Varying Effects of Cyclodextrin Derivatives on Aggregation and Thermal Behavior of Insulin in Aqueous Solution

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Maltosyl-β-cyclodextrin (G_2-β-CyD) suppressed the aggregation of insulin in neutral solution, while the sulfate of β-CyD (S-β-CyD) accelerated the aggregation. On the other hand, the sulfobutyl ether of β-CyD (SBE-β-CyD) showed varying effects on insulin aggregation, depending on the degree of substitution of the sulfobutyl group: *i.e.*, the inhibition at relatively low substitution and acceleration at higher substitution. Differential scanning calorimetric studies indicate that the self-association of insulin stabilized the native conformation of the peptide, as indicated by an increase in the mean unfolding temperature (T_m). G_2-β-CyD and SBE-β-CyD decreased the T_m value of insulin oligomers, while S-β-CyD increased the T_m value. ^{1}H-Nuclear magnetic resonance spectroscopic studies suggest that G_2-β-CyD includes accessible hydrophobic side chains of insulin within the CyD cavity, and hence perturbs the intermolecular hydrophobic contacts between aromatic side chains across the monomer–monomer interfaces. By contrast, the electrostatic interaction between the positive charges of insulin and the concentrated negative charges of the sulfate and sulfonate groups of the anionic β-CyDs seems to be more of a factor than the inclusion effects. These results suggest proper use of the CyD derivatives could be effective in designing rapid or long-acting insulin preparations.

Key words bovine insulin; cyclodextrin derivative; inclusion complexation; aggregation; differential scanning calorimetry; ^{1}H-NMR spectroscopy

The propensity of insulin to form reversible and irreversible aggregates in solution is of great concern as it may lead to the loss of biological potency, immunogenic reactions, blockage of infusion pumps or an unacceptable physical appearance in long-term therapeutic systems.[1] In the neutral solutions used clinically, insulin is mostly assembled as a zinc-containing hexamer and its self-association limits the rate of subcutaneous absorption, which is too slow to mimic the normal rapid increase of insulin in blood at the time of meal consumption.[2] These problems are further complicated by the tendency of insulin to adsorb onto the hydrophobic surfaces of containers and devices, perhaps by mechanisms similar to those inducing aggregations.[3] To overcome these drawbacks, several approaches have been proposed, including the use of amphiphatic excipients,[4] chemical modification[5] and site-directed mutation.[6]

An alternative to these strategies is complex formation with cyclodextrins (CyDs).[7–9] Our previous studies have shown that some hydrophilic CyDs, including maltosyl-β-CyD (G_2-β-CyD) and 2-hydroxypropyl-β-CyD, significantly inhibit the adsorption of bovine insulin to hydrophobic surfaces of containers and its aggregation by interacting with hydrophobic regions of the peptide.[10] Recently, the sulfates and sulfoalkyl ethers of CyDs have been evaluated as a new class of parenteral drug carriers because they are highly hydrophilic and less hemolytic than the parent and the other hydrophilic CyDs.[11,12] Following up on these studies, the effects of anionic β-CyD derivatives on the aggregation and thermal behavior of insulin in solution both at acidic and nearly neutral pHs were investigated and compared with that of G_2-β-CyD, with emphasis on the contribution of their inclusion ability and polyanionic character to the interaction with the peptide.

Experimental

Materials Bovine insulin was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), lot No. 26H170, with a nominal activity of 28.1 I.U./mg (bovine pancreas crystalline, I-5500), and was used without further purification. The content of zinc ion was about 0.5%, corresponding to approximately 2 mol of zinc ion per 1 mol of insulin hexamer. G_2-β-CyD was a generous gift from Ensuiko Sugar Refining Co., Ltd. (Yokohama, Japan). SBE-β-CyDs with average degrees of substitution of 3.9 and 6.2 (SBE4-β- and SBE7-β-CyDs) were donated by CyDex L.C. (Overland Park, KS, U.S.A.). S-β-CyD with an average degree of substitution of 10.7 was prepared by a non-regional selective method as described previously.[13] The structures and abbreviations of CyDs used are listed in Table 1. All other materials were of reagent grade, and deionized double distilled water was used.

