The Binding and Catalytic Properties of a Positively Charged Cyclodextrin

Yoshihisa MATSUI* and Akihiko OKIMOTO

Department of Agricultural Chemistry, Shimane University, Nishikawazu, Matsue 690

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In order to learn the significance of electrostatic interactions between charged groups in an enzymatic process, a positively charged cyclodextrin, mono-(6-trimethylammonio-6-deoxy)-β-cyclodextrin hydrogencarbonate (βCDtma), was prepared as a simple enzyme model, and its binding and catalytic properties were examined. The dissociation constant for an inclusion complex of βCDtma with an azo dye, sodium 4-(4-hydroxy-1-naphthylazo)-1-naphthalenesulfonate (**1**), was found to be 5.4×10^{-5} mol/dm^3 in an alkaline solution (pH 10.5, I_c=0.0 mol/dm^3) at 25 °C, the value being smaller than that for an inclusion complex of the parent β-cyclodextrin (βCD) with **1** by a factor of *ca.* 4. The catalytic effect of βCDtma on the alkaline hydrolyses of *o*-, *m*-, and *p*-acetoxybenzoic acids was significantly different from that of βCD. These observations were explained in terms of electrostatic and hydrophobic interactions between βCDtma and a guest molecule.

Cyclodextrin (CD) is a cyclic oligomer composed of six (αCD), seven (βCD), or more α-D-glucopyranose units linked 1→4 as in amylose. The interior cavity of the doughnut-shaped molecule provides a relatively hydrophobic environment into which an apolar molecule can be trapped.[1,2] Since the rate and stereochemistry of a reaction for an organic substrate are significantly changed by the formation of a CD–substrate adduct, CD has been utilized as a simple model for an enzymatic process.[2-4] However, an enzyme is a macromolecular electrolyte, whereas CD contains no charged group in the molecule. Electrostatic interactions between charged groups play an important role in an enzymatic system.[5-7] For example, they contribute not only to strengthening the binding of a specific substrate to an enzyme, but also to orienting the substrate properly in the active site of the enzyme.[8]

In the present work, a positively charged CD, mono-(6-trimethylammonio-6-deoxy)-β-cyclodextrin hydrogencarbonate (βCDtma), was prepared as a simple model of an enzyme containing a positively charged group in the vicinity of the active site. This molecule bears two binding sites, one being the hydrophobic cavity of the βCD ring, and the other, the cationic part of the ammonium ion. Focusing our attention on the cooperative action of the two binding sites, we examined the binding and catalytic properties of the modified CD in an aqueous solution. In this connection, Tabushi *et al.*[9] have recently reported that a CD flexibly capped with a metal ion can effectively bind a hydrophobic anion in such a manner as to facilitate cooperative double recognition.

Experimental

Materials. The βCD of a reagent grade was recrystallized from water and dried overnight *in vacuo* at 110 °C. Pyridine of a reagent grade was dried over calcium hydride and was distilled in the presence of fresh calcium hydride just before use. *p*-Toluenesulfonyl chloride of a reagent grade was used without further purification. *N,N*-Dimethylformamide (DMF) of a reagent grade was dried over K_2CO_3 and distilled in the presence of calcium hydride *in vacuo*. A solution of trimethylamine in DMF was prepared by introducing the gaseous trimethylamine to DMF. The concentration of trimethylamine in DMF was determined by titration with 0.1 mol/dm^3 oxalic acid. An azo dye, sodium 4-(4-hydroxy-1-naphthylazo)-1-naphthalenesulfonate (**1**), was prepared and purified as has been described previously.[10] *o*-Acetoxybenzoic acid (**2o**) of a reagent grade was used without purification. The *m*- and *p*-acetoxybenzoic acids (**2m** and **2p** respectively) were prepared by the reactions of the corresponding hydroxybenzoic acids with acetic anhydride in dry pyridine and were recrystallized from chloroform.

