Effects of Hydrophilic Cyclodextrins on Aggregation of Recombinant Human Growth Hormone

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Received June 16, 2004; accepted August 17, 2004

Purpose. To evaluate the effect of hydrophilic cyclodextrins (CyDs) on the aggregation induced by different stresses and on the oxidation and deamidation of recombinant human growth hormone (rhGH).
Methods. The aggregation of rhGH was induced by three denaturing techniques including chemical (4.5 M guanidine hydrochloride), thermal (differential scanning calorimetry), and interfacial denaturation (vortex agitation). The aggregates were characterized and quantified by UV spectrophotometry and size exclusion chromatography. The effects of hydrophilic CyDs on deamidation and oxidation rates of rhGH were studied by HPLC method.
Results. In both thermally and chemically induced aggregations, branched β-CyDs significantly inhibited the aggregation of rhGH compared with the other α- and γ-CyDs. This can explain that the β-CyD cavity with branched sugar moieties may be relatively preferable in preventing the aggregation of rhGH. In contrast, 2-hydroxypropyl β-CyD with surface activity was found to be effective in reducing the aggregation induced by interfacial denaturation compared with those of branched β-CyDs. On the other hand, these hydrophilic CyDs showed no noticeable inhibitory effect on the oxidation and deamidation rates of rhGH. The results suggested that CyDs interact preferably with exposed hydrophobic side chains rather than aliphatic side chains of rhGH, resulting in the inhibition of aggregation but not the oxidation and deamidation rates.
Conclusions. The different inhibitory effect of CyDs is dependent not only on the structure and property of CyD itself but also the nature of the denaturing stimulus. The current results suggested that hydrophilic β-CyDs can effectively inhibit the aggregation of rhGH. Thus, hydrophilic β-CyDs may be potentially useful excipients for parenteral preparation of rhGH.

KEY WORDS: aggregation; deamidation; hydrophilic cyclodextrin; hydrophobic interaction; oxidation; recombinant human growth hormone.

INTRODUCTION

Protein aggregation is an important phenomenon in biotechnology and human diseases (1–3). In biotechnology, the starting material for protein purification processes is often inclusion bodies, which are non-native, insoluble aggregates typically formed during production of foreign proteins in *Escherichia coli*. In Alzheimer and Parkinson diseases, protein folding, aggregation and disposition are responsible for their pathology.

Protein denaturation and aggregation can be induced by a variety of stress conditions. Principle factors contributing to protein denaturation in solution include exposure to extremes in temperature and pH, introduction of a high air/water or solid/water interface, and addition of organic or chaotropic solvents (4,5). Aggregation is often irreversible and may involve covalent bonds such as disulfide bridges or noncovalent forces such as hydrophobic interactions. Protein denaturation and aggregation are the major pathway leading to loss of valuable protein products in the biopharmaceutical industries. Thus, various excipients have been used for the solubilization or stabilization of proteins (6).

Cyclodextrins (CyDs) are the cyclic, nonreducing oligosaccharides produced by the action of cyclomaltodextrin glucanotransferase (CGTase) on α-glucans. α-CyD, β-CyD, and γ-CyD, which are respectively composed of 6, 7, and 8 D-glucosyl residues linked by α-1,4-linkages, are well-known and used in many fields especially in the pharmaceutical field (7,8). However, natural CyDs, particularly β-CyD, have relatively low solubility in water, and therefore various kinds of CyD derivatives with improved aqueous solubility have recently been prepared. In previous studies (9–11), we reported that some hydrophilic CyDs interact with accessible hydrophobic side chains of amino acids in peptides and proteins such as buserelin, insulin, and α-chymotrypsin. In this study, we investigated the effect of hydrophilic CyDs on the aggregation of recombinant human growth hormone (rhGH) under various stress conditions. Further, the effect of CyDs on oxidation and deamidation rates of rhGH was studied.

MATERIALS AND METHODS

Materials

β-CyD and hydroxypropylated CyDs (HP-CyDs) were supplied from Nihon Shokuhin Kako Co. (Tokyo, Japan). Various branched β-CyDs were obtained from Bio Research Corporation of Yokohama (Yokohama, Japan). rhGH (2.96 IU/mg) was kindly donated by Novo Nordisk Pharma Co. (Tokyo, Japan). Guanidine hydrochloride (GuHCl) was purchased from Nacalai Tesque Co. (Kyoto, Japan). Refolding CA kit was obtained from Takara (Tokyo, Japan). All other materials and solvents were of analytical grade. Deionized, double-distilled water was used throughout the study.

Refolding Aggregation Experiment

rhGH (4.73 mg/ml) was denatured by incubating in 4.5 M GuHCl in 10 mM phosphate buffer (pH 5, 50 μl) at 25°C for 12 h, at which the protein exists as molten globule-like intermediates. Refolding of rhGH was then initiated by adding pH 5.0 phosphate buffer (950 μl) containing various additives into the molten globule solution, and the solution was equilibrated at 25°C for 4 h. The samples were then analyzed for insoluble aggregates by monitoring the turbidity spectrophotometerically at 350 nm. The insoluble aggregates were removed by filtration through a 0.22 μm Millipore filter. The rhGH in the filtrate was analyzed to determine the monomeric and dimeric rhGH, using size exclusion HPLC (Tosoh TSK gel column G3000w, 7.5 mm i.d. × 600 mm) with a flow rate of 1.0 ml/min of 10 mM phosphate buffer (pH 7.4) at room temperature. The refolding CA kit was used according to the protocol from the supplier.

