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Editor's Choice paper

Homotropic cooperativity of cyclodextrin dimer as an artificial hydrolase

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ABSTRACT

Three kinds of cyclodextrin (CD) dimer (one homo-dimer of β-CD and two types of hetero-dimer of α-CD and β-CD) were synthesized as artificial hydrolases. The initial rates of the cleavage reaction of *p*-nitrophenyl methoxyethoxyethoxyacetate (**8**) by each of the three dimers were measured under the condition of excess of substrate at 25 °C in a pH 7.4 phosphate buffer solution. In a plot of initial rate against concentration of the substrate, the reaction with the homo-dimer (**βCβH 5**) gave a sigmoidal curve, whereas the reaction with either of the hetero-dimers gave a simple hyperbolic curve. Only **βCβH 5** showed homotropic cooperativity, with a Hill constant of 1.8.

1. Introduction

Natural enzymes have mechanisms to control their activities in response to the concentration of substrates, products or other effectors [1,2]. In contrast, there have not been many reports of artificial enzymes that have mechanisms to control their activities, although many successful artificial enzymes with high catalytic activity or high substrate selectivity have been reported [3–15]. Thus, an important area of research in the field of supramolecular chemistry is the development of chemical methods to control the activities of artificial enzymes [16,17].

Allosteric regulation is a sophisticated way in which the activity of an enzyme can be controlled [18]. Some artificial heterotropic allosteric enzymes, with a mechanism to control their activity in response to an effector, have been reported, but few artificial homotropic allosteric enzymes, with a mechanism to control their activity towards the substrate itself, have been described [19,20]. Allosteric enzymes are made up of several subunits and are classified as either heterotropic or homotropic enzymes. A heterotropic allosteric enzyme has a binding site for an effector as well as a substrate-binding site. Binding of an effector to the effector-binding site in the subunit of a heterotropic allosteric enzyme changes the affinity of the substrate-binding site in the neighboring subunit. By contrast, a homotropic allosteric enzyme consists of some identical subunits with identical binding sites located in symmetrical positions. Binding of a first substrate to a homotropic allosteric enzyme facilitates the binding of a second substrate. Each subunit of a homotropic allosteric enzyme changes its tertiary structure on substrate binding, which affects the structure and chemical activity of its neighboring subunits. This structural change causes a sigmoidal dependence of the reaction rate on the substrate concentration.

The Monod–Wyman–Changeux (MWC) model provides a simple description of allosteric proteins [18]. This model minimizes the number of intermediate states, and thus is only an approximation of reality; however, this simplification is also its virtue. The MWC model provides a simple framework to rationalize experiments or explain phenomena and can also assist in the molecular design of artificial allosteric proteins. The MWC model makes the following assumptions. (1) Allosteric proteins are oligomers composed of identical monomers (or 'protomers') that are associated in such a way that they all occupy equivalent positions. (2) Only one site on each protomer binds to each ligand that is able to form a stereospecific complex. (3) The protein exists in either of two conformational states, the T or R state. The two states are in equilibrium, but the T state predominates when the protein is unliganded. They differ in the energies and numbers of bonds between the subunits, with the T state being more constrained than the R state. (4) The T state has a lower affinity for ligands than the R state. (5) All binding sites in each state are equivalent and have identical binding constants. When the protein switches from one state to another, its molecular symmetry is conserved (the symmetry assumption).

The MWC model for a dimeric enzyme is illustrated in Fig. 1. Its homotropic activity can be explained by an equilibrium argument. In the absence of a substrate the enzyme is mainly in the T state; however, the conformational equilibrium is shifted to the right (the R state) when the substrate binds to the enzyme, because the binding ability of the R state is larger than that of the T state. This structural change induced by the substrate binding causes

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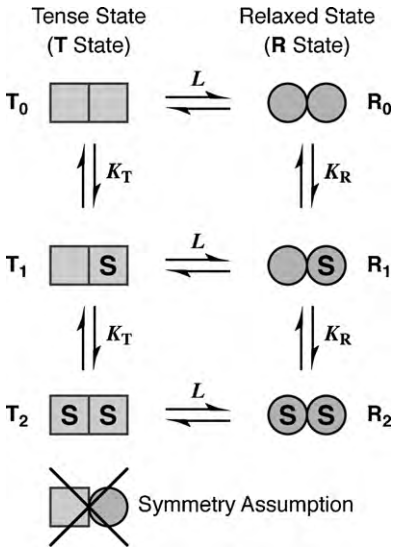


Fig. 1. MWC model for an allosteric enzyme.

sigmoidal dependence of the initial rate on the substrate concentration.

In terms of the MWC model, a host molecule that has two equal-sized binding sites at the symmetrical positions is expected to show an allosteric effect, if the binding of the first substrate to a binding site can improve the affinity for the second substrate. We previously prepared a cyclodextrin (CD) dimer (designated **βCβH**) that has a catalytic site located between two β-cyclodextrins (β-CDs) and acts as an artificial hydrolase [21]. **βCβH** shows a large acceleration ability and substrate specificity for the acyl chain length of the substrate, when *p*-nitrophenyl alkanoates are used as the substrate. This result indicates that there is some cooperativity between the two CD cavities of the CD dimer. New water-soluble substrates and two kinds of CD hetero-dimers were prepared and the catalytic activities of the CD homo-dimer and two kinds of CD hetero-dimers were studied. Here we have investigated the homotropic allosteric-like effect of **βCβH**, in comparison with cyclodextrin hetero-dimers that have a catalytic site located between a β-CD unit and an α-CD unit.

2. Experimental

2.1. General

Thin-layer chromatography (TLC) was carried out with silica gel 60 F_{254} (Merck Co.). Absorption spectra were recorded on a Shimadzu UV-3100 spectrometer. NMR spectra were recorded in D_2O at 25 °C on Varian VXR-500S and UNITY plus-400 spectrometers operating at 499.843 MHz and 399.973 MHz, respectively for ^{1}H. HDO (δ = 4.70 ppm) and $CDCl_3$ (δ = 7.26 ppm) were used as internal standards. Mass spectrometry was performed on a Shimadzu MALDI III mass spectrometer (TOFMS). Elemental analyses were performed by the Analytical Division in Research Laboratory of Resources Utilization of Tokyo Institute of Technology.

