Chiral Recognition by Cyclic Oligosaccharides. Enantioselective Complexation of Bilirubin with β-Cyclodextrin through Hydrogen Bonding in Water

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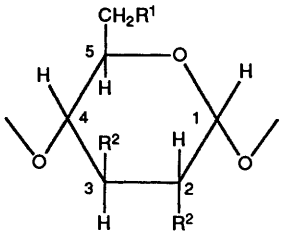
The mechanism for enantioselective complexation of bilirubin (BR) with β-cyclodextrin (β-CDx) in water (pH 10.8) has been studied by means of CD spectroscopy. The (−) to (+) bisignate CD signals observed for BR bound to β-cyclodextrin are enhanced upon addition of co-existing guests such as cyclohexanol and cyclooctanol which are completely included in the β-CDx cavity. Non-cyclic oligosaccharides such as maltoheptaose also induce the bisignate CD signals of BR. These results clearly indicate that a lipophilic cavity of β-CDx does not play an important role for enantioselective complexation of BR with β-CDx. The CD signals disappear in water at pH 13.0 where an electrostatic repulsion is expected between BR and β-CDx because either the carboxy groups of BR or the secondary hydroxy group of β-CDx are dissociated under these conditions. BR complexed with heptakis(6-deoxy)-β-CDx also shows the (−) to (+) bisignate CD Cotton effect. On the basis of these results, it has been concluded that optically active BR is bound to the rim of the β-CDx cavity and hydrogen bonding between the carboxylate ions of BR and the secondary hydroxy groups of β-CDx participates in the enantioselective complexation of BR with β-CDx.

(4*Z*,15*Z*)-Bilirubin-IXα (BR) is a yellow–orange bile pigment which is produced by the metabolism of heme.[1] X-Ray crystallography[2] and NMR spectroscopy[3] indicate that there are two conformational enantiomers of BR which are formed by intramolecular hydrogen bonds between the carboxy groups of the propionic acid residues and the carbonyl groups of the lactam functionalities as well as the NH groups of the pyrroles. In solutions, a rapid interconversion between (*R*)- and (*S*)-helix BRs occurs by breaking and reforming the intramolecular hydrogen bonds.[3a,e] BR when bound to deoxycholate micelles,[4] human serum albumin,[5] cyclodextrins (CDxs),[6] cinchona alkaloids,[7] *S*-(+)-2-aminobutane,[8] and nucleosides[9] in solutions exhibits bisignate Cotton effects on the CD spectra. The bisignate CD Cotton effects have been explained by the BR molecule selectively forming one of the diastereoisomers upon complexation with a chiral host molecule.[4–9] BR adopts a chiral 'ridge-tile' structure and an exciton coupling between two dipyrrinone chromophores splits the excited state of BR into two energy levels leading to a bisignate CD Cotton effect.[10] When the orientation of the transition dipoles of the two dipyrrinone chromophores adopts a (*S*)-helix configuration, the CD spectrum of BR is split into two bands at the wavelength region of electronic transition, where negatively and positively signed CD signals appear at longer and shorter wavelengths, respectively [(−) to (+) bisignate CD signals]. Oppositely-signed CD signals [(+) to (−)] are measured for the (*R*)-helix BR.

Lightner and his co-workers[6b] reported that BR (λ_{max} for UV–VIS = *ca.* 436 nm) bound to α-(cyclohexaamylose), β-(cycloheptaamylose), and γ-CDxs (cyclooctaamylose) in water shows the (−) to (+) bisignate CD signals, suggesting that the transition dipoles of the two dipyrrinone chromophores of BR take a (*S*)-helix configuration. It is surprising that no remarkable difference in the CD intensities has been observed between α-, β-, and γ-CDx complexes. In general, the stability of an inclusion complex of CDx greatly depends on the size of the CDx cavity. A guest molecule having a larger molecular size compared with the CDx cavity size cannot be included in the CDx cavity. A guest molecule which is too small to fill the CDx cavity does not form a stable inclusion complex because of a weak van der Waals stabilization.[11] We then started to study