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Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) measurements were made using an MC-2 differential microcalorimeter (MicroCal, Inc., Northampton, MA, USA) using the MicroCal Origin for data acquisition and analysis. All solutions were degassed under vacuum before being loaded into the calorimeter cells. The DSC scans were performed at a rate of 1°C/min in the temperature range from 10°C to 105°C under an excessive N_2 pressure at about 220 kPa. The calorimetric enthalpies of thermal folding of rhGH (1.18–4.73 mg/ml) in the absence or presence of CyDs in 10 mM phosphate buffer (pH 8.1, $I = 0.05$) were obtained from the DSC recordings of excess heat capacity changes. After subtracting the reference buffer data, the raw data obtained in the form of heat capacity as a function of measuring temperature were converted to excess molar heat capacity using the scan rate and the rhGH concentration.

Interfacial Denaturation

rhGH was dissolved in the absence or presence of CyDs in phosphate buffer (pH 8.1, $I = 0.05$) in a 12 × 75 mm borosilicate glass tube at a concentration of 1.18 mg/ml (1.0 ml), and the solutions were vigorously vortexed for 60 s to expand the air/water surface. Following the treatment, the samples were allowed to stand for 30 min at room temperature, after which amounts of the resulting insoluble aggregates were measured by changes in the turbidity, and those of the monomeric and dimeric rhGH in the filtrate were determined by HPLC, as described above.

Stability Constant and Thermodynamic Parameter

The stability constants of complexes of rhGH (0.23 mg/ml) with CyDs in phosphate buffer (pH 5.0, $I = 0.05$) containing 4.5 M GuHCl at 25°C were determined by the analysis of changes in tryptophan fluorescence intensity at λ_{em} 340 nm (λ_{ex} 277 nm), that is, the apparent stability constant ($K_{1:1}$) was obtained from the following Scott equation (12), assuming the 1:1 guest/host interaction:

$$ab/d = 1/(K_{1:1}\,\varepsilon_c) + b/\varepsilon_c$$

where a is the total concentration of rhGH, b is the total concentration of CyDs, ε_c is the difference in fluorescence intensity for free and complexed rhGH, and d is the change in tryptophan fluorescence intensity of rhGH by the addition of CyDs. The stability constants of the complexes were determined by circular dichroism spectroscopy (290 nm) at different temperatures (15°C, 25°C, 30°C, 40°C and 50°C), and thermodynamic parameters (enthalpy and entropy) were calculated from van't Hoff plots of the stability constants of the complexes *vs.* reciprocal absolute temperatures.

Oxidation

The oxidation of rhGH was carried out as described previously (13). Briefly, rhGH (9.45 mg/ml) was dissolved in 1.0 ml water. An aliquot of 7.5 μl of 3% hydrogen peroxide was added into 250 μl of rhGH solution. The reaction was carried out at 25°C and terminated by the addition of catalase. The oxidation of rhGH was assessed by reversed phase chromatography. A PLRS-S column (Polymer Laboratories) was operated at 40°C on a HPLC with a binary gradient consisting of 25 mM ammonium acetate (pH 7.5, solvent A) and *n*-propanol (solvent B). The gradient consisted of 34-39% solvent B in 100 min at a flow rate of 1.0 ml/min and the detection was 280 nm.

Deamidation

rhGH (1.0 mg/ml) was reconstituted in 0.05 M ammonium hydrogen carbonate and adjusted to pH 9.0 with ammonium hydroxide. The sample solution was incubated at 37°C in the absence and presence of CyDs (50.0 mM). The amount of deamidated rhGH was quantitated by anion exchange chromatography (14). A TSK DEAE-5PW column (75 × 7.5 mm i.d.) (Toso-Haas) was used with a binary solvent system consisting of 0.05 M ammonium acetate (solvent A) and 0.5 M ammonium hydrogen carbonate in solvent A (solvent B). The gradient elution was from 0% to 55% solvent B in 34 min at a flow rate of 1.0 ml/min, and the detection was 280 nm.

RESULTS AND DISCUSSION

Refolding Aggregation

Bam *et al.* (15) have found that at 4.5 M GuHCl, a molten globule intermediate of rhGH is stabilized and results in significant aggregations upon refolding. In this study, the effects of CyDs and various additives on the aggregation of rhGH upon refolding were studied by monitoring the turbidity due to the formation of the aggregates and by measuring the monomeric and dimeric rhGH in the filtrate solution using size exclusion chromatography (SEC).