Thin-layer chromatography (TLC) was performed on silica gel G (Merck) with a mixed solvent of acetic acid–chloroform–water (80: 10: 10 (v/v)). A solution of diphenylamine (0.1 g), aniline (0.1 ml), and 85% phosphoric acid (1 ml) in acetone (10 ml) was used as a spray reagent to detect the parent and the modified βCD.

Apparatus. The absorption and NMR spectra were recorded using a Hitachi Model 124 spectrophotometer and a JEOL Model JNM-MH-100 NMR spectrometer respectively. The pH of each aqueous solution was measured by means of an Orion Model 801A digital pH/mV meter.

Preparation of βCDtma. A solution of *p*-toluenesulfonyl chloride (3.65 g, 19.1 mmol) in dry pyridine (30 ml) was added to a solution of βCD (29.60 g, 26.1 mmol) in dry pyridine (300 ml) cooled below 5 °C. After stirring overnight at room temperature, the mixture was evaporated *in vacuo* at 40 °C to dryness. Diethyl ether (700 ml) was added to the residue. The precipitate was collected and recrystallized three times from water to afford 10.37 g (8.0 mmol; yield, 31% based on βCD) of mono-(6-*O*-*p*-tolylsulfonyl)-β-cyclodextrin (βCDots). The TLC of the product gave a spot with R_f 0.38 (*cf.* R_f 0.26 for βCD). The monosubstitution of βCD was confirmed by the PMR spectra of the product in DMSO-d_6.[11] The βCDots (10.27 g, 7.9 mmol) was dissolved in 300 ml of DMF containing 3.2 mol/dm^3 trimethylamine. After being heated overnight at 90—100 °C in sealed tube, the mixture was evaporated *in vacuo* to dryness. The residue was dissolved in 10 ml of water and then chromatographed on a 4.5×43 cm carboxymethylcellulose column, with 0.05 mol/dm^3 ammonium hydrogencarbonate buffer as the eluant. One hundred and sixty fractions were taken, each being 10 ml. The fractions were assayed for carbohydrate by TLC. Fractions which gave only one spot with R_f 0.15 (the fractions from No. 75 to No. 150) were combined and evaporated to dryness at 40 °C *in vacuo* to afford 4.07 g (3.3 mmol; yield, 42% based on βCDots) of the product, βCDtma, as a white powder. The NMR spectra of the product in D_2O showed absorptions at δ=3.05 due to the trimethyl hydrogens and at δ=4.97 due to the anomeric hydrogens of the glucopyranose rings. The relative areas of these peaks were about 9: 7, indicating the monosubstitution of βCD.

An apparent dissociation constant (K_d) for a βCDtma-**1**

inclusion complex and an apparent acid-dissociation constant (K_a) for **1** in both the absence and presence of βCDtma were spectrophotometrically determined as has been described previously.[10] The aqueous buffer solutions used were citric acid–Na_2HPO_4 (pH 3—7), KH_2PO_4–$Na_2B_4O_7$ (pH 6—9), and $Na_2B_4O_7$–NaOH (pH 9—12).

Kinetics. The rates of the alkaline hydrolysis of acetoxybenzoic acids (**2**) were measured by following the appearance of the absorptions of the corresponding phenoxide anions at 300, 310, and 280 nm for **2o**, **2m**, and **2p** respectively in alkaline aqueous solutions. The ionic strength (I_c) of the solution was maintained at 0.65 mol/dm³ with Na_2SO_4. In a typical run, 2.00 ml of a base solution was pipetted into a pair of 1.00-cm quartz cells, one of which was used as the reference cell, and the other, as the sample cell, in a spectrophotometer. After thermal equilibrium at 25.0±0.1 °C had been reached, 5 to 8 μl of a 0.06 to 0.14 mmol/dm³ ester solution in ethanol was added to the sample cell and the change in absorbance was followed. The results for various reaction times were treated according to the ordinary first-order rate equation. The rate constants were graphically determined. All the reactions examined obeyed good first-order kinetics with respect to substrates in both the absence and presence of βCDtma.