2.2. Materials

α- and β-Cyclodextrin were kind gifts from Nihon Shokuhin Kako Co., Ltd. All chemicals were of reagent grade and were used without further purification unless otherwise noted. Distilled water and acetonitrile used as solvents for spectroscopy were special fluorometry grade (Uvasol) from Kanto Chemicals. Deuterium oxide, with an isotopic purity of 99.95%, was purchased from Merck Co.

2.2.1. Synthesis of 6-carboxymethylthio-6-deoxy-α-cyclodextrin, α***C***, ***1***

Methyl sodium sulfidoacetate (2.27 g, 17.7 mmol) was added to a solution of 6-deoxy-6-iodo-α-cyclodextrin (6.33 g, 5.85 mmol) in DMF (90 mL). The resulting mixture was stirred at 80 °C for 72 h. After being cooled to room temperature, the reaction mixture was poured into acetone, and the precipitate was collected and dried in vacuo. A solution of the crude product in water (30 mL) was mixed with 1 M NaOH (10 mL), and was stirred at 0 °C for 1 h. After being warmed to room temperature, the reaction mixture was charged on SP Sephadex C-25 (H^+ form). The eluent was charged on QAE Sephadex A-25 (OH^- form). The eluent containing pure product was obtained with a linear gradient of 0–0.5 M of NH_4HCO_3 aqueous solution (2.20 g, 36% yield). ^{1}H NMR (500 MHz, D_2O, 25 °C): δ = 2.84 (dd, 1H, S-CH_2(6b)), 3.12 (dd, 1H, S-CH_2(6a)), 3.27 (d, 1H, S-CH_2-CO), 3.34 (d, 1H, S-CH_2-CO), 4.96–5.06 ppm (m, 6H, CH(1)); MS (TOFMS, *m*/*z*): $[M+Na]^+$ calcd 1069.3, found 1069.2; Anal. calcd for $C_{38}H_{62}O_{31}S.3H_2O$: C 41.45, H 6.23, S 2.91. Found: C 41.21, H 6.52, S 2.66.

2.2.2. Synthesis of 6-carboxymethylthio-6-deoxy-β-cyclodextrin, β***C***, ***2***

This compound was prepared by the condensation of 6-deoxy-6-iodo-β-cyclodextrin (13.4 g, 10.8 mmol) and methyl sodium sulfidoacetate (4.15 g, 32.4 mmol) by the same method used for **1** (8.22 g, 63% yield). ^{1}H NMR (500 MHz, D_2O, 25 °C): δ = 2.85 (dd, 1H, S-CH_2(6b)), 3.08 (dd, 1H, S-CH_2(6a)), 3.34 (s, 2H, S-CH_2-CO), 4.97–5.11 ppm (m, 7H, CH(1)); MS (TOFMS, *m*/*z*): $[M+Na]^+$ calcd 1231.3, found 1231.3; Anal. calcd for $C_{44}H_{72}O_{36}S.5H_2O$: C 40.68, H 6.36, S 2.47. Found: C 40.94, H 6.61, S 2.64.

2.2.3. Synthesis of 6-deoxy-6-(L-histidylamino)-α-cyclodextrin, α***H***, ***3***

N^{α}-*tert*-Butyloxycarbonyl-N^{im}-tosyl-L-histidine (3.30 g, 8.06 mmol) was added to a solution of 6-amino-6-deoxy-α-cyclodextrin (3.45 g, 3.55 mmol) in DMF (20 mL). After the solution was cooled below 0 °C, *N*,*N*-dicyclohexylcarbodiimide (900 mg, 4.36 mmol) and 1-hydroxybenzotriazole (343 mg, 2.54 mmol) were added. This resulting mixture was stirred at 0 °C for 2 h and then at room temperature for 2 days. The insoluble materials were removed by filtration, the filtrate was poured into acetone, and the precipitate was collected and dried in vacuo. The crude product was dissolved in a mixed solvent of methanol/water (5:1) and was refluxed for 2 h. The reaction mixture was poured into acetone, and the precipitate was collected and dried in vacuo. The crude product, 6-(N^{α}-*tert*-butyloxycarbonyl-N^{im}-tosyl-L-histidylamino)-6-deoxy-α-cyclodextrin, was dissolved with DMF (10 mL) and cooled to 0 °C. This solution was reacted with 1 M NaOH aqueous solution (5 mL) at 0 °C for 1 h. The reaction mixture was neutralized with 1 M HCl aqueous solution and poured into acetone, and then the precipitate was collected and dried in vacuo. The crude product, 6-(N^{α}-*tert*-butyloxycarbonyl-L-histidinylamino)-6-deoxy-α-cyclodextrin, was dissolved with 30 mL of CF_3COOH/CH_2Cl_2 (9:1) and stirred at 0 °C for 1 h. After evaporation of CF_3COOH and CH_2Cl_2, the crude product was dissolved in water (1 L) and charged on SP Sephadex C-25 (H^+ form). After washing with water (5 L), the crude product was eluted with 1 M NH_3 aqueous solution (750 mL). The crude product was purified by column chromatography on CM-Sephadex C-25 (NH^{4+} form) with a linear gradient of 0–0.09 M of NH_4HCO_3 aqueous solution. The eluent containing the pure product was concentrated to give the desired product (1.30 g, 33% yield). ^{1}H NMR (500 MHz, D_2O, 25 °C): δ = 2.85 (m, 2H, Im-CH_2), 3.04 (t, 1H, CH(4′)), 4.94–5.02 (m, 6H, CH(1)), 6.90 (s, 1H, Im), 7.66 ppm (s, 1H, Im); MS (TOFMS, *m*/*z*): $[M+Na]^+$ calcd 1131.4, found: 1131.3; Anal. calcd for

$C_{42}H_{68}O_{30}N_4.3H_2O$: C 43.37, H 6.41, N 4.82. Found: C 43.64, H 6.66, N 4.91.