When rhGH was refolded in a 4.5 M GuHCl solution, insoluble aggregates were formed, as indicated by an apparent increase in absorbance of the solution at 350 nm. At this wavelength, there is no absorption by the protein and CyDs. Figure 1 shows the effects of various CyDs on the aggregation of rhGH during the refolding from molten globule-like intermediates in 4.5 M GuHCl solution, determined from changes in the turbidity. Parent α-CyD and all other α-CyD derivatives inhibited the aggregation only about 30% of that of rhGH alone, and there was little difference in the inhibition between these α-CyD, that is, 6-*O*-α-maltosyl-α-CyD (G2-α-CyD), 6-*O*-α-(4-*O*-α-D-glucuronyl)-D-glucosyl-α-CyD (GUG-α-CyD), 6-*O*-α-D-glucosyl- α-CyD (G1-α-CyD), 6-*O*-α-D-mannosyl-α-CyD (Man-α-CyD), 6-*O*-α-D-galactosyl-α-CyD (Gal-α-CyD), 2-hydroxypropyl-α-CyD (HP-α-CyD), and 2,6-di-*O*-methyl α-CyD (DM-α-CyD). The structure of branched sugar units of the α-CyD derivatives, that is, number of glucose (G1 and G2), ionization (G2 and GUG), or orientation of hydroxyl group in branched sugar moiety (G1, Man and Gal), did not significantly affect the inhibition. On the other hand, branched β-CyDs and DM-β-CyD markedly inhibited the aggregation (about 90% inhibition). The structure of the branched sugar units did not affect the inhibition also in the β-CyD derivatives, although the effect of G1-β-CyD was slightly smaller than those of other β-CyDs. The inhibition effect of HP-β-CyD and sulfobutyl ether β-CyD (SBE-β-CyD) was significantly smaller than those of the branched β-CyDs, the effect of SBE-β-CyD being larger than that of HP-β-CyD. Unfortunately, the experiment for the parent β-CyD system at 50.0 mM was difficult because of its

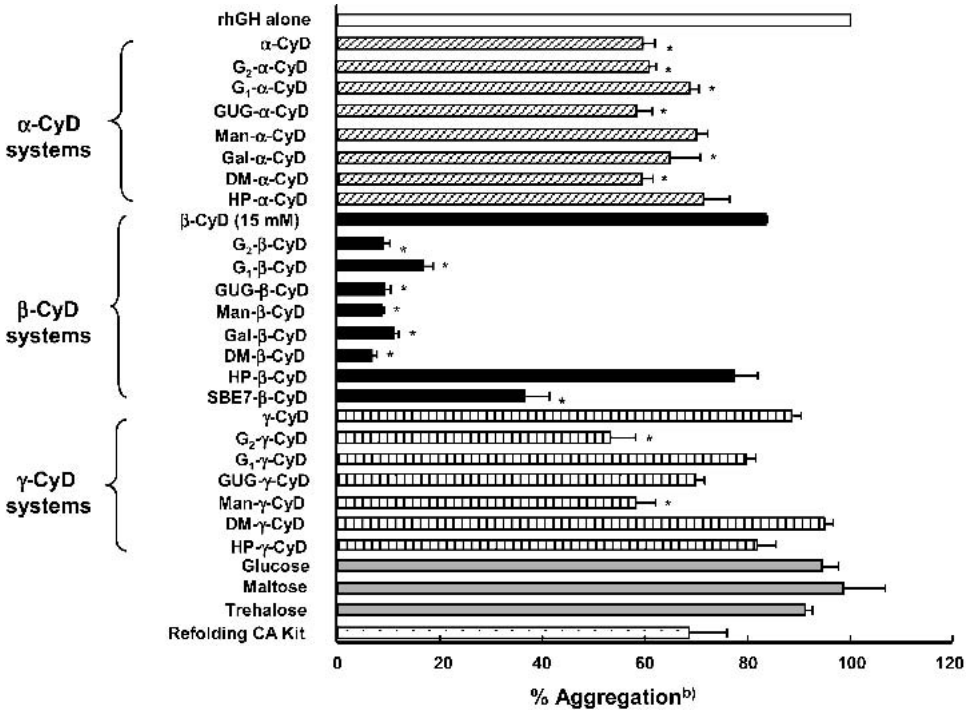

Fig. 1. Effects of additives (50 mM) on aggregation of rhGH during refolding from molten globule-like intermediates[a)] in phosphate buffer (pH 5.0) containing 4.5 M GuHCl at 25°C. (Note a: The initial concentration of rhGH was 4.73 mg/ml. Note b: Determined by measuring the absorbance at 350 nm.) Each value represents the mean ± SE of 3–4 experiments. *$p < 0.05$ vs. rhGH alone.

limited solubility in water (about 15 mM at 25°C). In the case of γ-CyDs, a statistically significant inhibition was observed only for G2-γ-CyD and Man-γ-CyD, whereas the inhibition of other γ-CyDs was statistically insignificant, although GUG-γ-CyD had a weak inhibiting effect. These results suggested that the structure of the branched sugar moieties in the γ-CyDs affects the aggregation of rhGH molecules. The non-cyclic sugars such as glucose, maltose and trehalose showed no reduction of the aggregation. These results indicate that the β-CyD cavity preferably interacts with amino acid residues of rhGH molecule that are primarily responsible for the aggregation (4,16) through the formation of a partial inclusion complex, and further the branched sugar moieties are involved in the interaction with the protein, such as the insertion of the branched moiety into small pockets in the protein. It is very interesting to note that the ability of the branched β-CyDs to inhibit the aggregation of rhGH is much greater than that of the commercially available refolding kit where molten globular states of the protein are stabilized by a surfactant, followed by extraction (inclusion) of the surfactant by a macrocyclic sugar to refold it to a native state. Further, handling our refolding method described here is easier than that of the commercial method.

