight glucopyranose units, respectively. The CD is able to include appropriate hydrophobic molecule like an alkyl group in the interior [9-11]. It is well known that contribution to the inclusion mechanism of CD is not only an enthalpic effect such as the hydrophobic interactions between CD and guest molecule but also an entropic effect such as the change in amount of hydration before and after the inclusion. In the view of this inclusion ability, CD can be an appropriate additive to perturb the stability of protein because CD would be able to influence the hydrophobic interactions of the protein. It is expected that CD molecule can include exposed hydrophobic part of protein especially at unfolded state by the inclusion ability of CD, which would decrease the Gibbs energy of unfolded state leading to destabilization of the folded state. It is difficult to estimate the respective enthalpic and entropic contribution in the CD binding to the protein because the obtained change in enthalpy and entropy will be included in the significant structural change of the protein. However, it is important to clarify the influence of CD on the protein structure and stability for revealing the role of hydrophobic interaction on the protein and the molecular recognition mechanism. Moreover, a substitution of CD is an effective approach for modifying the inclusion ability, therefore, to clarify the effects of the substituent can lead to make an arbitrary denaturant for protein by the arbitrary substitution of CD.

In this study, to clarify the effects of cavity size (α-CD, β-CD, γ-CD) and substitution on the stability and the conformation of cytochrome *c* which is a typical globular protein, thermal denaturations of cytochrome *c* were measured in several CD solutions at various concentrations by differential scanning calorimetry and circular dichroism. The substituent of CD used in this study was methyl group (CH_3-, Me-β-CD), 2-Hydroxypropyl group ($HOCH_3CHCH_2$-, HP-β-CD), and mono-acetyl group (CH_3CO-, Ac-β-CD) at hydrogen of hydroxyl group of CD, respectively. Each substituent has a characteristic property as follows; the Me group is hydrophobic and small conformation, the HP group has large hydrophobic part and small hydrophilic part, the Ac group has hydrophilic part which can make a hydrogen bond exists in secondary structure of protein.

2. Material and methods

2-1. Materials

Bovine heart cytochrome *c* was purchased from Sigma. Water was deionized with Millipore Simpli Lab. The acetic acid and sodium acetate 3 hydrate were special grade and purchased from Kishida Kagaku. The α-CD was purchased from Hayashibara Kogyo. The Ac-β-CD and Me-β-CD were purchased from Junsei Chemical. The γ-CD and HP-β-CD were purchased from Tokyo Kasei Kogyo. The substitution rate of Me-β-CD, HP-β-CD, and Ac-β-CD is 1.6-1.9, 0.6-0.9, and 0.8-1.2 per one glucose, respectively. All CDs are white powder with special grade. Unmodified β-CD is not adequate for this measurement because the solubility of β-CD is very low, 1.6 g·dL^{-1} at 25 °C. Therefore, in this study, Me-β-CD substituted by methyl group as a small substituent was used instead of β-CD.

2-2. Sample Preparation

A buffer solution was 50 mM acetate adjusting pH 4.04. A stock solution of cytochrome *c* was prepared with dialysis against the buffer solution. The concentration of cytochrome *c* was determined by absorbance measurement. A stock solution of each CD was prepared by dissolving an adequate gram of CD powder with the buffer solution. Sample solutions and solvents were prepared by mixing the two stock solutions of cytochrome *c* and CD with the buffer solution on adequate mixing ratio to get into the final concentration of cytochrome *c* is 0.6 mg·cm^{-3}, CD is 0 to 20 w/w%, respectively. Each sample solvent was prepared according to the same procedure without mixing the stock solution of cytochrome *c*. The final concentrations of cytochrome *c* were determined using dilution factors obtained from gravimetric and density data for the solvents and solutions. Cytochrome *c* was positively charged in this buffer pH4.04 because the isoelectric point of cytochrome *c* is near pH 10.