Aggregation Studies Insulin was dissolved in 10 mM sodium phosphate buffer (pH 6.8, $I=0.2$) in the absence and presence of additives. The aggregation of insulin was evaluated by measuring the insulin in the filtrate (filter: DISMIC-13CP045AN; Advantec Co., Tokyo, Japan) after freshly prepared insulin solutions stood in a silicone-coated glass tube at 25 °C; this container was used to minimize the adsorption of the peptide onto the surface. The concentration of insulin in the filtrate was determined by high-performance liquid chromatography (HPLC). The HPLC conditions were as follows: pump, LC-10AD (Shimadzu Co., Kyoto, Japan); detector, UVDEC 100 V-UV (210 nm, Jasco, Tokyo, Japan); column, YMC Pack C_8 AP-type, (4.6 mm i.d. × 150 mm; flow rate, 1.0 ml/min; 40 °C; YMC Co., Kyoto, Japan); internal standard, methyl *p*-aminobenzoate. The mobile phase consisted of 20% (v/v) acetonitrile and 0.05% (v/v) trifluoroacetic acid (TFA) in water as solvent A; 60% (v/v) acetonitrile and 0.05% (v/v) TFA in water as solvent B. A gradient profile: 0 to 100% B in 20 min, hold 100% B for 1 min, and return to initial condition immediately, was applied.

The volume–surface diameters of micron-sized insoluble insulin particles were measured using a Galai CIS-1 laser scan grading analyzer (Migdal Haemek, Israel). The distribution of submicron-sized soluble insulin oligomers was determined at 25 °C by quasielastic light scattering, using a submicron analyzer (NICOMP Model 370 EVHPL, Pacific Scientific Co., Palo Alto, CA, U.S.A.) equipped with 64 channels, a detector set at an angle of 90°, and an argon laser set at the blue-green 488 nm line, having a maximum power of about 280 mW (INNOVA 70, Coherent, Inc., Palo Alto, CA, U.S.A.). For each sample, light-scattering measurements were accumulated during about 10-min intervals to reduce random signal noise and to ensure a stable baseline.

Differential Scanning Calorimetry Scanning calorimetric measure-

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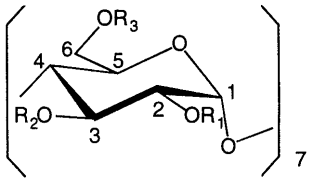
Table 1. Chemical Structures of Cyclodextrin Derivatives Used in This Study



Compound	Abbreviation	R_1	R_2	R_3	DS[a]
β-Cyclodextrin	β-CyD	H	H	H	—
6-*O*-Maltosyl-β-cyclodextrin	G_2-β-CyD	H	H	H or maltose	1
Sulfobutyl-β-cyclodextrin	SBE4-β-CyD	R_1, R_2, R_3 = H or $(CH_2)_4SO_3Na$			3.9
	SBE7-β-CyD				6.2
Sulfated β-cyclodextrin	S-β-CyD	R_1, R_2, R_3 = H or SO_3Na			10.7

a) The average degree of substitution.

ments were made using an MC-2 differential microcalorimeter (MicroCal, Inc., Northampton, MA, U.S.A.) using the MicroCal Origin for data acquisition and analysis. All solutions were degassed under a 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 5 to 95 °C under an excessive N_2 pressure at about 220 kPa. The calorimetric enthalpies of thermal unfolding, accompanied by the dissociation of insulin oligomers in the absence and presence of CyDs, 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 peptide concentration.

Proton-Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy The ^{1}H-NMR spectra of insulin (2 mM) in the absence and presence of CyDs (0.5 mM for SBE-β-CyDs or 100 mM for G_2-β-CyD) were taken on a JNM EX-400 spectrometer (Jeol, Tokyo, Japan), operating at 399.65 MHz at 25 °C, using 30%(v/v) CD_3COOD as a solvent. ^{1}H-NMR chemical shifts were given in parts per million (ppm) relative to that of the HOD signal or the CD_3COOD signal, with an accuracy of ±0.001. The ^{1}H-NMR signals of the aromatic region of insulin were assigned according to the reports of Hua and Weiss[14] and of Funke and co-workers.[15]

Results and Discussion

Effects of CyDs on Insulin Aggregation in Solution In solution, insulin exists in an equilibrium between monomers, dimers, hexamers and higher order aggregates, depending on environmental factors such as concentration, pH, ionic strength, temperature and metal ions. The two insulin molecules in the dimer are held together by predominantly hydrophobic forces, reinforced by four hydrogen bonds arranged in an antiparallel β-sheet structure between the two C-terminal strands of the B chain. In the presence of zinc ions three insulin dimers are assembled into a hexameric organization, in which the zinc ions are coordinated to B10 histidines.[1]

In neutral solutions, insulin is mostly polymerized as a zinc-containing hexamer, eventually leading to the precipitation of higher order aggregates. A substantial aggregation of insulin occurred at concentrations of more than 0.05 mM in a phosphate buffer (pH 6.8, I=0.2) at 25 °C, as indicated by the increased optical density at 600 nm and the decreased concentration of the peptide remaining in the solution.[10] The HPLC analyses of the redissolved insulin aggregates revealed no fragmentation of the peptide and the non-covalent nature of the aggregates.