The insoluble aggregates were separated and removed from soluble aggregates by filtering through a 0.22 μm Millipore filter, and the filtrate was analyzed by size exclusion chromatography (SEC) to determine the amounts of monomer and dimer of rhGH molecules. Figure 2 shows the percentages of the monomer, dimer and soluble aggregates of rhGH in the filtrates after the refolding. The amount of soluble aggregates (higher order oligomers) was estimated by subtracting the amount of the monomer and the dimer from the total rhGH amount. In the absence of the additives, rhGH existed as monomer and dimer in approximately 50% and 8%, respectively, and the remaining rhGH (42%) as soluble aggregates. In the case of the α-CyD, γ-CyD, HP-β-CyD and SBE-β-CyD systems, rhGH existed as monomer, dimer and soluble aggregates. By the addition of branched β-CyDs and DM-β-CyD, the percentage of the retained monomer after refolding increased up to 65–75%. Furthermore, the refolding of rhGH in the presence of branched β-CyDs and DM-β-CyD had the maximum propensity to form rhGH monomer and dimer, whereas the other CyDs tended to form large amounts of rhGH monomer and higher order oligomers, leading to the formation of insoluble aggregates. The non-cyclic sugars, glucose, maltose and trehalose, showed no difference in the effect on suppression of aggregation upon refolding in comparison with that of rhGH alone. Much higher concentrations may be necessary for these linear sugars to inhibit the aggregation of rhGH, because an optimum concentration of sugars to stabilize protein structure is known to be 1–2 M (17). The treatment by the commercially available refolding kit gave a large fraction of rhGH monomer and dimer with a small fraction of higher oligomers in the filtrate after separation of the insoluble aggregates.

It can be concluded from Figs. 1 and 2 that the cavity dimensions as well as the presence of chemical substituents on the CyD ring have a profound effect on its interaction with the exposed hydrophobic surfaces of molten globule-like intermediates of rhGH that may reduce the protein-protein interactions, resulting in the inhibition of aggregation, although the inclusion mode may be different among CyDs.

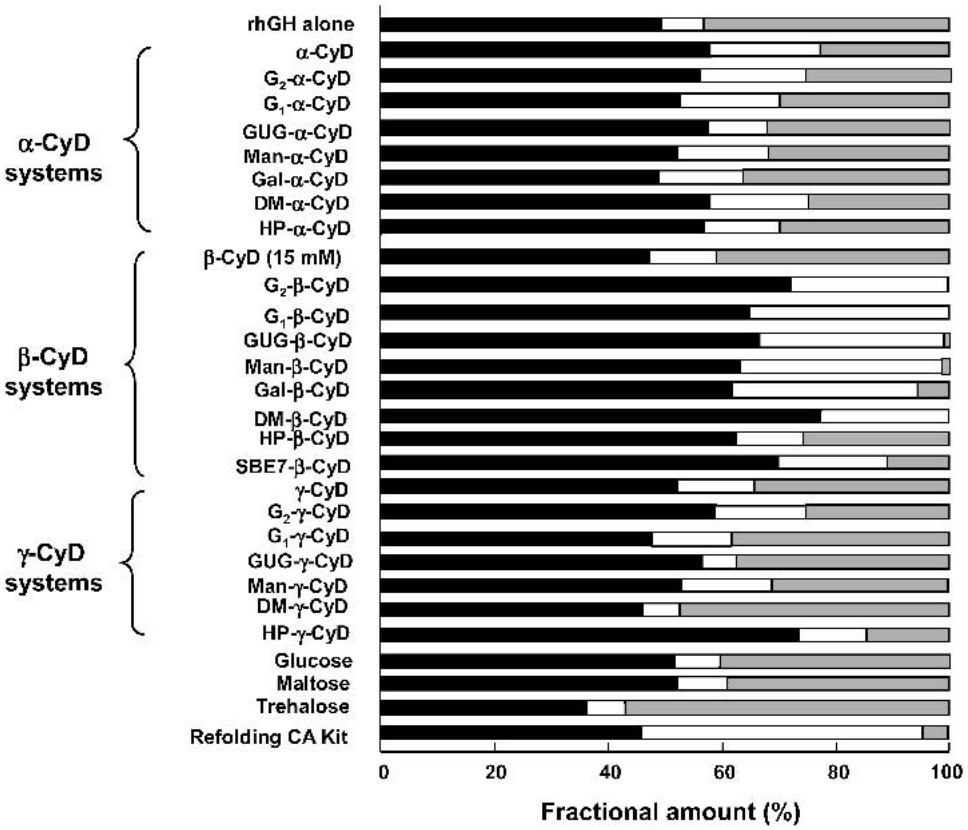

Fig. 2. Percentages of monomer, dimer, and soluble aggregates of rhGH[a)] formed after refolding in the absence or presence of additives (50 mM) in phosphate buffer (pH 5.0) containing 4.5 M GuHCl at 25°C. (Note a: The initial concentration of rhGH was 4.73 mg/ml.) Each value represents the mean of 3–7 experiments. Key: (■) monomer, (□) dimer, and (▩) soluble aggregate.