2-3. Differential scanning calorimetry (DSC)

Thermal stability of cytochrome *c* was monitored with a high sensitivity differential scanning calorimeter, MicroCal MCS, at a scanning rate of 1 K·min^{-1}. The protein concentrations of solution were kept close to 0.6 mg·cm^{-3}. All sample solutions and reference solvents were degassed at least 3 min before DSC measurements. A solvent blank was measured before and after each set of the sample experiments. Thermal denaturation temperature, T_m, calorimetric enthalpy change, ΔH, and half-value width of the denaturation, $T_{1/2}$, were calculated with a program attached to this instrument. The ΔT_m is obtained from T_m-$T_{m,0}$ reflecting the destabilization effects, where $T_{m,0}$ is T_m in absent of CD. The $T_{1/2}$ reflects the cooperatively for thermal denaturation; the smaller $T_{1/2}$ means higher cooperatively.

2-4. Circular dichroism

Circular dichroisms of cytochrome *c* in CD solutions were taken on a J-720 (JASCO) with temperature control system PTC-348WI at a scanning rate of 1 K·min^{-1}. The cell length was 1.0 mm and the protein concentrations of solution were kept close to 0.6 mg·cm^{-3}. The circular dichroism spectra of cytochrome *c* were measured before and after heating to check the reversibility.

3. Results and Discussion

3-1. Effects of internal diameter of CD

CD can include hydrophobic guest molecule in the cavity and the cavity size is significantly related to the molecular recognition of the guest molecule [12,13]. For revealing the influence of CD on the hydrophobic interaction that contributes to the protein structure, it is important to clarify the influence of the cavity size on the protein stability. Fig. 1 shows the DSC thermograms of cytochrome *c* in about 8 w/w% CD solutions, α-CD, β-CD (Me-β-CD), and γ-CD, whose internal diameter is different each other. The same percent of mass for each CDs means the molarity of glucose composes CD is almost equal, but the molarity of CD is different.

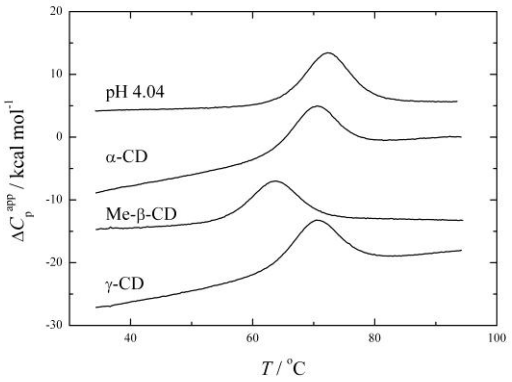

Fig. 1 DSC thermograms of cytochrome *c* in buffer (50 mM acetate, pH 4.04) with α-, Me-β-, and γ-CD at about 8 w/w%. The concentration of cytochrome *c* is 0.6 mg·cm^{-3} corresponding to about 50 μM.

Obviously, a significant endothermic peak was observed in all CD solutions, indicating that cytochrome *c* unfolds by heating. All transitions were almost reversible at least over 95 %. Thermodynamic parameters, T_m, ΔH, and $T_{1/2}$, which were determined with an assumption of two states model, are listed in Table 1.

Table 1 Thermodynamic parameters of cytochrome *c* in various CD solutions.

CD	conc. w/w%	T_m K	ΔT_m K	ΔH kJ/mol	$T_{1/2}$ K
	0	345.40	0.00	324.0	8.62
α	8	343.08	-2.32	294.2	8.46
γ	8	343.60	-1.80	335.1	8.98
Me-β	5	340.32	-5.08	260.6	8.94
	10	336.78	-8.62	242.0	9.03
	15	334.43	-10.97	235.1	9.10
	20	331.34	-14.06	229.5	9.19
Hp-β	20	339.39	-6.01	253.9	8.83
Ac-β	20	323.48	-21.92	130.6	9.49

It should be emphasized that the endothermic peak was shifted to lower temperature by all CD adding, although the shift was small in α-CD and γ-CD systems, suggesting that cytochrome *c* was destabilizing by CDs. It is known that the addition of glucose composes CD stabilizes the folded protein [14]. The destabilization effect of CD opposite to glucose suggests that the presence of the inclusion ability with ring-shaped structure of CD greatly take part in the destabilization effect. There is a large interest in studying how such a difference in destabilization effect was occurred. The destabilization mechanism of CD would be explained as follows. The CD molecule includes an exposed hydrophobic part in the unfolded protein. The inclusion would stabilize the unfolded state due to decrease in Gibbs energy of the unfolded state, because accessible surface area of the hydrophobic part would decrease by the inclusion of CD. Consequently, the binding of CD to the unfolded state would lead to relatively destabilize the folded state. The difference of

destabilization effect between α-CD, β-CD (Me-β-CD), and γ-CD would be caused by the difference of inclusion ability derived from the cavity size. Fig. 2 shows a correlation of ΔT_m of cytochrome *c* estimated at same molarity of CD (82 mM, corresponding to 8 w/w% of α-CD) to the internal diameter of CD.