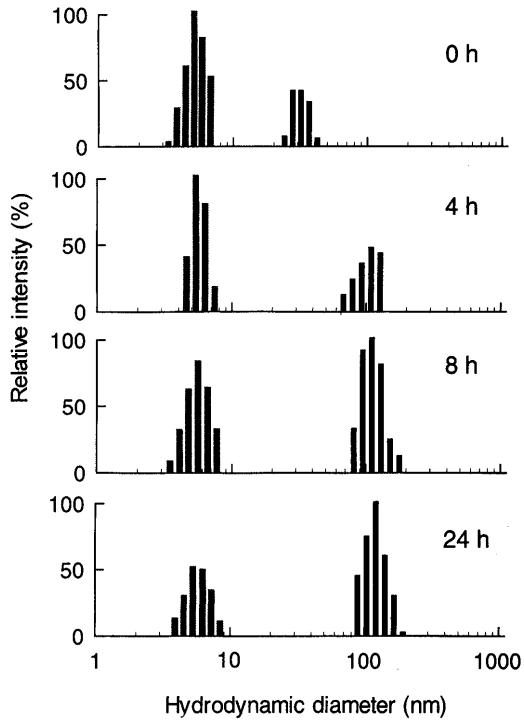


Fig. 1. Changes in Intensity-Weighted Distribution for Soluble Insulin Particles after Preparation of Insulin Solution (0.15 mM) in Phosphate Buffer (pH 6.8, I=0.2) at 25 °C

Figure 1 shows changes with time in intensity-weighted distribution for soluble insulin particles after preparation of the insulin solution (0.15 mM) at 25 °C, where micron-sized insoluble particles corresponding to highly aggregated insulin (>0.22 μm) were filtered out prior to analysis. A freshly-prepared insulin solution showed bimodal distribution of particles with mean hydrodynamic diameters of 5.3 nm and 31.2 nm, respectively. The first peak, at 5.3 nm in diameter, seems to be primarily an insulin hexamer, as judged by crystallographic dimensions for the hexamer, showing an almost cylindrical structure with a diameter of 5 nm and a height of about 3.5 nm.[1] The second peak, at 31.2 nm in diameter, may indicate intermediate assemblies, the diameter of which increased with the elapse of time and reached a critical size over 100 nm 24 h after preparation. Furthermore, the intensity ratio of the second peak to the first one increased from

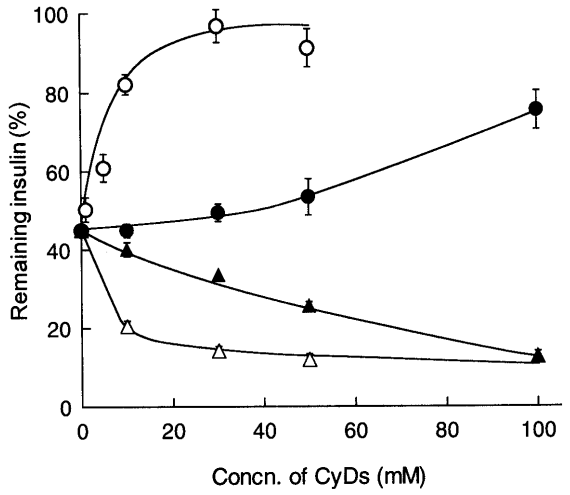


Fig. 2. Effects of CyDs on Aggregation of Insulin (0.15 mM) 24 h after Preparation of Insulin Solution in Phosphate Buffer (pH 6.8, $I=0.2$) at 25 °C, as a Function of CyD Concentrations

○, with G_2-β-CyD; ●, with SBE4-β-CyD; △, with SBE7-β-CyD; ▲, with S-β-CyD. Each point represents the mean ± S.E. of 4 experiments.

0.4 at time zero to 1.7, 24 h after preparation. Sluzky *et al.* have proposed a mathematical model describing the kinetics of insulin aggregation in aqueous solution upon agitation in the presence of hydrophobic surfaces, in which partially unfolded insulin monomers associate and form the initial nucleating species, eventually leading to the insulin aggregation.[16] In the same way, the intermediate species observed here would serve as precursors to micron-sized aggregates *via* successive kinetic processes.