2.2.4. Synthesis of 6-deoxy-6-(L-histidylamino)-β-cyclodextrin, βH, 4

This compound was prepared from 6-amino-6-deoxy-β-cyclodextrin (4.50 g, 3.97 mmol) by the same method used for **3** (1.82 g, 36% yield). ^{1}H NMR (500 MHz, D_2O, 25 °C): δ = 2.78 (m, 2H, Im-CH_2), 3.10 (t, 1H, H(4′)), 3.23 (dd, 1H, NH-CH_2(6b)), 4.92–5.00 (m, 7H, CH(1)), 6.77 (s, 1H, Im), 7.57 ppm (s, 1H, Im); MS (TOFMS, *m*/*z*): $[M+Na]^+$ calcd 1293.4, found: 1293.4; Anal. calcd for $C_{48}H_{78}O_{35}N_4.5H_2O$: C 42.35, H 6.52, N 4.12. Found: C 42.25, H 6.24, N 4.07.

2.2.5. Synthesis of 6-{N-[6-(6-deoxy-β-cyclodextrinylthio) acetyl]-L-histidylamino}-6-deoxy-β-cyclodextrin, βCβH, 5

N,*N*-Dicyclohexylcarbodiimide (80.3 mg, 0.389 mmol) and 1-hydroxybenzotriazole (52.7 mg, 0.390 mmol) were added to a solution of 6-deoxy-6-(L-histidylamino)-β-cyclodextrin (450 mg, 0.354 mmol) and 6-carboxymethylthio-6-deoxy-β-cyclodextrin (855 mg, 0.707 mmol) in DMF (10 mL) at 0 °C. This solution was stirred at 0 °C for 2 h and then at room temperature for 48 h. The insoluble materials were removed by filtration, the filtrate was poured into acetone, and the precipitate was collected and dried in vacuo. The crude product was purified by column chromatographies on CM-Sephadex C-25 (H^+ form and NH^{4+} form) to give the desired product (573 mg, 66% yield). ^{1}H NMR (500 MHz, D_2O, 25 °C): δ = 2.71 (dd, 1H, S-CH_2(6b)), 2.90 (dd, 1H, S-CH_2(6a)), 2.96 (dd, 1H, Im-CH_2), 3.03 (dd, 1H, Im-CH_2), 3.19 (t, 1H, CH(4′)), 3.29 (d, 1H, S-CH_2-CO), 3.34 (d, 1H, S-CH_2-CO), 3.37 (dd, 1H, NH-CH_2(6b)), 4.90–5.04 (m, 14H, CH(1)), 6.92 (s, 1H, Im), 7.67 ppm (s, 1H, Im); MS (TOFMS, *m*/*z*): $[M+Na]^+$ calcd 2483.8, found: 2483.7; Anal. calcd for $C_{92}H_{148}O_{70}N_4S.15H_2O$: C 40.44, H 6.57, N 2.05, S 1.17. Found: C 40.36, H 6.54, N 1.98, S 1.13.

2.2.6. Synthesis of 6-{N-[6-(6-deoxy-α-cyclodextrinylthio) acetyl]-L-histidylamino}-6-deoxy-β-cyclodextrin, αCβH, 6

This compound was prepared by the condensation of **1** (489 mg, 0.467 mmol) and **4** (300 mg, 0.236 mmol) by the same method used for **5** (419 mg, 77% yield). ^{1}H NMR (500 MHz, D_2O, 25 °C): δ = 2.73 (dd, 1H, S-CH_2(6b)), 3.01 (dd, 1H, S-CH_2(6a)), 2.95 (dd, 1H, Im-CH_2), 3.03 (dd, 1H, Im-CH_2), 3.17 (t, 1H, CH(4′)), 3.30 (d, 1H, S-CH_2-CO), 3.36 (d, 1H, S-CH_2-CO), 3.39 (dd, 1H, NH-CH_2(6b)), 4.89–5.04 (m, 13H, CH(1)), 6.92 (s, 1H, Im), 7.67 ppm (s, 1H, Im); MS (TOFMS, *m*/*z*): $[M+Na]^+$ calcd 2321.7, found: 2321.9; Anal. calcd for $C_{86}H_{138}O_{65}N_4S.14H_2O$: C 40.47, H 6.56, N 2.20, S 1.26. Found: C 40.44, H 6.30, N 2.26, S 1.44.

2.2.7. Synthesis of 6-{N-[6-(6-deoxy-β-cyclodextrinylthio) acetyl]-L-histidylamino}-6-deoxy-α-cyclodextrin, βCαH, 7

This compound was prepared by the condensation of **2** (436 mg, 0.361 mmol) and **3** (300 mg, 0.271 mmol) by the same method used for **5** (439 mg, 71% yield). ^{1}H NMR (500 MHz, D_2O, 25 °C): δ = 2.69 (dd, 1H, S-CH_2(6b)), 2.91 (dd, 1H, S-CH_2(6a)), 2.94 (dd, 1H, Im-CH_2), 3.05 (dd, 1H, Im-CH_2), 3.12 (t, 1H, CH(4′)), 3.29 (d, 1H, S-CH_2-CO), 3.34 (d, 1H, S-CH_2-CO), 4.85–5.02 (m, 13H, CH(1)), 6.96 (s, 1H, Im), 7.66 ppm (s, 1H, Im); MS (TOFMS, *m*/*z*): $[M+Na]^+$ calcd 2321.7, found: 2321.6; Anal. calcd for $C_{86}H_{138}O_{65}N_4S.10H_2O$: C 41.65, H 6.42, N 2.26, S 1.29. Found: C 41.70, H 6.12, N 2.27, S 1.06.