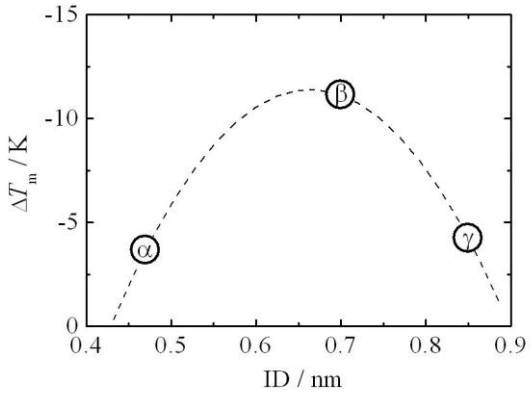

Fig. 2 Dependence of ΔT_m of cytochrome *c* on the inner diameter of CDs.

There was a maximum in the correlation, though the data is a little, significantly indicating that a moderate internal diameter is necessary to have large destabilization effect. Aachmann *et al.* determined the dissociation constant for the CD interaction with unfolded Chymotrypsin Inhibitor 2 by stopped flow fluorescence measurements at 288 K to be 26.9 ± 3.3 mM for α-CD, 18.9 ± 0.7 mM for β-CD, and 47.7 ± 9.0 mM for γ-CD [15]. The small value for β-CD indicates that β-CD can bind with the unfolded state more strongly than other CDs, showing consistency with the difference of destabilization effect obtained in our DSC results. These results indicate that CD molecules relatively destabilized folded state of cytochrome *c* by stabilizing the unfolded state due to inclusion of hydrophobic side chains of amino acids into CDs and the destabilization effects were dependent on the cavity size of CD.

3-2. Effects of substitution of CD

The β-CD with an appropriate internal diameter shows the largest destabilization effect for globular protein. But the solubility of normal β-CD is remarkably low compared with other CDs because of high stability of crystal structure. Therefore the modified β-CDs were well used for study and industry [16, 17]. because the solubility is improved by the substitution at hydrogen of hydroxyl group due to a destabilization of the crystal structure. The modification of CD is expected as an effective approach to give CD new functions because the modification influence on the inclusion ability.

DSC thermograms of cytochrome *c* in 20 w/w% Me-β-CD, HP-β-CD, and Ac-β-CD solutions are shown in Fig. 3. All transitions were almost reversible at least over 95 %. Obviously, a significant endothermic peak was shifted to lower temperature by addition of CDs. The obtained thermodynamic parameters, T_m, ΔH, and $T_{1/2}$, are listed in Table 1. It is noted that ΔT_m was significantly different each other, suggesting that each CD had a different destabilization effect. The order of higher destabilization effect was Ac-β-CD > Me-β-CD > HP-β-CD which is same order as previous results of lysozyme [18]. The fact that the ΔT_m changes by the substitution suggests the substituent directly interacts with protein and/or influences the inclusion ability probably due to the substituent properties such as hydrophobicity and steric exclusion. As shown in Table 1, in all systems, the ΔH decreases with decreasing in the T_m. Especially in Ac-β-CD system, the ΔH significantly reduced from 324 kJ·mol^{-1} (0 w/w%) to 130 kJ·mol^{-1} (20 w/w%). What is the reason why Ac-β-CD has such higher destabilization effect than others? It is thought that the destabilization effects of Ac-β-CD would have two systems. One is that CD includes hydrophobic part of unfolded state with hydrophobic cavity of CD leading to stabilize the unfolded state preferentially. The other is that the C=O in acetyl group would alternatively break the hydrogen bond between N-H and C=O of amino acids which is essential bond to make a secondary structure of protein. Contrary, the HP-β-CD has the smallest destabilization effect in modified β-CD systems probably because the steric exclusion of HP group inhibits the binding of CD to protein. The fact that the each modified CDs has different destabilization effect shows a possibility to make an arbitrary denaturant for protein by the arbitrary substitution of CD.