Figure 2 shows the effects of CyDs on the aggregation of insulin 24 h after preparation of the insulin solution (0.15 mM) in phosphate buffer (pH 6.8) at 25 °C, as a function of the CyD concentrations. The CyDs did not affect the mean volume–surface diameter of insoluble insulin particles, which was determined to be 28.4 ± 2.6 μM. Unfortunately, the presence of the CyDs at higher concentrations made it impossible to determine the submicron-range particle distribution for insulin, because of the overlapping of extremely large scattering intensity peaks corresponding to the CyDs and their hydrates.[17] G_2-β-CyD suppressed the aggregation of insulin in a concentration-dependent manner. Under the present condition, a minimal concentration of G_2-β-CyD which was necessary to achieve statistically significant inhibition of insulin aggregation was 5 mM, at which the molar ratio of G_2-β-CyD to insulin was 33 : 1, and complete inhibition was observed at concentrations more than 30 mM. G_2-β-CyD may interact with hydrophobic amino acid residues of insulin, and thus prevent the aggregation by eliminating intermolecular hydrophobic contacts.[10] This view was confirmed by a dilution microcalorimetric study, in which CyDs increased the dissociation of insulin oligomers in a manner consistent with their binding to the dissociated form of the peptide molecule.[18] On the other hand, S-β-CyD accelerated the insulin aggregation. Since S-β-CyD has highly concentrated negative charges located near the entrance of the cavity and shows limited inclusion ability, its polyanionic character may contribute to the accelerated aggregation of insulin.

As shown in Fig. 2, SBE-β-CyD showed varying effects on insulin aggregation, depending on the degree of substitution; *i.e.* inhibition at relatively low substitution and acceleration at higher substitution. Since the sulfonate groups in SBE-β-CyD are appropriately spaced from the CyD cavity with a butyl chain and do not interfere with the inclusion process, SBE4-β-CyD may inhibit the insulin aggregation in a manner similar to G_2-β-CyD. In the case of SBE7-β-CyD, the electric effects seem to be more of a factor than the inclusion effects, eventually leading to the acceleration of the insulin aggregation.

Under the present condition with pH 6.8 higher than the isoelectric point of insulin (p$I=5.3$),[19] the net charge of the peptide is negative. The charged and polar groups on the insulin surface would be surrounded by water molecules *via* ionic hydration and/or hydrogen bondings. High concentrations of sulfates such as $(NH_4)_2SO_4$ and Na_2SO_4 are commonly used to precipitate or crystallize polypeptides in the native form, which can be ascribable to the loss of the hydration layer on the peptide surface.[20] In fact, some neutral salts accelerated the aggregation of insulin, with the efficacy increasing in the order: NaCl $\ll CH_3COONa < Na_2SO_4$, a sequence which corresponds to their salting-out potency.[21] For instance, under the same condition as in Fig. 2, the remaining percentages of insulin in the presence of 100 mM NaCl, CH_3COONa and Na_2SO_4 were 41.5 ± 1.9%, 22.9 ± 1.3% and 18.6 ± 1.0%, respectively. Therefore, the sulfate and sulfonate groups in S-β-CyD and SBE-β-CyD would remove the hydration layer from the insulin molecule in a manner similar to these lyotropic anions, a situation which makes the intermolecular interaction of the peptide stronger, eventually leading to the accelerated association or aggregation of the peptide. The logarithm of peptide solubility (S) is known to decrease almost linearly with the ionic strength (I) of salts according to the following equation[22]:

$$\log S=\beta-KsI \quad (1)$$

where β and Ks are empirical constants, Ks being the salting-out constant. The Ks values increased in the order: S-β-CyD (0.386) < Na_2SO_4 (0.736) < SBE7-β-CyD (0.952).

As another aspect of the effects of the anionic CyDs on the insulin aggregation, S-β-CyD and SBE-β-CyD are likely to induce a conformational transition in the insulin hexamer in a manner similar to lyotropic anions, which partially transform an extended chain from the B1 to B9 residues (T-state) into an α-helix (R-state) *via* electrostatic screening of an ion pair interaction between the B1-phenylalanine and the A17-glutamic acid at the dimer–dimer interface.[23] This may also contribute to the accelerated association of the peptide.