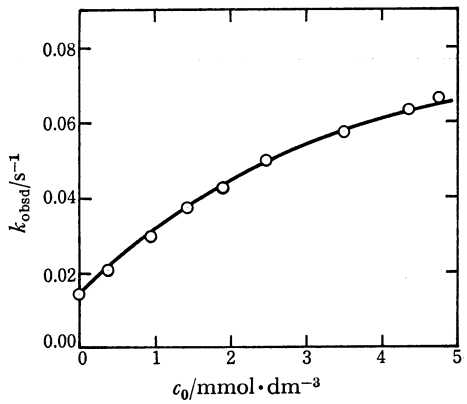

Fig. 1. The plot of k_{obsd} *vs.* c_0 for a βCDtma–**2m** system in 0.0105 mol/dm³ NaOH at 25 °C and I_c=0.65 mol /dm³.

Kinetic Determination of the Dissociation Constants and Rate Constants of βCDtma–2 Complexes. As is shown in Fig. 1 as an example, the observed first-order rate constant (k_{obsd}) for each substrate examined approached a maximum value as the βCDtma concentration (c_0) increased. This saturation behavior suggests that the rate process involves the prior formation of an inclusion complex of βCDtma with each substrate. Upon the assumption of the formation of a 1:1 βCDtma–**2** complex, k_{obsd} is represented by the following equation if c_0 is much higher than that of a substrate (s_0):

$$k_{obsd} = -\frac{K_d(k_{obsd}-k_{un})}{c_0} + k_c. \quad (1)$$

In this equation, k_d is the dissociation constant of an inclusion complex, and k_{un} and k_c, the rate constants for free and complexed substrates respectively. The plot of k_{obsd} *vs.* $(k_{obsd}-k_{un})/c_0$ was virtually linear for each βCDtma–**2** system; the values of K_d and k_c were calculated from the slope and intercept respectively of the line obtained by the use of the least-squares method.

Results and Discussion

Apparent Dissociation Constants for βCDtma–Azo Dye Inclusion Complexes. In order to learn the role of a positively charged group in the binding properties of βCDtma, the apparent dissociation constants (K_d) were determined for the βCDtma– and βCD–azo dye systems. Sodium 4-(4-hydroxy-1-naphthylazo)-1-naphthalenesulfonate (**1**) was used as an azo dye, since the dye shows a large spectral change upon being included within the βCD cavity.[10] In fact, the addition of βCDtma to an aqueous solution of **1** caused a large spectral change, one which is quite similar to that caused by the addition of the parent βCD. An isosbestic point was observed at 438 nm for a solution at pH 4.0 or at 445 nm for a solution at pH 10.5. These observations suggest that **1** forms a 1:1 inclusion complex with βCDtma in a manner similar to that with βCD in an aqueous solution. Table 1 shows the K_d values for the βCDtma– and βCD–**1** systems at the pH values of 4.0 and 10.5. The pK_a value of **1** is *ca.* 7.5 (see Table 2), so the acidic hydroxyl group of **1** is mostly undissociated at pH 4.0, while it is mostly dissociated at pH 10.5. The K_d value for a βCDtma–**1** system is larger than that for a βCD–**1** system at pH 4.0, whereas the former is smaller than the latter at pH 10.5.

Table 1. The K_d values for βCDtma– and βCD–**1** systems at 25 °C and I_c=0.03 mol/dm³

pH	K_d/(mmol/dm³)		$\frac{K_d(\beta CD)}{K_d(\beta CDtma)}$
	βCDtma	βCD	
4.0[a]	0.767	0.508	0.66
10.5[b]	0.096	0.195	2.03

a) Citric acid–Na_2HPO_4 buffer. b) $Na_2B_4O_7$–NaOH buffer.