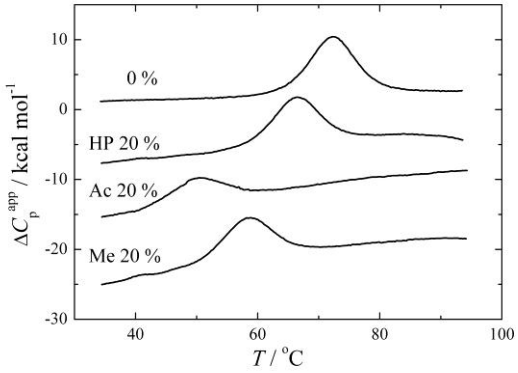

Fig. 3 DSC thermograms of cytochrome *c* in buffer (50 mM acetate, pH 4.04) with each modified β-CD at 20 w/w%.

There is a large interest in whether the destabilization effect of CD is dependent on kind of proteins. Interestingly, the ΔT_m of cytochrome *c* which was -21.9 K (Ac-β-CD), -14.1 K (Me-β-CD), and -6.0 K (HP-β-CD) at 20 w/w% of CD, was larger than that of lysozyme, -17.4 K (Ac-β-CD), -8.2 K (Me-β-CD), and -5.5 K (HP-β-CD), respectively [18]. The significant difference of ΔT_m for proteins would be attributed from own stability of protein at native state which is easily influenced by the environment like pH and solvent. More systematical analysis for several proteins should be necessary to understand the detailed relationship between stability, CD, and temperature.

3-3. Concentration dependence of Me-β-CD

DSC thermograms of cytochrome *c* in several Me-β-CD solutions are shown in Fig. 4. Obviously, a significant endothermic peak was shifted to lower temperature with increasing concentration of CD. The obtained thermodynamic parameters are listed in Table 1 and plotted against the concentration in Fig. 5. T_m and ΔH was decreasing with an increase in the CD concentration indicating that cytochrome *c* was destabilized by addition of CD. On the other hand, the $T_{1/2}$ was increased with an increase in the CD concentrations. Interestingly, a significant positive correlation, r=0.894, is shown in the relationship between T_m and $T_{1/2}$ (figure not shown). This result suggests that CD would stabilize the denaturation process by covering the exposed hydrophobic part of cytochrome *c*.

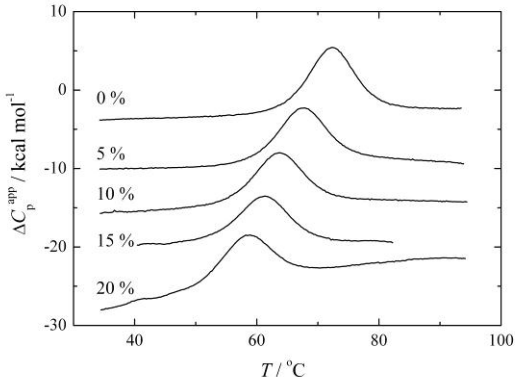

Fig. 4 DSC thermograms of cytochrome *c* in buffer (50 mM acetate, pH 4.04) with Me-β-CD (0-20 w/w%).

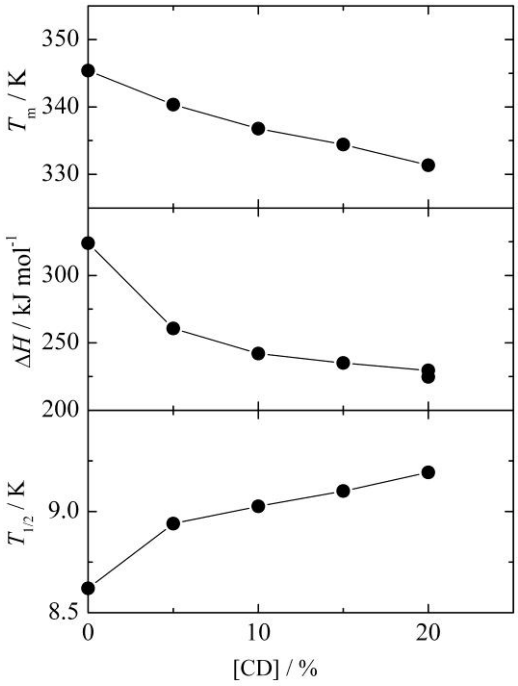

Fig. 5 CD concentration dependence of T_m, ΔH, and $T_{1/2}$ for thermal denaturation of cytochrome *c* in Me-β-CD solutions.