β-CDx: $R^1 = R^2 = OH$
TMe-β-CDx: $R^1 = R^2 = OCH_3$
amino-β-CDx: $R^1 = NH_3Cl$, $R^2 = OH$
deoxy-β-CDx: $R^1 = H$, $R^2 = OH$

the mechanisms for the enantioselective complexation of BR with cyclic and non-cyclic oligosaccharides. In a previous communication,[12] we reported that heptakis(2,6-di-*O*-methyl)-β-CDx (DMe-β-CDx) and heptakis(2,3,6-tri-*O*-methyl)-β-CDx (TMe-β-CDx) do not induce the bisignate CD Cotton effect and non-cyclic oligosaccharides such as maltose, maltotriose, and maltoheptaose selectively complex with BR having a (*S*)-helix configuration. These results suggest that hydrogen bonding between host and guest participates in the enantioselective complexation of BR with β-CDx. The present paper deals with a more detailed mechanism including the participation of intermolecular hydrogen-bond formation in water and in the binding site.

Results and Discussion

CD Spectra of BR.—As shown in Fig. 1, the (−) to (+) bisignate CD Cotton effect is observed in the BR–β-CDx system, which is in good agreement with the result reported by Lightner *et al.*[6b] Exciton-coupling theory[13] can be applied to understand the (−) to (+) bisignate CD Cotton effect and indicates the formation of a β-CDx complex with (*S*)-helix BR. The intensities of the CD signals for the BR–β-CDx complex ($\Delta\varepsilon = -7.4$ and $+4.9$ dm^3 mol^{-1} cm^{-1} at 454 and 401 nm, respectively)

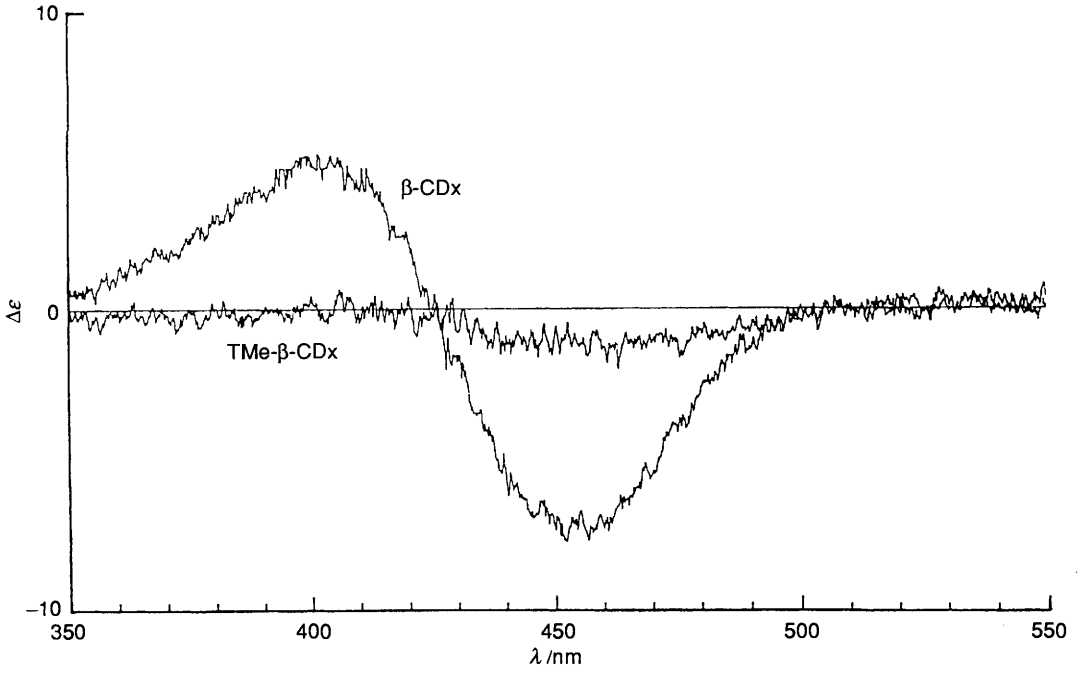
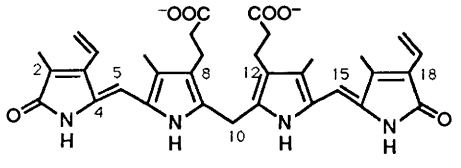


Fig. 1 CD spectra of BR (2.5×10^{-5} mol dm^{-3}) in aqueous β-CDx and TMe-β-CDx (0.01 mol dm^{-3}) solutions at pH 10.8 and 20 °C

are much weaker than those of the oppositely-signed CD signals reported for the BR–human serum albumin complex ($\Delta\varepsilon$ = *ca.* +50 and −23 dm^3 mol^{-1} cm^{-1} at 460 and 407 nm, respectively).[5e] In the presence of human serum albumin, a fairly intense fluorescence of BR was observed at 522 nm while a very weak fluorescence was detected at the same wavelength region for the BR–β-CDx system. The differences in the CD and fluorescence spectra between the albumin and β-CDx complexes of BR may be interpreted in terms of relatively weak complexation of BR with β-CDx (*vide infra*).