The varying effects of the CyD derivatives on the insulin association were confirmed by the ultrafiltration experiments shown in Fig. 3. G_2-β-CyD and SBE4-β-CyD facilitated the permeation of insulin through an ultrafiltration membrane with a nominal molecular weight cut-off of 50 kDa, the former being more effective. By contrast, S-β-CyD and SBE7-β-CyD reduced the membrane permeation of insulin, as reflected by the accelerated association of the peptide. Our previous studies have dem-

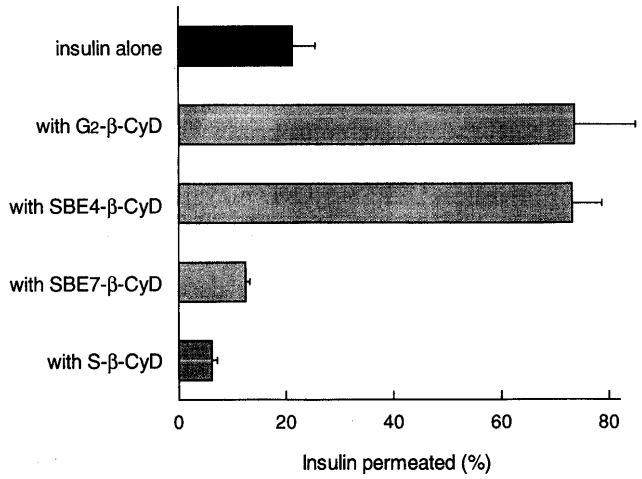


Fig. 3. Effects of CyDs (100 mM) on Permeation of Insulin (0.1 mM) through Ultrafiltration Membrane (XM 50) in Phosphate Buffer (pH 6.8, $I=0.2$) at 25 °C

Each point represents the mean ± S.E. of 2—9 experiments.

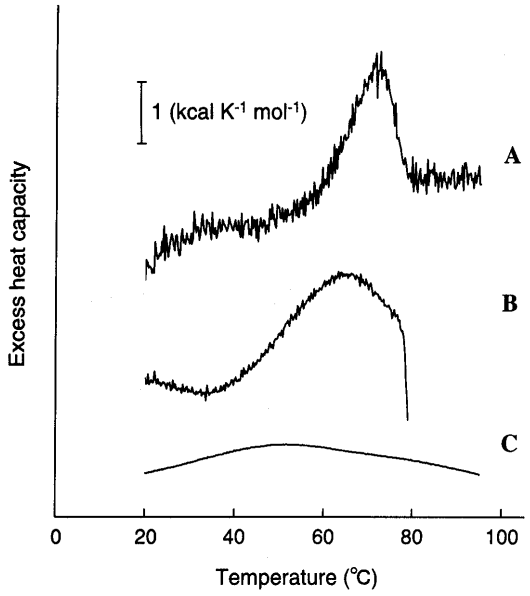


Fig. 4. Typical Excess Heat Capacity Curves for Thermal Unfolding of Insulin at Different Association States

A, insulin (0.1 mM) in phosphate buffer (pH 6.8, $I=0.2$); B, insulin (0.3 mM) in phosphate buffer (pH 2.0, $I=0.2$); C, insulin (5 mM) in 30% (v/v) acetic acid solution.

onstrated that the permeation of insulin mediated by G_2-β-CyD was much greater than that by ethylenediaminetetraacetic acid (EDTA), which is known to sequester zinc ions from insulin oligomers and dissociate them into the dimer.[24] Furthermore, G_2-β-CyD facilitated the permeation of insulin through the membrane in an acidic solution at pH 2.0, in which the peptide exists primarily as a zinc-free dimer.[10] These results indicate that G_2-β-CyD shifts the equilibrium in favor of the monomeric form.

Effects of CyDs on Thermal Behavior of Insulin Figure 4 shows the typical excess heat capacity curves for thermal unfolding of insulin at different association states, after base-line subtraction and concentration normalization. The concentration of insulin used was chosen here based on the solubility of the peptide at different association