Stability Constants and Thermodynamic Parameters

To evaluate the interaction strength of CyDs to rhGH in a molten globule state, the apparent 1:1 stability constants of the rhGH/CyD complexes were determined by measuring the changes in the fluorescence intensity (340 nm) of rhGH by the addition of CyDs at different concentrations in 4.5 M GuHCl, and analyzing the titration curves of the fluorescence intensity *vs.* CyD concentrations using the Scott's equation (12). Parent β-CyD was not used because of its poor aqueous solubility. As shown in Table I, the branched β-CyDs which significantly reduced the aggregation of rhGH gave the higher stability constants, whereas γ-CyD with the small inhibiting effect showed the lower stability constant. These results suggested that aromatic amino acids of rhGH, responsible for the aggregate formation, fit tightly into the cavity of β-CyDs, compared to that of γ-CyDs which are too large to include such aromatic residues. In the case of the branched γ-CyDs, the branched moieties may play an important role in the inhibition of the aggregation. On the other hand, α-CyD with the small inhibiting effect had the large stability constant, whereas DM-β-CyD gave the small stability constant in spite of its large inhibiting effect. The amino acid residues that are involved in the interaction may differ between the α- and DM-β-CyD complexes, that is, α-CyD may include some small side chains of amino acids which has a minor role in the aggregation whereas DM-β-CyD may interact with much more important amino acids for the aggregation, leading the efficient inhibition. HP-β-CyD gave a stability constant of moderate magnitude, but its inhibiting effect was small enough to be statistically insignificant, and was similar to those of HP-α-CyD and HP-γ-CyD. The substitution of the secondary hydroxyl groups of CyDs (i.e. wider rim of the cavity) may hinder the inclusion of hydrophobic side chains of amino acids of the protein (18).

Table I. Apparent (1:1) Stability Constants[a] of Complexes of rhGH (0.23 mg/ml) with CyDs in Phosphate Buffer (pH 5.0, $I = 0.05$) Containing 4.5 M GuHCl at 25°C

System	Stability constant ($K_{1:1}$, M^{-1})
α-CyD	168 ± 57
γ-CyD	19 ± 4
HP-β-CyD	80 ± 13
DM-β-CyD	20 ± 6
G_2-β-CyD	173 ± 37
GUG-β-CyD	223 ± 47

[a] Determined by fluorescence method (340 nm), excitation wavelength was 277 nm. Each value represents the mean ± SE of 3–9 experiments.

Thermodynamic parameters of the complex formation in 4.5 M GuHCl solution were obtained from van't Hoff plots (10–60°C) for branched CyDs, G2-α-, -β-, and -γ-CyDs, and GUG-β-CyD. The plots gave very small negative enthalpy changes ($\Delta H = -0.01$ to 0.03 kJ/mol) and large positive en-

tropy changes ($\Delta S = 28$ to 31 kJ/mol), suggesting that hydrophobic interaction (19) plays an important role in the rhGH/branched CyDs complexation, together with a destruction of water structure around rhGH and CyD molecules. Therefore, the inhibiting mechanism of CyD in the refolding aggregation of rhGH seems to be rather complicated, and many factors such as inclusion complexation with side chains of amino acids of the protein, interaction of the branched sugar moieties with protein pockets, effect of CyDs on water structure around the protein etc may be involved in the inhibition.

Thermal Denaturation

The thermal denaturation of rhGH was studied by differential scanning calorimetry (DSC). The calorimetric profile for rhGH in phosphate buffer (pH 8.1) displayed a single endotherm, and the mean unfolding temperature (T_m) of rhGH reduced with increasing protein concentrations (data not shown). These results may be explained by an increase in protein-protein interaction at higher protein concentrations.

The ratio of the van't Hoff enthalpy to the calorimetric enthalpy ($\Delta H_v/\Delta H_c$) gives useful information on the mechanism of protein folding and transition (20). If the ratio of $\Delta H_v/\Delta H_c$ is close to 1, it indicates the two-state transition. The ratio is less than 1, indicating the presence of intermediate states in the unfolding process, whereas the ratio is more than 1, suggesting the aggregation of protein during the unfolding process.

Table II shows the thermodynamic parameters of rhGH in the absence or presence of CyDs in phosphate buffer (pH 8.1) as well as the ratios of $\Delta H_v/\Delta H_c$. The ratios of $\Delta H_v/\Delta H_c$ are about 3.2–3.9. It has been known that rhGH exists as a monomer when stored at room temperature. Thus, the $\Delta H_v/\Delta H_c$ ratio of > 1.0 suggested that the heat-induced disruption of native structure leads to aggregation of rhGH. These results agree with the previous report that above pH 3.5 the thermal denaturation is irreversible due to the aggregation of rhGH upon unfolding (21). The hydrophilic CyDs increased the unfolding temperature (Tm) of rhGH. Interestingly, branched β-CyDs not only increased the unfolding temperature of rhGH, but also decreased the $\Delta H_v/\Delta H_c$ ratio in comparison with that of rhGH alone. In contrast to the case of HP-CyDs, the branched α- and γ-CyDs showed an increased or an equal $\Delta H_v/\Delta H_c$ ratio when compared with those in the absence of CyDs. The present results suggested that the interaction of the branched β-CyDs with accessible hydrophobic side chains in the rhGH molecule leads to a less compact conformation of the protein and reduces its aggregation during the unfolding process.

Interfacial Denaturation

Shaking of protein solutions exposes the molecules to the air/water interface which has high interfacial tension. This exposure to the interface causes the globular protein to unfold, thus eventually exposing the hydrophobic core and leading to self-association as the hydrophobic groups of one molecule interact with those of another. rhGH is known to be subject to aggregation and precipitation from the solution upon shaking. This aggregation is not induced by the shear itself but by denaturation at an aqueous/hydrophobic surface (22).