No CD signals were detected when β-CDx was replaced by TMe-β-CDx as well as DMe-β-CDx. A Corey–Pauling–Koltun (CPK) molecular model suggests that a dipyrrinone moiety of BR can be included in both cavities of β-CDx and TMe-β-CDx. Unfavourable size matching, therefore, may not explain the result that BR in the aqueous TMe-β-CDx solution does not show the CD signal. In a hydrogen-bonding interaction, TMe-β-CDx can only act as an electron donor (Lewis base) while β-CDx has both natures of Lewis acid and base. The accurate values for acid dissociation of two propionic acid residues of BR have not been determined.[14] At pH 10.8 (pH of the present conditions), however, BR should be in a dianion form.[15] The BR dianion has the possibility of interacting with β-CDx through hydrogen bonding between the $-CO_2^-$ groups of BR and the –OH groups of β-CDx. TMe-β-CDx cannot, however, associate with the BR dianion through hydrogen bonding. The results exhibited in Fig. 1 can therefore be understood by assuming hydrogen bonding between the $-CO_2^-$ groups of BR and the –OH groups of β-CDx as a force for the enantioselective complexation. At pH 7.7, the CD intensities were slightly weakened ($\Delta\varepsilon$ = −4.6 and +3.9 dm^3 mol^{-1} cm^{-1} at 457 and 404 nm, respectively). The CD signals were completely diminished when the pH was lowered to 5.5. This may be ascribed to a much weaker hydrogen bonding between the $-CO_2H$ groups



of BR and the –OH groups of β-CDx compared with that between the $-CO_2^-$ groups and the –OH groups. Similar intermolecular hydrogen-bond formation has been demonstrated for the complex of albumin and optically-active BR from the results of resonance Raman[15] and CD spectroscopies.[5f] We have found that non-cyclic oligosaccharides such as maltose, maltotriose and maltoheptaose also induce the bisignate Cotton effect on the CD spectra of BR in water at pH 10.8.[12] This strongly suggests that the lipophilic cavity of β-CDx does not participate in the enantioselective complexation.

Effects of Coexisting Guests.—If the lipophilic cavity of a cyclic oligosaccharide does not play an important role for enantioselective complexation of BR, a coexisting guest, which is tightly included in the CDx cavity, may not diminish the CD signals in the BR–β-CDx system. It is known that cyclooctanol is strongly bound to β-CDx (K = 2000 dm^3 mol^{-1} at 25 °C).[16] In order to confirm whether cyclooctanol is located inside the β-CDx cavity, the ^{1}H NMR spectrum of an equimolar solution of β-CDx and cyclooctanol (0.01 mol dm^{-3}) in D_2O was measured. The proton signals for H-1 (d, 5.03 ppm), H-2 (dd, 3.62 ppm), H-4 (t, 3.55 ppm), and H-6 (m peaked at 3.84 ppm) of β-CDx scarcely shift upon addition of cyclooctanol. The signals due to H-3 (t, 3.93 ppm) and H-5 (the signals are hidden under the H-6 proton signal in the absence of cyclooctanol, *ca.* 3.82 ppm) protons which are located inside of the cavity, however, markedly shift to upper magnetic fields in the presence of cyclooctanol, $\Delta\delta$ being 0.05 and *ca.* 0.06 ppm for H-3 and H-5, respectively. The NMR data clearly indicate that cyclooctanol is included in the cyclic cavity of β-CDx.