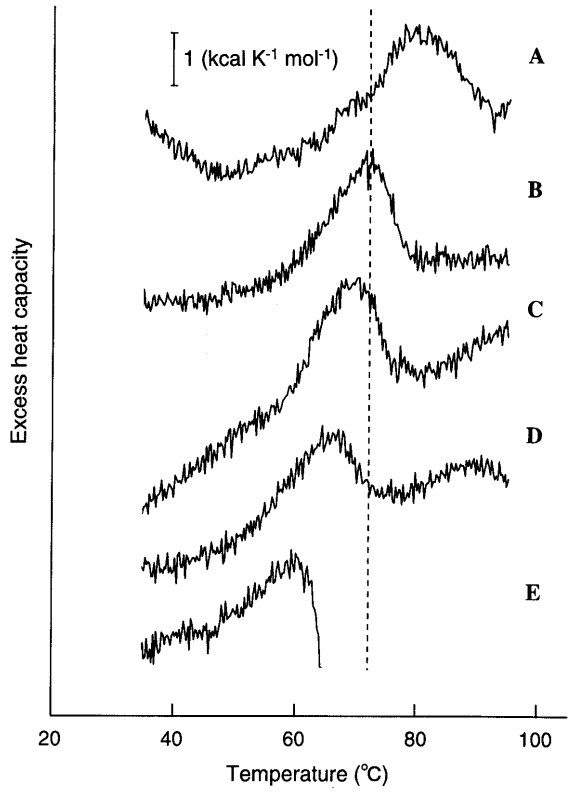


Fig. 5. Effects of CyDs (100 mM) on Excess Heat Capacity Curve for Thermal Unfolding of Insulin (0.1 mM) in Phosphate Buffer (pH 6.8, $I=0.2$)

A, with S-β-CyD; B, insulin alone; C, with SBE7-β-CyD; D, with SBE4-β-CyD; E, with G_2-β-CyD. The dotted line represents the mean unfolding temperature of insulin without CyDs.

states. Under the present condition, the thermal unfolding of insulin was completely irreversible, since no endothermic peak was observed by rescanning the sample after it was cooled from the first run. In 30% (v/v) acetic acid solution, insulin exists mostly as the monomer[10] and gives a broad endothermic peak around 50 °C. In phosphate buffer (pH 2.0), insulin is predominantly dimeric and shows an endothermic peak over 60 °C, following an irregular exothermic pattern due to the precipitation of insulin. By contrast, the insulin hexamer and higher order oligomers in phosphate buffer (pH 6.8) showed a mean unfolding temperature (T_m) of approximately 70 °C, where large changes in the excess heat capacity may be due to the dissociation of insulin oligomers, a process which is known to be endothermic.[18] These results clearly indicate that self-association of the insulin molecules stabilized the native conformation of the peptide, and the T_m value of the peptide was a diagnostic measure for the conformational changes of the peptide.

Figure 5 shows the effects of CyDs on the excess heat capacity curves of insulin solution at pH 6.8. G_2-β-CyD and SBE4-β-CyD significantly reduced the T_m value of insulin oligomers, the former being more effective. These CyD derivatives may shift the equilibrium in favor of the unfolded insulin by dissociating the oligomers and/or binding to hydrophobic side chains exposed on the unfolded peptide.[25] Although SBE7-β-CyD promoted the insulin aggregation, it either did not affect or actually reduced slightly the T_m value of insulin. This indicates

that the conformational energy of the unfolded insulin is reduced by incorporating the exposed hydrophobic groups in the unfolding peptide into the CyD cavity, which may compensate fully for the thermal stabilization arising from the accelerated association of the peptide. On the other hand, S-β-CyD, having a limited inclusion ability, increased the T_m value of insulin by approximately 10 °C, resulting solely from the higher degree of association of the peptide.

Effects of CyDs on the ^{1}H-NMR Spectrum of Insulin In solution, an insulin monomer is in equilibrium with the dimer and hexamer.[1] Of the three insulin species, the monomer is the most likely to interact with the hydrophobic cavity of CyDs, and the primary targets for complexation would be the aromatic side chains in the peptide. In this study, further insight into the interaction mode of insulin with the CyD derivatives was gained by employing ^{1}H-NMR spectroscopy in deuterium oxide (D_2O) containing 30% (v/v) deuterated acetic acid (CD_3COOD). This solvent system weakened the self-association of insulin, enabling the monomer to be the predominant species.[10]

Figure 6 shows the effects of G_2-β-CyD (100 mM) and SBE7-β-CyD (0.5 mM) on the ^{1}H-NMR spectrum of the aromatic region of insulin (2 mM) in 30% (v/v) CD_3COOD solution at 25 °C. The concentration of SBE7-β-CyD was limited to 0.5 mM or less because of the precipitation of insulin due to the neutralization of cationic charges in the peptide by anionic sulfonate groups of the CyD. Similarly, even with lower concentrations of S-β-CyD, insulin was precipitated and the ^{1}H-NMR data could not be obtained under the acidic condition used. Table 2 summarizes the ^{1}H-NMR chemical shift displacements of assignable aromatic protons of insulin upon the addition of CyDs. As shown in Fig. 6-A, the C2 protons of the B5- and B10-histidines were well dislocated from the main aromatic envelope (6.2—7.0 ppm), and the peak height ratio of the C2 proton signal of the B10-histidine to that of the B5-histidine (B10/B5 ratio) is known to be affected by changes in the concentration of insulin in a manner that reflects the self-association of insulin.[22] Judging from the B10/B5 ratio in Fig. 6-A, insulin is confirmed to be primarily monomeric at a concentration of 2 mM.[10]