2.2.8. Synthesis of p-nitrophenyl 2-[2-(2-methoxyethoxy) ethoxy]acetate, 8

p-Nitrophenol (5.0 g, 35.9 mmol), 2-[2-(2-methoxyethoxy) ethoxy]acetic acid (8.3 g, 46.6 mmol), and 1,3-dicyclohexylcarbodiimide (14.8 g, 71.7 mmol) were reacted in tetrahydrofuran (100 mL) at 0 °C overnight. A precipitate that formed in the reaction mixture was filtered off. After evaporation of the solvent, the product was purified with a column chromatography on silica gel (elution with chloroform/ethyl acetate (10:1)) to give the desired product (9.25 g, 86% yield). ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 3.38 (s, 3H, OCH_3), 3.56 (m, 2H, OCH_2), 3.66 (m, 2H, OCH_2), 3.74 (m, 2H, OCH_2), 3.84 (m, 2H, OCH_2), 4.46 (s, 2H, $COCH_2$), 7.33 (d, 2H, Ar), 8.28 ppm (d, 2H, Ar); Anal. calcd for $C_{13}H_{17}O_7N$: C 52.17, H 5.73, N 4.68. Found: C 52.15, H 5.70, N 4.68.

2.2.9. Synthesis of m-nitrophenyl 2-[2-(2-methoxyethoxy) ethoxy]acetate, 9

m-Nitrophenol (5.0 g, 35.9 mmol), 2-[2-(2-methoxyethoxy) ethoxy]acetic acid (8.3 g, 46.6 mmol), and 1,3-dicyclohexylcarbodiimide (14.8 g, 71.7 mmol) were reacted by the same method used for **8** (9.0 g, 84% yield). ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 3.39 (s, 3H, OCH_3), 3.57 (m, 2H, OCH_2), 3.68 (m, 2H, OCH_2), 3.74 (m, 2H, OCH_2), 3.85 (m, 2H, OCH_2), 4.43 (s, 2H, $COCH_2$), 7.49 (m, 1H, Ar), 7.57 (t, 1H, Ar), 8.02 (t, 1H, Ar), 8.12 ppm (m, 1H, Ar); Anal. calcd for $C_{13}H_{17}O_7N$: C 52.17, H 5.73, N 4.68. Found: C 52.16, H 5.74, N 4.66.

2.2.10. Synthesis of p-nitrophenyl {2-[2-(2-methoxyethoxy) ethoxy]ethyl} ether, 10

2-[2-(2-Methoxyethoxy)ethoxy]ethyl *p*-toluensulfonate (5.7 g, 17.9 mmol), *p*-nitrophenol (2.49 g, 17.9 mmol), and potassium carbonate (4.96 g, 35.9 mmol) were refluxed in acetone (130 mL) for 24 h. After potassium carbonate was filtered off and the solvent was evaporated, the product was purified with a column chromatography on silica gel (elution with chloroform/ethyl acetate (7: 1)) to give the desired product (7.08 g, 90% yield). ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 3.38 (s, 3H, OCH_3), 3.55 (m, 2H, OCH_2), 3.65 (m, 2H, OCH_2), 3.68 (m, 2H, OCH_2), 3.89 (m, 2H, OCH_2), 4.22 (s, 2H, $COCH_2$), 6.98 (d, 2H, Ar), 8.20 ppm (d, 2H, Ar); Anal. calcd for $C_{13}H_{19}O_6N$: C 54.73, H 6.71, N 4.91. Found: C 54.72, H 6.71, N 4.91.

2.3. Kinetics

Hydrolysis reactions were followed by monitoring the appearance of *p*-nitrophenol or *m*-nitrophenol spectrophotomerically using a Shimadzu UV-3100 spectrometer. The reaction was conducted in a quartz cell in the water-jacketed cell holder of UV-3100. Temperature was maintained at 25 °C by a HAAKE F3 circulating water bath. The reaction was initiated by adding a stock solution of an ester in acetonitrile to a buffer solution in the quartz cell using a HAMILTON microliter syringe. The total amount of acetonitrile in the cell was adjusted to be same in all runs by adding an amount of acetonitrile before addition of the substrate. The pH of the reaction mixture did not change during the course of the reaction. The rates used in the calculation of rate constants were averages of at least three determinations that agreed within 3%.

3. Results and discussion

3.1. Catalytic activities of artificial enzymes (4–7)

In our previous results, cooperativity between the two CD cavities of the CD homo-dimer (**βCβH**) was implied by the dependence of the reactivity of the homo-dimer on the acyl chain length of the substrate in the cleavage reaction of *p*-nitrophenyl alkanoates [21], however, the cooperativity of this reactivity with the alkanoate esters could not be examined in detail owing to the low solubility of the alkanoate esters in water. We therefore synthesized a new substrate ester **8** (Chart 1) that is more soluble in water, and studied the catalytic activity of the CD homo-dimer (**βCβH**) under the condition of high substrate concentration. We also synthesized two kinds of hetero-dimer (**αCβH 6** and **βCαH 7**) by the same method

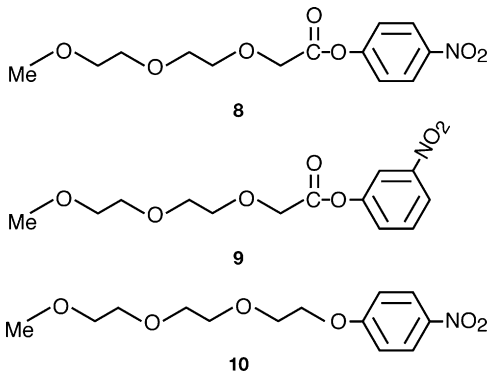
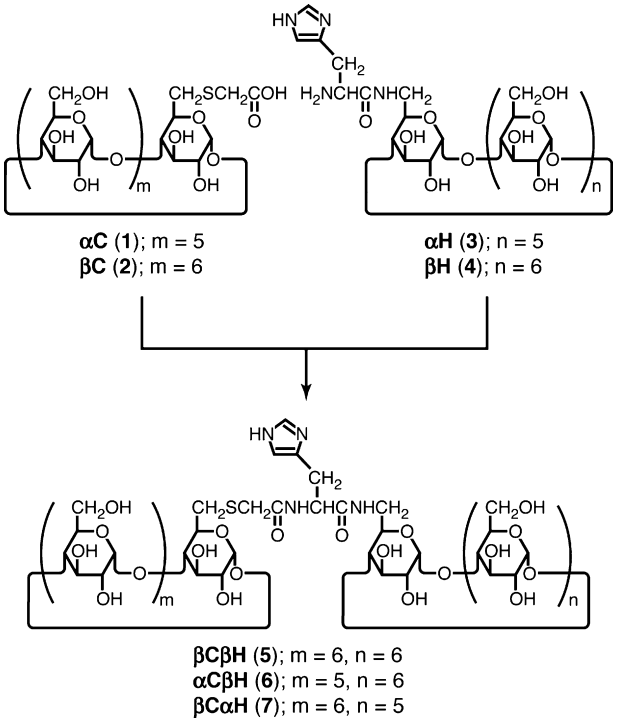