The effect of cyclooctanol on the bisignate CD spectrum of BR in the aqueous β-CDx solution at pH 10.8 is shown in Fig. 2. Coexistence of cyclooctanol enhances the CD signals, $\Delta\varepsilon$ in the presence of 0.01 mol dm^{-3} cyclooctanol being −29.4 and +23.2 dm^3 mol^{-1} cm^{-1} at 458 and 406 nm, respectively. The effects of other coexisting guests are summarized in Table 1. Hexan-1-ol, cyclohexanol, octan-1-ol, and 1-adamantanecarboxylate ion, which are hydrophobic guests, also enhance the CD signals whereas smaller guests such as methanol, ethanol, and butan-1-ol as well as the hydrophilic carboxylate ions such as acetate, propionate, butyrate and valerate ions scarcely affect the CD

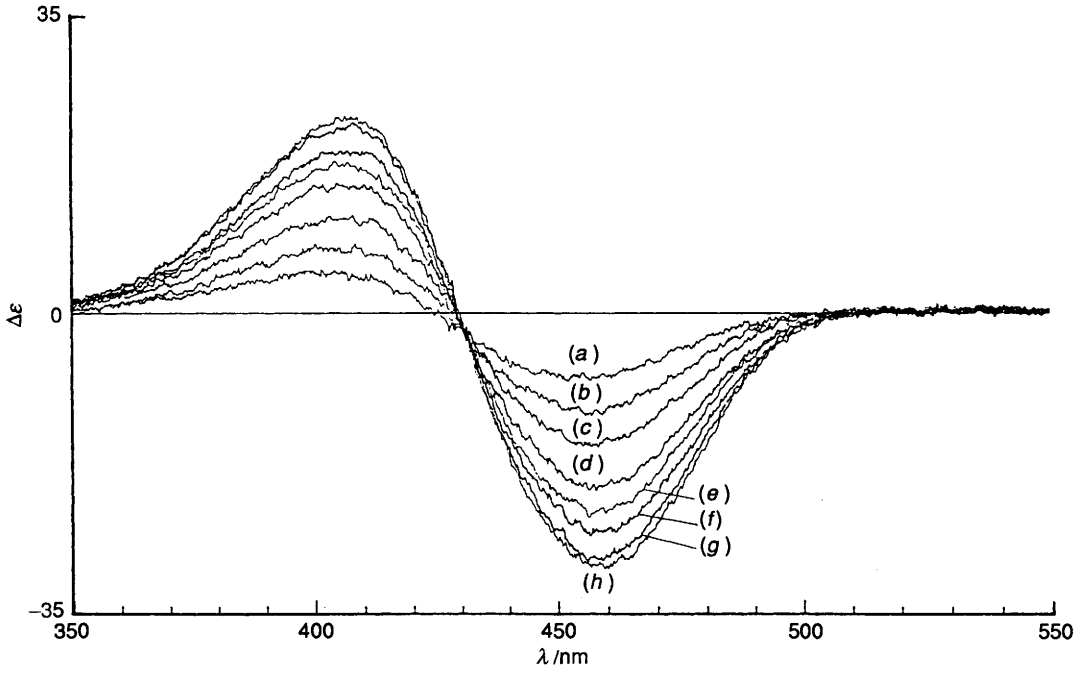


Fig. 2 Effect of cyclooctanol on the CD spectrum of BR (2.5×10^{-5} mol dm^{-3}) in aqueous β-CDx (0.01 mol dm^{-3}) solution at pH 10.8 and 20 °C. [Cyclooctanol]/10^{-3} mol dm^{-3}: (*a*) 0; (*b*) 1; (*c*) 2; (*d*) 4; (*e*) 5; (*f*) 6; (*g*) 8; (*h*) 10.

Table 1 Effects of coexisting guests on CD spectra of BR (2.5×10^{-5} mol dm^{-3}) in aqueous β-CDx solutions (0.01 mol dm^{-3}) at pH 10.8 and at 20 °C

Coexisting guest ([C]/mol dm^{-3})	$\Delta\varepsilon_1(\lambda_{ext}/nm)$	$\Delta\varepsilon_2(\lambda_{ext}/nm)$
Non	−7.4(454)	+4.9(401)
Methanol (0.01)	−7.8(453)	+5.4(402)
Ethanol (0.01)	−7.8(453)	+5.4(405)
Butan-1-ol (0.01)	−9.5(455)	+6.8(405)
Pentan-1-ol (0.01)	−12.9(456)	+9.8(406)
Hexan-1-ol (0.01)	−16.6(457)	+13.7(407)
Cyclohexanol (0.001)	−10.5(456)	+6.5(402)
Cyclohexanol (0.01)	−16.7(456)	+11.5(406)
Cyclohexanol (0.04)	−18.4(457)	+14.1(407)
Octan-1-ol (0.001)	−12.2(456)	+7.7(404)
Octan-1-ol (0.005)	−17.3(457)	+13.2(405)
Cyclooctanol (0.001)	−11.4(457)	+7.4(404)
Cyclooctanol (0.005)	−23.3(458)	+17.7(406)
Cyclooctanol (0.01)	−29.4(459)	+23.2(407)
Acetic acid (0.5)	−8.6(453)	+3.8(402)
Propionic acid (0.5)	−7.5(453)	+3.6(400)
Butyric acid (0.5)	−8.3(455)	+4.5(403)
Valeric acid (0.5)	−7.5(457)	+4.5(406)
1-Adamantanecarboxylic acid (0.01)	−22.0(458)	+17.6(406)