Upon the addition of G_2-β-CyD, the ^{1}H-NMR signals

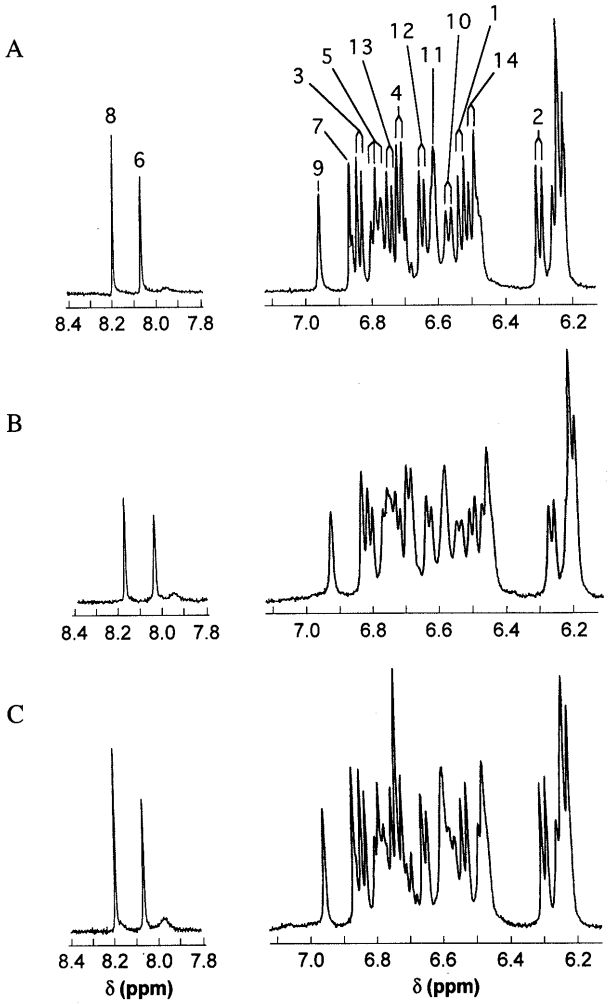


Fig. 6. Effects of G_2-β-CyD (100 mM) and SBE7-β-CyD (0.5 mM) on ^{1}H-NMR Spectrum of Aromatic Region of Insulin (2 mM) in D_2O Containing 30% (v/v) CD_3COOD at 25 °C

A, insulin alone; B, with G_2-β-CyD; C, with SBE7-β-CyD. The numbers above the peaks are the same as in Table 2.

Table 2. Effects of G_2-β-CyD (100 mM) and SBE-β-CyDs (0.5 mM) on ^{1}H-NMR Chemical Shifts of Insulin (2 mM) in 30% (v/v) Deuterated Acetic Acid at 25 °C

Number	Side chain	Position	Insulin alone (2 mM) Chemical shift (δ)	With β-CyDs, $\Delta\delta$[a] (ppm)		
				With G_2-β-CyD	With SBE4-β-CyD	With SBE7-β-CyD
1	Tyrosine (A14)	C2 and 6	6.530	0.000	0.001	0.008
2	Tyrosine (A14)	C3 and 5	6.295	0.001	0.001	0.009
3	Tyrosine (A19)	C2 and 6	6.833	0.004	0.001	0.007
4	Phenylalanine (B1)	C2 and 6	6.713	0.006	0.004	0.018
5	Phenylalanine (B1)	C4	6.784	−0.001	0.000	0.007
6	Histidine (B5)	C2	8.063	−0.003	−0.001	0.008
7	Histidine (B5)	C4	6.864	−0.001	0.000	0.005
8	Histidine (B10)	C2	8.195	−0.001	0.002	0.006
9	Histidine (B10)	C4	6.955	0.000	−0.001	0.004
10	Tyrosine (B16)	C2 and 6	6.568	0.002	0.001	0.008
11	Phenylalanine (B24)	C3 and 5	6.611	−0.001	−0.006	−0.012
12	Phenylalanine (B25)	C2 and 6	6.647	0.012	0.001	0.009
13	Phenylalanine (B25)	C3 and 5	6.743	0.010	−0.001	0.003
14	Tyrosine (B26)	C2 and 6	6.498	−0.005	−0.004	−0.010

a) $\Delta\delta = \delta_{\text{with CyD}} - \delta_{\text{insulin alone}}$. Negative signs indicate upfield displacement.