In this study, aggregation of rhGH was induced by introducing an extensive air/water interface by vortexing. The extent of aggregation increased as a function of vortexing time (0-60 s) and was accompanied by a significant increase in the absorbance at 350 nm (i.e. increase in turbidity). Figure 3A shows the changes in absorption at 350 nm after vortexing rhGH solutions for 60 s in the presence of G2-β-CyD, GUG-β-CyD, HP-β-CyD, and DM-β-CyD, in comparison with the absorption (100%) of rhGH in the absence of CyDs. Figure 3B shows the percentages of the monomer and dimer contents in the filtrates after separation of insoluble aggregates. In the presence of HP- and DM-β-CyD, the formation of insoluble aggregate was almost completely suppressed even at 10 mM of the hosts, and the amount of monomer increased up to about 100%. On the other hand, the suppressing effect of the branched β-CyDs with the low surface activity (23) was lower than those of HP-β-CyD and DM-β-CyD. It is of interest to note that the inhibiting effect of HP-β-CyD was in contrast to that of the refolding aggregation described above. The suppressing effect of HP-β-CyD and DM-β-CyD may be due to the prevention of rhGH adsorption on the air/water surface, because of their surface active property, i.e., these results may be due to the surface activity of HP- and DM-β-CyD which can partially include rhGH molecule resulting in the decrease in the concentration of free rhGH in the air/water surface, and in addition, the presence of the β-CyDs at

Table II. Thermodynamic Parameters of rhGH (3.54 mg/ml) in the Absence and Presence of CyDs (100 mM) in Phosphate Buffer (pH 8.1, $I = 0.05$)[a]

	System	Tm (°C)	ΔHcal (kcal/mol)	ΔHv (kcal/mol)	ΔHv/ΔHcal
	rhGH alone	74.0 ± 0.1	60 ± 1	193 ± 4	3.22 ± 0.10
α-CyD systems	with HP-α-CyD	76.1 ± 0.0	62 ± 2	183 ± 3	2.98 ± 0.13
	with G_2-α-CyD	74.5 ± 0.0	52 ± 3	219 ± 3	4.47 ± 0.23
β-CyD systems	with HP-β-CyD	77.0 ± 0.1	54 ± 1	194 ± 2	3.58 ± 0.08
	with G_1-β-CyD	76.6 ± 0.1	62 ± 3	153 ± 2	2.49 ± 0.15
	with G_2-β-CyD	76.6 ± 0.0	53 ± 1	133 ± 2	2.58 ± 0.17
	with GUG-β-CyD	75.5 ± 0.1	65 ± 2	167 ± 5	2.53 ± 0.08
	with DM-β-CyD[b]	—	—	—	—
γ-CyD systems	with HP-γ-CyD	76.1 ± 0.0	57 ± 1	207 ± 1	3.65 ± 0.03
	with G_2-γ-CyD	76.0 ± 0.0	56 ± 1	202 ± 2	3.60 ± 0.06

[a] Each value represents the mean ± SE of 3–4 experiments.
[b] Could not be determined due to precipitation of DM-β-CyD at higher temperature.

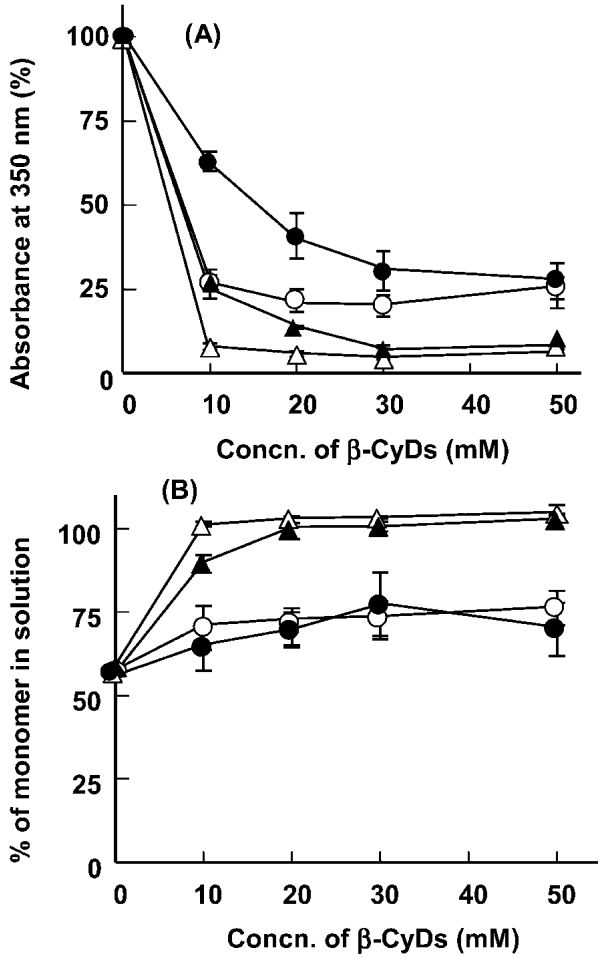

Fig. 3. Effects of CyDs on formation of insoluble aggregate[a)] (A) and percent of monomer[b)] (B) in solution[c)] after agitation in phosphate buffer (pH 8.1, $I = 0.05$) at 25°C. [Note a: Determined by UV at 350 nm. Note b: Determined by size exclusion chromatography. Note c: rhGH solutions (1.0 ml, 1.18 mg/ml) were agitated at high speed with a vortex mixer for 60 s.] Each point represents the mean ± SE of 4–6 experiments. Key: (○) G_2-β-CyD, (●) GUG-β-CyD,(△) DM-β-CyD, and (▲) HP-β-CyD.