Chart 1. New water-soluble substrates and potential inhibitor for hydrolysis reaction.



Scheme 1. Syntheses of the homo-dimer (**βCβH 5**) and the hetero-dimers (**αCβH 6** and **βCαH 7**).

used for the homo-dimer (Scheme 1) and their reactivities were compared with the homo-dimer (**βCβH 5**).

The initial rates for the cleavage reaction of the *p*-nitrophenyl ester **8** catalyzed by a CD monomer (**βH 4**) and the CD homo-dimer (**βCβH 5**) were measured under the condition of excess substrate (a 5- to 50-fold excess over the artificial enzymes) at 25 °C in a pH 7.4 phosphate buffer solution. The reaction was followed by monitoring the appearance of *p*-nitrophenolate at 400 nm. The plot of the initial rate of the cleavage reaction of the substrate ester **8** by

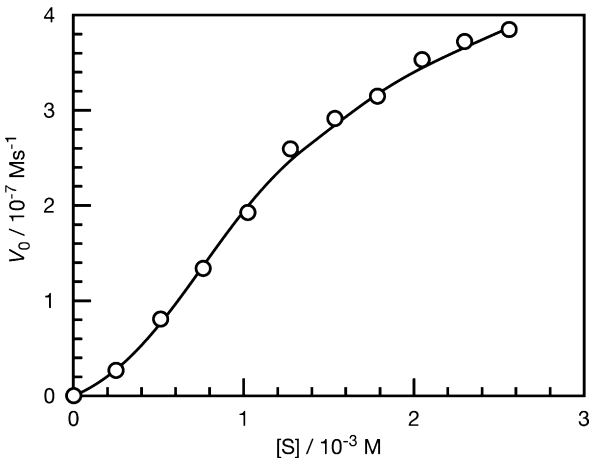


Fig. 2. Dependence of the initial rate on the substrate concentration for the cleavage of the substrate **8** by **βCβH 5** (4.8×10^{-5} M) in pH 7.4 phosphate buffer (0.1 M) at 25 °C.

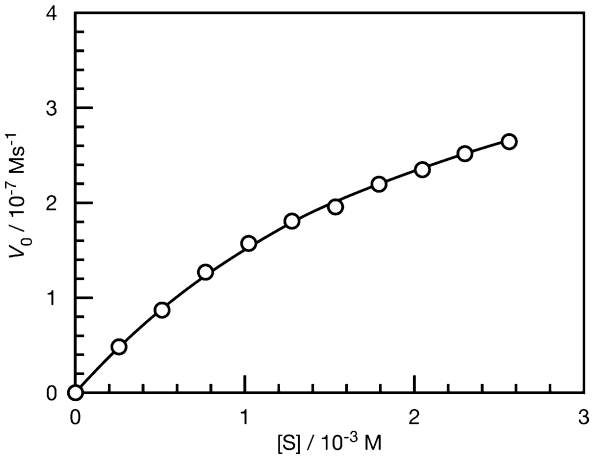


Fig. 3. Dependence of the initial rate on the substrate concentration for the cleavage of the substrate **8** by **βH 4** (4.8×10^{-5} M) in pH 7.4 phosphate buffer (0.1 M) at 25 °C.

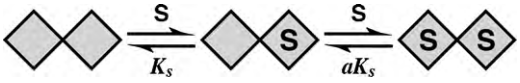
the homo-dimer **βCβH 5** against substrate concentration gave a sigmoidal curve, as shown in Fig. 2, whereas the plot of the initial rate of the cleavage reaction by **βH 4** gave a simple hyperbolic curve, as shown in Fig. 3. Thus, the observed sigmoidal behaviour is due to the reactivity of **βCβH 5** rather than to that of the ester **8**, because **βH 4** shows simple saturation behaviour for the same substrate.

The kinetic data for **βCβH 5** were fitted to the Hill equation (Eq. (1)) [22,23] as shown in Fig. 2, to give a Hill constant of 1.8 (Table 1). This result means that high cooperativity exists between the two β-CD cavities of the homo-dimer **βCβH 5**, and the Hill constant of 1.8 indicates that a 1:2 complex of the homo-dimer and the substrate **8** is the most reactive intermediate. The kinetic data for **βH 4** were fitted to the Michaelis–Menten equation (Eq. (2)) and were also

Table 1
Michaelis–Menten's parameters and Hill's parameters for the cleavage of the ester (**8**) by **βH**, **βCβH**, **αCβH**, or **βCαH**[a].