of the B26-tyrosine were shifted upfield, while those of the A19-tyrosine and the B1- and B25-phenylalanines were shifted downfield. The G_2-β-CyD-induced chemical shift displacements and line broadening would be ascribable to the shielding effect due to the inclusion of the aromatic side chains within the CyD cavity[26] and/or the concomitant conformational changes in the peptide. β-CyD derivatives are reported to have relatively small binding constants of less than $200\,M^{-1}$ with free aromatic amino acids at nearly neutral pH, with the efficacy decreasing in the order of tyrosine > phenylalanine ≫ histidine, depending on their hydrophobicity[27] and on the complementary strength between host and guest molecules.[28,29] Under the acidic condition used in this study at a pH meter reading of 1.9, the imidazole moiety of the B5 and B10 histidines was completely protonated since its pK_a is *ca.* 7.0[30]; this may further destabilize such complexation.

Lovatt *et al.* have demonstrated calorimetric dilution data for insulin: CyD systems (25 °C, pH 2.5) fitted to a model assuming a sequential binding of CyDs to at least two possible sites on the insulin monomer, with corresponding binding constants of 10—$20\,M^{-1}$ and $<5\,M^{-1}$ for high and low affinity sites, respectively.[18] Based on the results in Table 2 and on the inclusion ability for the three amino acids, G_2-β-CyD may include accessible hydrophobic amino acid residues such as phenylalanine and tyrosine in the N-terminal end (B1) and C-terminal region (B25 and B26) of the B-chain, these side chains having a high motional freedom,[31] while the side chains in the α-helices (A14 and B16) are not significantly perturbed in the presence of G_2-β-CyD.

In general, the topological constraints of the peptide backbone may reduce the formation of inclusion complexes.[28] The B24-phenylalanine is known to be directed toward the hydrophobic interior of the insulin molecule and its ring rotation is considerably restricted,[32] as indicated by a remarkable line broadening of the resonances (Fig. 6-A). Therefore, no noticeable change in the ^{1}H-NMR signal of the B24-phenylalanine by the addition of G_2-β-CyD may be ascribable to the difficulty of the CyD in gaining access to the folded side chain. Also evident was the deshielding of the C2 and C6 protons of the A19-tyrosine in the presence of G_2-β-CyD. Two-dimensional NMR studies have demonstrated that the B25-phenylalanine is flexible in solution and turns transiently toward the A-chain, touching a stably folded A19-tyrosine.[14] Accordingly, a possible explanation for the deshielding may reside in the perturbation of such intramolecular interaction between the side chains, probably through the inclusion of the B25-phenylalanine within the CyD cavity. These results suggest that G_2-β-CyD includes accessible hydrophobic side chains within the CyD cavity and hence perturbs the intermolecular hydrophobic contacts between aromatic side chains across the monomer-monomer interfaces, eventually leading to the inhibition of self-association of the peptide.

As the concentration of insulin increased, the B10/B5 ratio decreased, along with a remarkable line broadening of the main aromatic resonances. Furthermore, the proton signals for the B10, B16, B24 and B26 residues were shifted upfield, while those for the A14, B1, B5 and B25 residues were shifted in the opposite direction. These spectral changes suggest the dimerization of insulin forming an antiparallel β-sheet structure between the two C-terminal strands of the B chain.[1] Similar spectral changes are expected for the insulin: SBE7-β-CyD system, because SBE7-β-CyD enhanced the self-association of insulin at neutral pH. As shown in Fig. 6 and Table 2, even with the low concentration of SBE7-β-CyD, large chemical shift changes were observed for the B24 and B26 residues (upfield-shift) and the B1 residue (downfield-shift), while the B10/B5 ratio was not much changed. SBE4-β-CyD at a concentration of 0.5 mM showed a small but essentially identical pattern of ^{1}H-NMR chemical shift changes of insulin, as compared with those observed for SBE7-β-CyD (Table 2). These spectral changes may arise from the electrostatic interaction between the positive charges in insulin and the negative charges in SBE-β-CyD rather than the inclusion complexation, and are somewhat different from those derived from the concentration-dependent dimerization of the peptide. Further NMR studies are under way to elucidate the detailed mode of interaction of insulin with the CyD derivatives.

In conclusion, the present results clearly suggest that G_2-β-CyD and anionic β-CyDs interact with insulin in a varying manner, modifying the self-association and thermal behavior of the peptide, and hence a proper use of the CyD derivatives could be effective in designing rapid or long-acting insulin preparations.

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