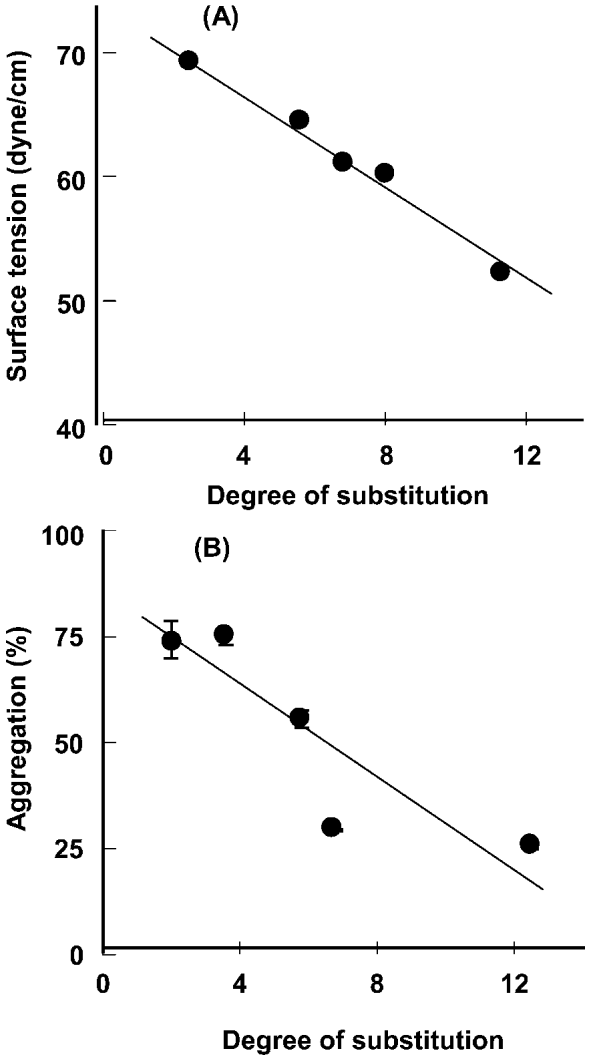

Fig. 4. (A) Plots of surface tension vs. degree of substitution of HP-β-CyD[a)], and (B) plots of aggregation[b)] of rhGH vs. degree of substitution of HP-β-CyD (10 mM). [Note a: 0.1% w/v at 25°C. Note b: rhGH solutions (1.0 ml, 1.18 mg/ml) were agitated at high speed with a vortex mixer for 60 s.] Each point represents the mean ± SE of 3 experiments.

the interface would reduce the available sites for protein adsorption (24). To confirm the relationship between surface activity and anti-aggregating properties of surface active CyD, we used HP-β-CyD with different surface activities owing to the different degrees of substitution (D.S.) of the 2-hydroxypropyl group. The surface tension of HP-β-CyD solution decreased with an increase in the D.S. (Fig. 4A), and the aggregation of rhGH decreased with the D.S. (Fig. 4B). Thus, the effectiveness of surface active CyDs in reducing the interfacial denaturation may be related to its surface activity in a manner analogue to that proposed for surfactants (22).

Oxidation and Deamidation

A number of amino acid side chains in proteins are susceptible to oxidations, such as methionine, tryptophan, histidine and tyrosine residues (4). However, the major oxidative reaction of rhGH occurs at the methionine residues. rhGH possesses three methionine residues: Met-14, Met-125, and Met-170. The oxidation has been detected at both Met-14 and Met-125 which exist on surface of the protein, whereas Met-170 may be buried in the interior of the protein and hence is unable to react with peroxide (25). In our study, the oxidation of rhGH in solution was generated using 3% hydrogen peroxide. Figure 5A shows the time profiles of remaining rhGH monitored by HPLC. The first-order rate constant for the oxidation of rhGH in the absence of CyDs was 0.509 h^{-1}, and all CyDs used (50.0 mM) showed insignificant differences in oxidation rates compared with native rhGH, (0.532, 0.597, 0.661, 0.568 and 0.458 h^{-1} for G_2-β-CyD, GUG-β-CyD, DM-β-CyD, HP-β-CyD and SBE7-β-CyD, respectively), suggesting that these CyDs do not interact with methionine residue of rhGH or scavenge superoxide species occurring during oxidation process.

Deamidation is the primary mode of degradation of rhGH in solution (26). rhGH has 9 asparagine and 13 glutamine residues. Previous studies identified Asp-149 as the principle site of deamidation for rhGH stored under alkaline