	Michaelis–Menten's Parameters			Hill's Parameters			
	k_{cat} [10^{-2} s^{-1}]	K_m [10^{-3} M]	*r*	k_{cat} [10^{-2} s^{-1}]	K' [10^{-4} M]	*n*	*r*
βH	1.10 ± 0.10	2.43 ± 0.40	0.998	0.900 ± 0.238	5.92 ± 0.22	1.16 ± 0.25	0.999
βCβH	–	–	–	1.05 ± 0.11	0.0656 ± 0.0012	1.80 ± 0.22	0.999
αCβH	1.48 ± 0.56	5.08 ± 0.26	0.997	0.824 ± 0.048	12.5 ± 0.8	1.15 ± 0.53	0.998
βCαH	0.837 ± 0.026	4.77 ± 0.20	0.997	0.612 ± 0.053	13.0 ± 0.6	1.13 ± 0.47	0.998

[a] At 25 °C in pH 7.4 phosphate buffer (0.1 M); [**8**] = 2.5×10^{-4}–2.5×10^{-3} M, [**βH**] = [**βCβH**] = [**αCβH**] = [**βCαH**] = 4.8×10^{-5} M.



Scheme 2. Cooperative binding of substrates to the enzyme.

fitted to the Hill equation (Table 1). The Hill constant for **βH 4** is 1.1, which means that the 1:2 complex does not form in the case of **βH 4**.

$$V_0 = \frac{k_{cat}[E]_0[S]^n}{K' + [S]^n} \quad (1)$$

$$V_0 = \frac{k_{cat}[E]_0[S]}{K_m + [S]} \quad (2)$$

The dissociation constant of the homo-dimer and the second substrate is given by aK_s, and K' of Eq. (1) is given by $a^{n-1}K_s^n$, where K_s is the dissociation constant of the homo-dimer for the first substrate and a is the cooperativity coefficient (Scheme 2) [22,23]. If K_s for **βCβH 5** is equal to K_m for **βH 4**, i.e., 2.43×10^{-3}, then a is calculated to be 0.25 for **βCβH 5**. This value shows that the second dissociation constant (aK_s) of **βCβH 5** is 0.25 times the value of its first dissociation constant (K_s); in other words, the second binding constant ($1/aK_s$) of **βCβH 5** is four times larger than the first binding constant ($1/K_s$). Thus, the cooperative behaviour of the two cavities makes the binding ability of **βCβH** greater for the second substrate than for the first substrate. The following experiments were performed to elucidate the mechanism of this allosteric-like reaction behaviour of **βCβH**.

The initial rates of the cleavage reaction of the *p*-nitrophenyl ester **8** by hetero-dimers **αCβH 6** and **βCαH 7** were measured under the same conditions used for **βH 4** and **βCβH 5**. The hetero-dimers **αCβH 6** and **βCαH 7** showed simple saturation behaviour rather than sigmoidal behaviour (Fig. 4). Table 1 shows the kinetic parameters of **4**–**7** for the cleavage reactions, which were obtained by fitting the kinetic data to ether the Hill equation (Eq. (1)) or the Michaelis–Menten equation (Eq. (2)). The Hill constants of **αCβH 6** and **βCαH 7** are approximately 1. The k_{cat} values of **4**–**7** do not differ much, but the Hill constant of **5** is twice as large as that of the others. The overall apparent binding ability ($1/K'$) of **βCβH 5** is 200 times larger than that of **αCβH 6** or **βCαH 7**. Among the artificial enzymes, only **βCβH 5** has a large binding ability for the second substrate and demonstrates high cooperativity for substrate binding. During the reaction, the *p*-nitrophenyl moiety is located in one of the two cavities and the ethylene glycol chain is located in the other cavity of the dimer. The ethylene glycol chain of the ester acts as a spacer to stabilize the complex of the homo-dimer with the second substrate. Because the α-CD cavity is too small to hold both the phenyl moiety and the ethylene glycol chain, the hetero-dimer cannot make an inclusion complex with the second substrate. The important point of these results is that two β-CD cavities caused the sigmoidal behaviour observed for the cleavage reaction of the ester **8**, in contrast, α- and β-CD cavities did not cause such a behaviour. This means that the formation of the 1:2 host/guest complex is important for the sigmoidal behaviour. k_{cat} of **αCβH 6** or **βCαH 7** are not same, because the imidazole moiety is not located at the center of the dimer **6** or **7**. The reactivities of the reaction intermediates depend on both the distance and the angle between the imidazole moiety and the ester carbonyl group of the substrate **8**. Therefore, the reactivity of the dimer **6** is different from that of the dimer **7**, and their k_{cat} are not same, because k_{cat} depends on the reactivity of the reaction intermediate. On the other hand, the binding ability of the dimer mainly depends on its cavity size. So, K_m of the dimer **6** is similar to that of the dimer **7**. The Hill constant is correlated to the binding ability in this case and the Hill constant of the dimer **6** is similar to that of the dimer **7**.

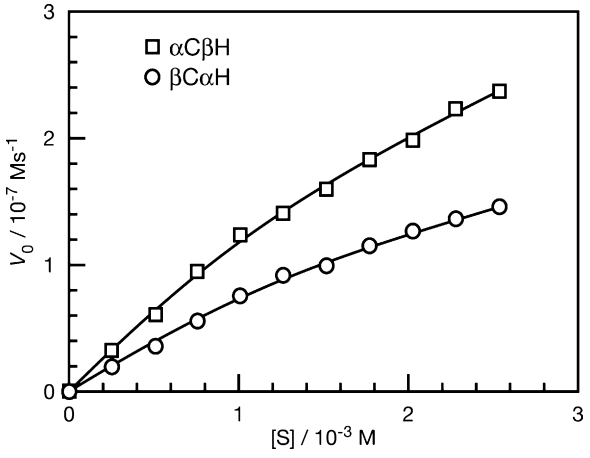


Fig. 4. Dependence of the initial rate on the substrate concentration for the cleavage of the substrate **8** by **αCβH 6** (4.8×10^{-5} M) and **βCαH 7** (4.8×10^{-5} M) in pH 7.4 phosphate buffer (0.1 M) at 25 °C.