With an assumption that the destabilization effect is induced only by CD binding, the effect was determined as using a number of bound CD molecule and a binding constant to unfolded state according to eq. 1 [19].

$$\frac{\Delta T_m}{T_m} = -\left(\frac{nRT_{m,0}}{\Delta H_0}\right)\ln(1+[\mathrm{CD}]K) \quad (1)$$

where ΔH_0 is enthalpy change for denaturation of protein in absent CD, *n* is the difference number of bound CD to between folded and unfolded protein, *K* is binding constant of CD to the protein, [CD] is molarity of CD, and *R* is gas constant. The *K* and *n* were determined by non-linier least squares method in Fig. 6 and Table 2 together with the previous results of lysozyme [18].

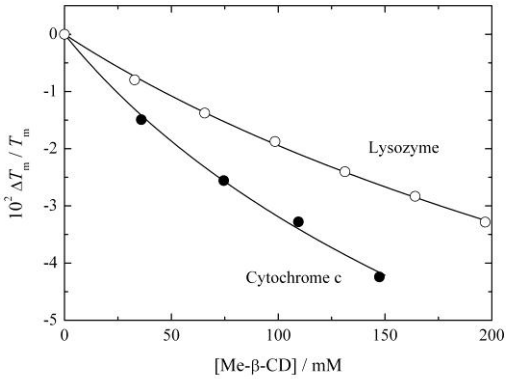

Fig. 6 Plots of $\Delta T_m/T_m$ of cytochrome *c* and lysozyme against Me-β-CD concentration. The solid lines were obtained by non-linear least squared method with eq. (1).

Table 2 The bound number and binding constant of Me-β-CD to the unfolded state of cytochrome *c* and lysozyme.

Protein	$T_{m,0}$ /K	K/M^{-1}	n
Cytochrome *c*	345.4	10.3 ± 2.9	5.0 ± 1.0
Lysozyme	350.4	5.5 ± 0.8	6.7 ± 0.7

The obtained *n* and *K* of CD for cytochrome *c* is 5.0 ± 1.0 and 10.3 ± 2.9 M^{-1}, respectively. Pace *et al*. estimated the binding constant of usual denaturants for globular proteins to be 0.1 M^{-1} and 0.6 M^{-1} of urea and guanidine hydrochloride (Gdn-HCl) at 300 K, respectively [20]. The fact that the *K* of Me-β-CD is significantly larger than that of usual denaturants indicates that CD molecule can more strongly bind to unfolded state than usual denaturants. However, the estimated midpoint concentration of CD, C_m, for the denaturation at 300 K from equation 1 was about 2.7 M which means same denaturant efficiency as urea, C_m = 3.0 M, less than Gdn-HCl, C_m = 1.25 M [21]. There is a large interest that CD showed same denaturant efficiency as urea despite having the significant larger *K*. As shown in Fig. 6, the slope becomes small with an increase in the CD concentration. These results suggest that CD has a strong destabilization effect in the low concentration as reflected to large ΔT_m and has a weak unfolding effect in the high concentration as reflected to high C_m. The probable reason why CD has such inconsistent effects is that the binding site on protein is limited compared with the usual denaturants because CD is far steric molecule than urea and Gdn-HCl and CD influences especially not non-hydrophobic interactions like hydrogen bonds and electrostatic interaction of the protein but hydrophobic interaction due to the inclusion ability.

It is noted that, as shown in Table 2, the binding constant *K* for cytochrome *c* (10.3 ± 2.9 M^{-1}) is larger than lysozyme (5.5 ± 0.8 M^{-1}) though *n* is not different so much. Aachmann *et al*. determined the dissociation constant of CD with unfolded chymotrypsin inhibitor 2 whose value is 18.9 ± 0.7 mM for β-CD, corresponding to 52.9 ± 2.0 M^{-1} of *K* at 288 K [15]. The difference in *K* by the difference in a kind of protein would be primarily caused by own protein stability as mentioned above. More systematical analysis for several proteins should be necessary to understand the detailed relationship between stability, CD, and temperature.