signals. On the basis of these results, it can be concluded that an inclusion of the secondary guest in the cavity of β-CDx does not prevent the enantioselective complexation of BR with β-CDx and optically active BR, therefore, may be bound to the rim(s) of the toroidal CDx cavity. The effects of the coexisting guests support the theory that the hydrogen-bonding interaction contributes to the enantioselective complexation of BR with β-CDx. The results of Lightner *et al.*[6b] show that no remarkable difference in the CD spectra of BR is observed between the α-, β- and γ-CDx complexes and this can be interpreted in terms of formation of the 'outside-complexes' of BR and CDxs.

Binding Site.—The β-CDx cavity has primary and secondary hydroxy group sides. Both sides of the cavity can act as hydrogen bonding sites for BR. Intramolecular hydrogen bonding between a hydroxy group (–OH) and an adjacent hydroxylate ion ($-O^-$) lowers the pK_a of the secondary hydroxy groups of CDxs.[17,18] The pK_a value of the secondary hydroxy group of β-CDx has been reported to be 12.2 at 25 °C.[18a] We measured the CD spectrum of BR in an aqueous alkaline solution of β-CDx at pH 13.0 and did not observe any CD signal. Since BR is in the dianion form at pH 13.0, an electrostatic repulsion between the host and the guest seems to prevent the formation of the BR–β-CDx complex. In other words, the secondary hydroxy group side of β-CDx may be the binding site for the enantioselective complexation.

Heptakis(6-deoxy)-β-CDx (deoxy-β-CDx) has only a secondary hydroxy group side as a hydrogen-bonding site. Since the solubility of this CDx in water is considerably low, we could measure the CD spectra only in dilute deoxy-β-CDx solutions. BR (2.5×10^{-5} mol dm^{-3}) in aqueous deoxy-β-CDx (1×10^{-3} mol dm^{-3}) at pH 10.8 shows weak, (−) to (+) bisignate CD signals ($\Delta\varepsilon = \pm 0.5$ dm^3 mol^{-1} cm^{-1}) which is almost the same as that for the BR–β-CDx system ($\Delta\varepsilon = \pm 0.8$ dm^3 mol^{-1} cm^{-1}) under the same conditions. This strongly supports the theory that the chiral-recognition site is the secondary hydroxy group side of CDx.

The CD spectrum of BR in the aqueous heptakis(6-amino-6-deoxy)-β-CDx (amino-β-CDx) at pH 7.2 was measured. An oppositely signed, (+) to (−) bisignate CD Cotton effect was observed ($\Delta\varepsilon = +11.4$ and -8.0 dm^3 mol^{-1} cm^{-1} at 466 and 409 nm, respectively). At pH 7.2, a predominant species of BR may be the monoanion and/or dianion forms and the amino groups of amino-β-CDx are protonated. Therefore, it is quite reasonable to assume that BR is mainly bound to amino-β-CDx through an electrostatic interaction and that most secondary hydroxy groups do not participate in the complexation. The difference in enantioselectivity between β-CDx and amino-β-CDx also supports the assumption that the enantioselective complexation of BR with β-CDx occurs at the secondary hydroxy group side of β-CDx. Why does BR select the secondary hydroxy groups as the binding sites? The hydroxymethyl groups of β-CDx can rotate freely whereas the rotational freedom of the secondary hydroxy groups is severely restricted. The fixed binding sites may be preferable for a weak interaction such as hydrogen bonding.