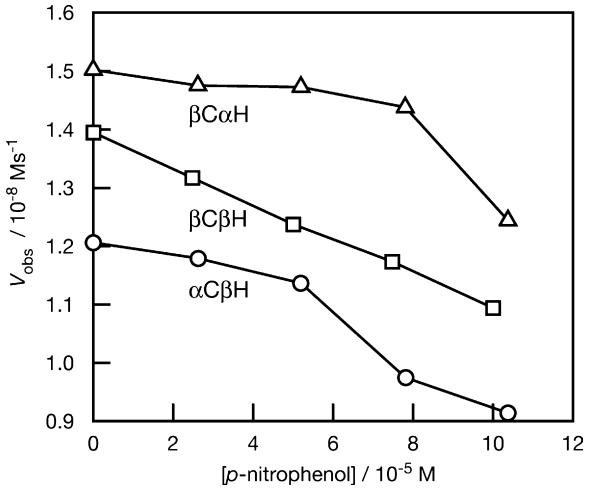


Fig. 5. Effect of *p*-nitrophenol on the initial rates of cleavage of the substrate **8** (4.3×10^{-5} M) by the CD dimers **5**–**7** (4.0×10^{-5} M) in pH 7.4 phosphate buffer (0.1 M) at 25 °C.

We carried out an inhibition experiment using *p*-nitrophenol to confirm that both two cavities in the dimer are involved in the reaction. The initial rate of the cleavage reaction of the *p*-nitrophenyl ester **8** catalyzed by **βCβH 5** linearly decreased with an increasing concentration of *p*-nitrophenol (Fig. 5). The initial rate of the reaction catalyzed by α**CβH 6** or **βC**α**H 7** scarcely changed at low concentrations of *p*-nitrophenol, but linearly decreased at high concentrations of *p*-nitrophenol (Fig. 5). These results indicate that both cavities in the dimers take part in the reactions and that the two cavities in **βCβH 5** participate in the reaction to the same degree. These results also show that the binding abilities of the two cavities for *p*-nitrophenol are not equivalent in the hetero-dimers, whereas the two cavities of **βCβH 5** have same binding ability for *p*-nitrophenol. This can be explained as follows. pK_a of *p*-nitrophenol is 7.08 and *p*-nitrophenol already dissociates in the experiment condition. It is reported that the binding constants of α-CD and β-CD for *p*-nitrophenolate are 3550 and 944 M^{-1}, respectively [24]. Therefore, occupation of the β-CD cavity of the hetero-dimer by the substrate will not be affected by a low concentration of *p*-nitrophenol and the reaction rate will not be decreased. When a larger quantity of *p*-nitrophenol is added, however, the β-CD cavity is filled with *p*-nitrophenol and the reaction rate is slowed. By contrast, the rate of the reaction catalyzed by the homo-dimer is slowed even by low concentrations of *p*-nitrophenol, because the forma-

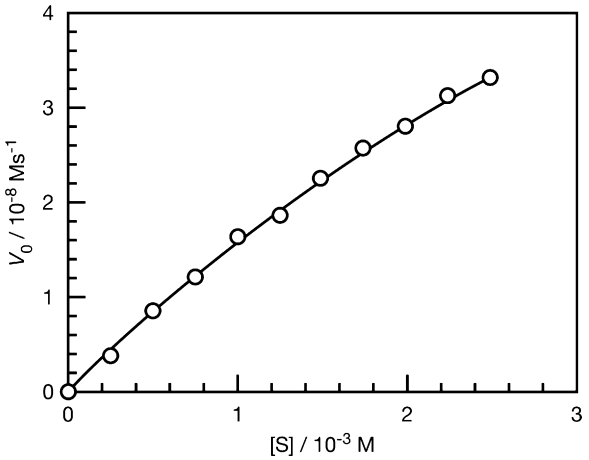


Fig. 6. Dependence of the initial rate on the substrate concentration for the cleavage of *p*-nitrophenyl acetate by **βCβH 5** (4.8×10^{-5} M) in pH 7.4 phosphate buffer (0.1 M) at 25 °C.

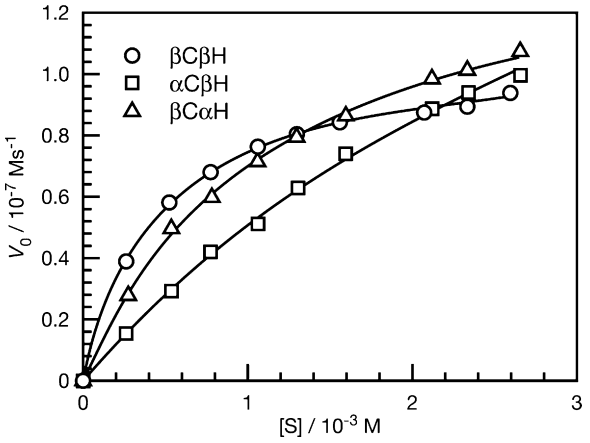


Fig. 7. Dependence of the initial rate on the substrate concentration for the cleavage of the substrate **9** by the CD dimers **5**–**7** (4.8×10^{-5} M) in pH 7.4 phosphate buffer (0.1 M) at 25 °C.

tion of the major reaction intermediate, i.e., the 1:2 host/guest complex, is inhibited by low concentrations of *p*-nitrophenol.

By comparing the reaction behaviour of the ester **8** with **βCβH 5** and that of *p*-nitrophenyl acetate (**pNPA**) with **βCβH 5**, it is possible to determine whether the ethylene glycol chain is essential for the sigmoidal behaviour. The plot of the dependence of the initial rates for the cleavage reaction of **pNPA** by the homo-dimer **βCβH 5** gave a simple saturation curve rather than a sigmoidal curve, as shown in Fig. 6. The kinetic data were fitted to the Michaelis–Menten equation (Table 2). This result suggests that the ethylene glycol chain is required for the sigmoidal behaviour, and that both the *p*-nitrophenyl moiety and the ethylene glycol chain of the ester **8** occupy the cavities of the dimer during the reaction. Unfortunately, it is difficult to determine whether the ethylene glycol chain has a specific property that gives rise to cooperative behaviour, because the reactivity of the ester **8** cannot be compared in the same reaction conditions with that of simple alkanoate esters, owing to the low solubility of alkanoate esters in water.