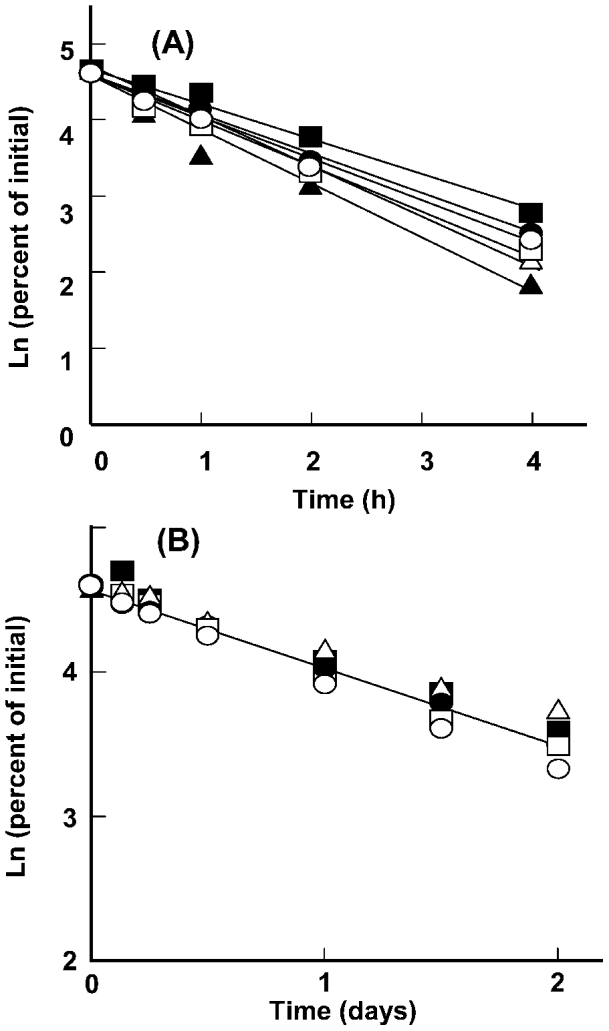

Fig. 5. (A) First-order plots for oxidation of rhGH (9.45 mg/ml) in the absence or presence of β-CyDs (50 mM) in 3% hydrogen peroxide at 25°C, and (B) first-order plots for deamidation of rhGH (1.0 mg/ml) in the absence or presence of β-CyDs (50 mM) in 0.05 M ammonium bicarbonate buffer (pH 9.0) at 37°C. Each value represents the mean of 3 experiments. Key: (○) alone, (●) G_2-β-CyD, (△) GUG-β-CyD, (▲) DM-β-CyD, (□) HP-β-CyD, and (■) SBE-β-CyD.

conditions, with only a minor amount of deamidation occurring at Asn-152. In this study, the deamidation of rhGH was performed in alkaline pH using ammonium hydrogen carbonate (pH 9.0) in the absence or presence of CyDs (50.0 mM). As shown in Fig. 5B, the first-order rate constant of the deamidation of rhGH in the absence of CyDs was 0.583 day^{-1}, and there was no significant decrease in the deamidation rate of rhGH after the addition of these CyDs (0.510, 0.423, 0.535, 0.585, and 0.537 day^{-1}, for G2-β-CyD, GUG-β-CyD, DM-β-CyD, HP-β-CyD, and SBE7-β-CyD, respectively). Taken together, these results agreed with the oxidation data that CyDs may rather interact with the exposed hydrophobic side chains than aliphatic side chains of rhGH, resulting in inhibition of aggregation but not the oxidation and deamidation rates.

CONCLUSIONS

Aggregation is a serious problem in the pharmaceutical development of proteins. Some hydrophilic CyDs significantly inhibit the aggregation and thermal inactivation of some peptides and its aggregation by interacting with accessible hydrophobic side chains of peptides. In this study, we investigated the effect of hydrophilic CyDs on the chemically induced aggregation of rhGH. rhGH forms molten globule intermediate in 4.5 M GuHCl and results in significant aggregation upon refolding. Branched β-CyDs and DM-β-CyD significantly reduced the aggregation of rhGH during refolding from the molten globule-like intermediates, while parent and branched α- and γ-CyDs and HP-CyDs showed no noticeable inhibitory effects. Furthermore, the size exclusion chromatographic analysis revealed that the concentration of rhGH monomer remaining in solution after refolding was highest in the presence of branched β-CyDs and DM-β-CyD. DM-β-CyD and that branched β-CyDs are most preferable to prevent the aggregation of rhGH, although the inhibiting mechanism seems to be different between these β-CyDs.

Calorimetric studies for thermal unfolding of rhGH at a neutral pH demonstrated that hydrophilic CyDs can stabilize the native rhGH, as indicated by an increase in the mean unfolding temperature (Tm) and inhibit the aggregation of rhGH. Among the hydrophilic CyDs, branched β-CyDs increase the Tm of rhGH and decrease the $\Delta H_v/\Delta H_c$ ratio. These results suggest that the interaction of the branched β-CyDs with accessible hydrophobic side chains in the rhGH molecule leads to less compact conformation of the protein and reduces its aggregation during the folding process.

In contrast to the other stress-induced denaturations, DM-β-CyD and HP-β-CyD were found to be highly effective in preventing the aggregation, compared with those of branched β-CyDs due to their surface activities which decrease the driving force for protein adsorption and reduce the available sites for protein adsorption. Thus, surface active CyDs may stabilize rhGH by a surface phenomenon like mechanism of surfactant.

Taken together from various stress-induced aggregations, the results emphasize that what is effective as a stabilizer for one stress on protein solutions may not be applicable to another stress. Thus, the use of CyDs for protein stabilization is dependent not only on the structure and property of CyD itself but also on the nature of the denaturing stimulus. The present results suggested that hydrophilic β-CyDs can effectively inhibit the aggregation of rhGH. Thus hydrophilic β-CyDs may be potentially useful excipients for parenteral preparation of rhGH. Further studies may be necessary to elucidate the detailed anti-aggregation mechanism and the sites of interaction between CyDs and rhGH.

ACKNOWLEDGMENTS

We would like to thank Ms. Tomoko Nakatsuka, Mr. Yuki Katou, Mr. Taishi Higashi, and Ms. Seiko Takeda for their technical assistance.

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