3-4. Heat capacity change

The ΔC_P, which is the difference in heat capacity of protein between at folded state and unfolded state, can reflect the difference in the hydration. The value of ΔC_P is usually positive which means that heat capacity for the unfolded state is larger than that for the folded state because unfolding induces increase in hydration due to increase in the accessible surface area of protein [22, 23]. The ΔC_P can be determined from one DSC curve in principle because DSC measures the apparent heat capacity of protein. Unfortunately, it was difficult to determine a precise ΔC_P from one DSC curve in this study because of experimental error. Under these circumstances, in this study, the ΔC_P were determined thermodynamically from the temperature dependence of ΔH according to eq. 2.

$$\left(\frac{\partial \Delta H}{\partial T}\right)_P = \Delta C_P \qquad (2)$$

The obtained ΔC_P was 9.7 ± 2.0, 4.4 ± 0.6, and 2.7 ± 0.5 ($kJ \cdot mol^{-1} \cdot K^{-1}$) at 5, 10, and 20 w/w% of Me-β-CD, respectively. It is noted that the ΔC_P were decreasing with increase in the CD concentration. The decreasing ΔC_P suggests that CD molecule preferentially bound to unfolded state and the binding induced dehydration of the unfolded state.

3-5. Secondary structure change of cytochrome *c*

Thermodynamic parameters obtained with DSC for thermal denaturation of protein is macroscopic information including all interaction changes between folded and unfolded state. Therefore, DSC is an effective measurement to reveal the overall conformational changes, but it is quite not easy to specify where and how of the protein unfolds. Circular dichroism of protein is an effective measurement to reveal the change in secondary structure of protein in solution. Especially the value at 222 nm can reflect the content of α-helix of protein[24]. The conformational information obtained by circular dichroism can support the thermodynamic parameters by DSC to reveal the conformational changes.

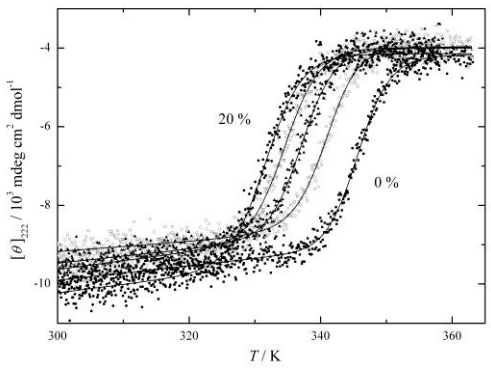

Fig. 7 Temperature dependences of $[\theta]_{222}$ of cytochrome *c* in Me-β-CD solutions at 0, 5, 10, 15, and 20 w/w%.

Fig. 7 shows temperature dependences of molar ellipticity of cytochrome *c* at 222 nm, $[\theta]_{222}$, in Me-β-CD solutions at various concentrations. In all cases, the intensity of $[\theta]_{222}$ was decreased with increasing temperature, indicating that the content of α-helix of cytochrome *c* was decreasing and cytochrome *c* was unfolding by temperature. It is noted that all transitions were reversible and the transition curve was shifted to lower temperature with an increase in the Me-β-CD concentration. These results indicated that the secondary structure of cytochrome *c* was destabilizing by Me-β-CD as shown in DSC results. The T_m obtained from the transition with an assumption of two states model was 345.8 ± 0.1, 340.8 ± 0.1, 337.2 ± 0.2, 334.7 ± 0.1, and 331.8 ± 0.1 K at 0, 5, 10, 15, and 20 w/w% of Me-β-CD concentration, respectively, which were almost same to the results in DSC measurements. These results suggest that the secondary structure and the tertiary structure of cytochrome *c* were unfolded cooperatively in the thermal denaturation.

4. Conclusion

As shown in this study, CD destabilizes the folded state of cytochrome *c* by stabilizing the unfolded state due to inclusion of hydrophobic part of unfolded state into the hydrophobic cavity of CDs. CD molecule can strongly bind to the unfolded state but the binding site is less than usual denaturants. The destabilization effects depend on the cavity size, substitution, and concentration of CD. These results indicated that CD will be used as a new arbitrary denaturant for protein with the combination of arbitrary cavity size and substitution of CD.

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