Comparing the reaction behaviour of the *p*-nitrophenyl ester **8** with that of the *m*-nitrophenyl ester **9** yields information about the structure of the inclusion complex that the dimer forms with the substrate during the reaction, because the *m*-nitrophenyl moiety and the ethylene glycol chain of the *m*-nitrophenyl ester **9** cannot both occupy each cavity of the same dimer molecule at the same time, whereas the *p*-nitrophenyl moiety and the ethylene glycol chain of the *p*-nitrophenyl ester **8** can simultaneously occupy each cavity. When the *m*-nitrophenyl ester **9** was used as a substrate, the plot of the dependence of the initial rate of the cleavage reaction catalyzed by dimers **5**–**7** on the substrate concentration gave a simple saturation curve, as shown in Fig. 7. The kinetic data could be fitted to the Michaelis–Menten equation (Table 3). This result therefore strongly indicates that the sigmoidal behaviour of **βCβH 5** arises from the inclusion of both the nitrophenyl moiety and the ethylene glycol chain of the ester **8** in each cavity of the same homo-dimer molecule.

Table 2
Michaelis–Menten's parameters for the cleavage of **pNPA** by **βCβH**[a].

	k_{cat} [10^{-3} s^{-1}]	K_m [10^{-3} M]	*r*
βCβH	2.99 ± 0.95	8.45 ± 0.32	0.995

[a] At 25 °C in pH 7.4 phosphate buffer (0.1 M); [**βCβH**] = 4.8×10^{-5} M, [**pNPA**] = 2.5×10^{-4}–2.5×10^{-3} M.

Table 3
Michaelis–Menten's parameters for the cleavage of the ester (**9**) by **βCβH**, **αCβH**, or **βCαH**[a].

	k_{cat} [10^{-3} s^{-1}]	K_m [10^{-3} M]	*r*
βCβH	2.28 ± 0.41	0.470 ± 0.028	0.999
αCβH	5.42 ± 0.97	4.13 ± 0.10	0.997
βCαH	3.05 ± 0.38	1.18 ± 0.36	0.990

[a] At 25 °C in pH 7.4 phosphate buffer (0.1 M); [9] = 2.5×10^{-4}–2.5×10^{-3} M, [**βCβH**] = [**αCβH**] = [**βCαH**] = 4.8×10^{-5} M.

3.2. Estimation of the reaction mechanism

A mechanism for the observed sigmoidal behaviour was derived on the basis of the above data as shown in Fig. 8. The homo-dimer can take various conformations in aqueous solution in the absence of a guest. In order to simplify the mechanism, therefore, two main conformations were assumed: a *cis* conformation and a *trans* conformation. In the *cis* conformation (**C Form**) the two CD cavities of the homo-dimer face each other, whereas in the *trans* conformation (**T Form**) the two CD cavities of the homo-dimer face away from each other. Our computational conformational studies indicate that if the linker is long enough to rotate the CD cavity, then the **T Form** is more stable than the **C Form** in aqueous solution in

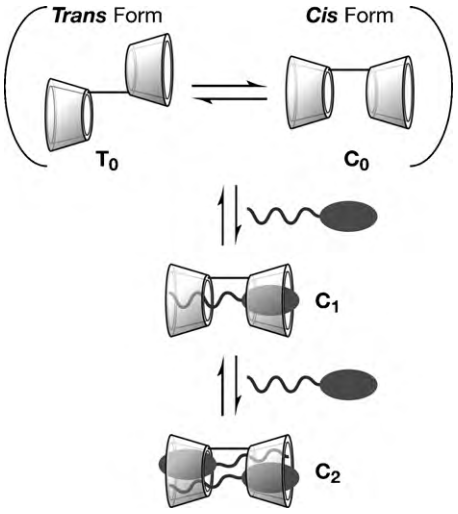


Fig. 8. Schematic representation of proposed mechanism for the reaction of **βCβH 5** with the substrate **8**.

the absence of a guest, because hydroxy groups of CD tend to make hydrogen bonds with waters in aqueous solution and the dimer in the **T Form** can make hydrogen bonds with more waters than can the dimer in the **C Form** [14]. If the CD cavity in the **C Form** accommodates the first substrate, the dimer immediately proceeds to the state of a 1:2 host/guest complex, because the first substrate fixes the conformation as the **C Form** and the guest in the CD dimer in the **C Form** acts a spacer against the binding of a second guest. The first guest to bind in a CD cavity in the **T Form** slightly affects the binding of the second guest in the CD cavity in the **T Form**. Therefore, the formation of this inclusion complex shifts the equilibrium from the **T Form** to the **C Form**, and this conformational change causes the observed sigmoidal behaviour.

To verify this proposed mechanism, a non-reactive material (the ether **10**) that would act as a spacer was added to the reaction system. The initial rates for the cleavage reaction of the *p*-nitrophenyl ester **8** catalyzed by the CD homo-dimer (**βCβH 5**) were increased 1.2- and 1.3-folds by the addition of the ether **10** at 5×10^{-4} and 1×10^{-3} M, respectively under the conditions of the substrate **8** at 5×10^{-4} M and **βCβH 5** at 4.7×10^{-5} M in pH 7.4 phosphate buffer (0.1 M) at 25 °C. This result indicates that one of the substrates in the 1:2 complex of **βCβH 5** and the substrate **8** acts as a spacer or a potential inhibitor [20,25,26].

4. Conclusions

The cooperativity of the two β-CD cavities in the homo-dimer molecule gives rise to the sigmoidal behaviour of the dependence of the initial rate for the ester cleavage on the substrate concentration. This cooperativity is caused by the formation of a 1:2 complex between the homo-dimer and the substrate. In this 1:2 complex, both the phenyl moiety and the ethylene glycol chain occupy the two cavities of the same dimer molecule, with the ethylene glycol chain of one substrate and the phenyl moiety of the other substrate together in same cavity. This 1:2 complex is the most reactive intermediate formed during the reaction. Such behaviour is similar to that of a natural allosteric enzyme, which is made up of identical subunits.

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