The modification of βCD to the positively charged form resulted in a decrease in the stability of an inclusion complex with the undissociated form of **1**. A decrease in the hydrophobicity of the interior cavity of the CD ring, as well as a change in the geometry of the inclusion complex, with the modification of βCD may be the cause of the decrease in the stability of the inclusion complex. In this connection, we previously suggested[10] that the 4-hydroxy-1-naphthylazo moiety of **1**, rather than the 4-sulfonato-1-naphthylazo moiety, is bound to the βCD cavity, mainly through hydrophobic interactions. If βCDtma also includes the 4-hydroxy-1-naphthylazo moiety of **1**, a decrease in the hydrophobicity of the CD cavity, accompanied by the close location of a positive charge, may be responsible for the decrease in stability. In such a mode of binding, the negatively charged sulfonate group is too remote in position from the positive charge of βCDtma to enhance effectively the association of **1** with the host by means of the electrostatic force of attraction. It is also possible that the 4-sulfonato-1-naphthylazo moiety of **1**, instead of the 4-hydroxy-1-naphthylazo moiety, is included in the cavity of βCDtma. In this case, electrostatic interactions may contribute considerably to the association, while

the contribution of hydrophobic interactions to the association may significantly decrease. In either event, electrostatic interactions can scarcely cooperate with hydrophobic interactions in the present host-guest system.

On the other hand, the dissociated form of **1** is bound to the βCDtma *ca.* two times more strongly than the parent βCD. This fact suggests that electrostatic interactions between the positive charge of βCDtma and the negative charge of the naphthyloxide anion of **1** cooperate with hydrophobic interactions between the βCD cavity and the naphthylazo moiety in this case. A similar extent of binding enhancement due to electrostatic interactions has recently been reported in differently modified CD-substrate systems.[9]

Effect of the Ionic Strength on the K_d Values for the βCDtma- and βCD–1 Systems. In order to determine which electrostatic or hydrophobic interactions contribute more to the association of the modified βCD with **1**, the effect of the ionic strength (I_c) on the K_d values was examined for inclusion complexes at the pH values of 4.0 and 10.5. An increase in I_c generally weakens ion-ion and ion-dipole interactions,[12] while it strengthens hydrophobic interactions (salting-out effect).[13] Figures 2 and 3 show the plots of log K_d *vs.* $I_c^{1/2}$ for complexes of βCDtma and the parent βCD with **1** at pH 4.0 and at pH 10.5 respectively. Each plot is virtually linear. The K_d values increase with an increase in I_c for the βCDtma–**1** systems, whereas they decrease with an increase in I_c for the parent βCD–**1** systems at both pH's. These observations suggest that electrostatic, rather than hydrophobic, interactions play a dominant role in the association of βCDtma with **1**, while the reverse is true for the βCD–**1** systems. The extrapolation of the linear log K_d *vs.* $I_c^{1/2}$ plot to the intercept gives the K_d value ($K_d°$) at $I_c=0$ mol/dm³. The $K_d°$ values for the βCDtma– and βCD–**1** systems at pH 10.5 were equal to 5.4×10^{-5} and 2.2×10^{-4} mol/dm³ respectively, indicating that the βCDtma binds the anionic **1** four times more strongly than the parent βCD does at $I_c=0$ mol/dm³.

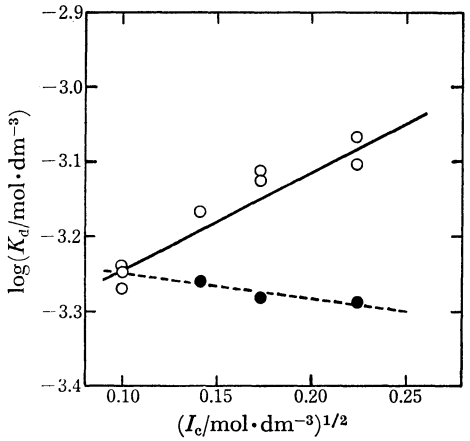

Fig. 2. The effect of I_c on the K_d value for βCDtma–**1** (○) and βCD–**1** (●) systems at pH 4.0 and 25 °C.

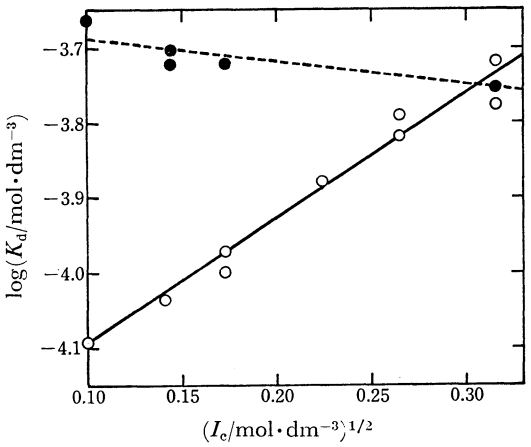

Fig. 3. The effect of I_c on the K_d value for βCDtma–**1** (○) and βCD–**1** (●) systems at pH 10.5 and 25 °C.

Table 2. The effect of βCDtma on the pK_a value of **1** at 25 °C and I_c=0.04 mol/dm³

[βCDtma] / mmol/dm³	pK_a
0.00	7.50
0.48	7.00
0.95	6.87

Effect of βCDtma on the Apparent pK_a *Values of* **1**. In a protein–[14] or micellar–[15] dye system, it has frequently been shown that charged groups around a binding site significantly affect the pK_a value of a bound dye. The apparent pK_a values for **1** in the absence and in the presence of βCDtma were spectrophotometrically measured at I_c=0.04 mol/dm³ (Table 2). The observed pK_a values decrease with an increase in the βCDtma concentration. A similar decrease in pK_a has been observed in a βCD–**1** system at I_c=0.05 mol/dm³.[10] However, the extent of the decrease in pK_a is greater in a βCDtma–**1** system than in a βCD–**1** system. For example, the addition of only 0.95 mmol/dm³ βCDtma lowered the pK_a from 7.50 to 6.87, while the addition of 2.02 mmol/dm³ βCD decreased the pK_a only to 7.02. The pK_a value for an inclusion complex between βCDtma and **1** was calculated, by the use of Eq. 7 in Ref. 10, to be 6.60 at I_c=0.04 mol/dm³ (*cf.* pK_a=6.97 for a βCD–**1** complex at I_c=0.05 mol/dm³ [10]). It is apparent that the acid dissociation of **1** is facilitated to a greater extent by the formation of an inclusion complex with βCDtma than by the formation of a complex with the parent βCD. This fact may also be related to the electrostatic action of a positive charge in βCDtma.

Effect of βCDtma on the Alkaline Hydrolysis of **2**. In order to learn the effect of a positive charge on the catalytic properties of βCDtma, kinetic parameters were determined for the alkaline hydrolysis of **2** in the absence and in the presence of βCDtma. The substrates bear a carboxylate anion in alkaline solutions, so that it can be anticipated that the catalytic action of the positively charged βCDtma is significantly different from that of the parent βCD. The parameters for βCDtma and βCD systems are compared with each other in Table 3.

Table 3. Kinetic and equilibrium parameters for βCDtma- and βCD-acetoxybenzoic acid systems in alkaline solutions at 25 °C and I_c=0.65 mol/dm³

Substrate	Catalyst	$10^3 k_{un}$ / s⁻¹	$10^3 k_c$ / s⁻¹	k_c/k_{un}	K_d / mmol/dm³
2o[a]	βCDtma	1.89	4.25	2.25	2.29
2o	βCD	1.89	14.5	7.67	12.4
2m[b]	βCDtma	14.4	136	9.44	6.39
2m	βCD	14.4	58.1	4.03	4.40
2p[c]	βCDtma	1.51	1.99	1.32	0.528
2p	βCD	1.51	1.93	1.28	2.08

a) 0.0098 mol/dm³ NaOH. b) 0.0105 mol/dm³ NaOH. c) pH 10.84 ($Na_2B_4O_7$–NaOH buffer).

The effects of the modification of βCD on the kinetic and equilibrium parameters for the alkaline hydrolyses of **2o**, **2m**, and **2p** are markedly different from one another. The K_d values for the βCDtma–**2o** and –**2p** systems are smaller than those for the parent βCD–**2o** and –**2p** systems respectively, while the K_d value for a βCDtma–**2m** system is larger than that for the corresponding βCD system. An increase in the electrostatic attraction force does not always result in an increase in the stability of a host-guest complex. It seems that the K_d value depends on the geometry of the inclusion complex, in which electrostatic interactions contribute either favorably or unfavorably to the stabilization of the complex.

The k_c value for a βCDtma–**2o** system is smaller than that for a parent βCD–**2o** system, while that for a βCDtma–**2m** system is larger than that for the corresponding βCD system. The k_c values for βCDtma- and βCD–**2p** systems are virtually equal to each other. The value of k_c/k_{un} is a measure of the proximity between the catalytic and the reaction sites of an inclusion complex.[16] The catalytic site of the complex is an alkoxide ion derived by the acid dissociation of a secondary hydroxyl group of βCD. The reaction site is an ester carbonyl carbon. The significant change in the k_c/k_{un} ratio of the **2o** and **2m** systems with the modification of βCD indicates that the geometries of the βCDtma–**2o** and –**2m** complexes are considerably different from those of the parent βCD–**2o** and –**2m** complexes respectively. The geometry of a βCDtma–**2p** complex may be virtually the same as that of a βCD–**2p** complex. In this connection, the geometries of the βCD–**2o**, –**2m**, and –**2p** inclusion complexes, as well as the changes in the geometries accompanied by the modification of βCD to βCDtma, were presumed to be as is shown in Figs. 4, 5, and 6 respectively; these results

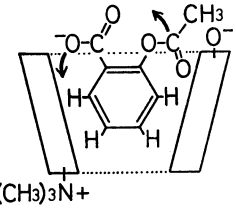

Fig. 4. The geometry of **2o** in the βCD cavity, suggested by the CPK molecular models.

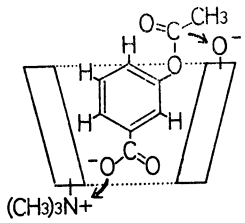

Fig. 5. The geometry of **2m** in the βCD cavity, suggested by the CPK molecular models.

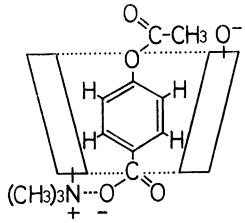

Fig. 6. The geometry of **2p** in the βCD cavity, suggested by the CPK molecular models.

were obtained by the use of the Corey-Pauling-Koltum molecular models. The acetyl groups of **2o** and **2m** may be located in the vicinity of the catalytically active alkoxide ion in their βCD complexes (the k_c/k_{un} values for these complexes are fairly large). In βCDtem–**2o** and –**2m** complexes, the carboxylate anions of the substrates may be electrostatically attracted to the cationic group of βCDtma, as is shown by the arrows in Figs. 4 and 5 respectively. At the same time, the acetyl group of **2o** may go far off from the alkoxide ion on the βCD ring, while that of **2m** may be brought closer to the alkoxide ion. In a βCD–**2p** system, the carboxylate anion of the substrate may lie close to the primary hydroxyl groups of βCD. The modification of one of the hydroxyl groups to the trimethylammonium group may cause little change in the geometry of the substrate in the βCD cavity. However, the electrostatic interaction between the ammonium cation and the carboxylate anion may serve to stabilize a complex of **2p** with βCDtma, compared with that of **2p** with βCD. Therefore, the above observations indicate that electrostatic interactions between a positive charge of βCDtma and a negative charge of a substrate affect not only the stability of the host-guest complex, but also the orientation of the guest in the host molecule. Although the detailed geometry of inclusion complexes remains to be determined, the present host-guest systems are interesting as simple model systems of an enzymatic process.

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