Table 2 Binding constants (K) as a function of temperature and thermodynamic parameters (ΔH and ΔS) for complexation of BR with β-CDx in the absence and presence of cyclooctanol at pH 10.8

Temperature/K	K/dm^3 mol^{-1}	ΔH/kJ mol^{-1}	ΔS/J mol^{-1} K^{-1}
BR–β-CDx system			
278	47.1	−27.4 ± 1.8	−66.0 ± 6.5
283	35.5		
288	29.0		
293	26.9		
298	23.1		
303	16.4		
308	14.3		
BR–β-CDx–cyclooctanol system			
284.5	108.7	−39.5 ± 3.2	−96.8 ± 9.9
286	96.0		
288.9	88.8		
291.8	77.5		
293.6	67.9		
295.8	57.4		
297	52.8		

Thermodynamic Parameters.—Binding constants (K) for the BR–β-CDx and BR–β-CDx–cyclooctanol systems were determined as a function of temperature by measuring CD spectral changes of BR upon addition of β-CDx or the cyclooctanol–BR complex. Because of the low solubility of cyclooctanol in water, the conditions were constructed such that 95% of the β-CDx molecules form the cyclooctanol–β-CDx complex. We used $K = 2000$ dm^3 mol^{-1} for formation of the cyclooctanol–β-CDx complex[15] to evaluate the amounts of cyclooctanol which should be added. The K values at various temperatures were determined by Benesi–Hildebrand plots[19] for changes in the CD intensities at 454 or 459 nm. In all cases, a good linear relationship between $\Delta\varepsilon^{-1}$ and [β-CDx]$^{-1}$ or [β-CDx–cyclooctanol complex]$^{-1}$ was observed, where $\Delta\varepsilon$ is the molar circular dichroism measured in the presence of β-CDx. In the absence of β-CDx, $\Delta\varepsilon$ is null. Linear Benesi–Hildebrand plots indicate the formation of the 1:1 complex of BR and β-CDx and the 1:1:1 complex of BR, β-CDx, and cyclooctanol. The results are listed in Table 2 together with the enthalpic (ΔH) and entropic changes (ΔS) for complexation.

It is revealed that the BR–β-CDx complex is considerably unstable. The lability of this complex is due to a negative and small ΔH and a negative and relatively large ΔS. In the cases of small amides in water, positive entropy changes due to the release of bound water molecules mainly promote the formation of hydrogen-bonded dimers of amides.[20] It can be assumed that complexation between large molecules greatly reduces translational and rotational entropies. In particular, a large molecule has a lot of freedom to rotate when it is dissolved in solution in the monomer form. The rotations may be restricted by complexation leading to negative and large ΔS_{rot}, where ΔS_{rot} denotes the rotational entropy change. The negative and large ΔS_{trans}, the translational entropy change, and ΔS_{rot} may overcome the entropy gain due to release of water molecules from solute molecules. In the presence of cyclooctanol, ΔS is smaller than that for the system in the absence of cyclooctanol. The binding of BR with β-CDx, however, becomes enthalpically favourable in the presence of cyclooctanol. The hydrophobic nature of β-CDx should increase when its cavity is filled with cyclooctanol. Inclusion of cyclooctanol may cause partial release of bound water molecules from the hydroxy groups of β-CDx. Indeed, positive entropy changes have been determined for the association of alkyl alcohols with β-CDx.[16] ΔS determined in the present system does not include a contribution of this dehydration process because the K values were evaluated by changing the concentration of the cyclooctanol–β-CDx complex. The partial dehydration should be favourable for the hydrogen-bonding interactions between the secondary hydroxy groups of β-CDx and the carboxylate ions of BR.

The hydrogen-bonding interaction may not be the sole binding force for the BR–β-CDx complex because addition of methanol reduces the CD signal intensities. It has been demonstrated that van der Waals interactions between apolar solute molecules are strongest in water.[21] At the present stage, we believe that the cooperation of the van der Waals and hydrogen-bonding interactions promotes the enantioselective complexation of BR with β-CDx.

Conclusions

We have found that BR bound to β-CDx preferentially takes the (S)-helix configuration and that the hydrogen-bonding interaction between the CO_2^- groups of BR and the secondary −OH groups of β-CDx is essential for the enantioselective complexation which occurs outside of the CDx cavity.

Hsieh and Morris[15] measured the resonance Raman spectra of the BR–human serum albumin and BR–α- and –β-CDx complexes. They concluded that the intramolecular hydrogen bonds of BR are broken upon complexation with albumin and that the intermolecular hydrogen bonds and the ring stacking between BR and the protein promote the tight binding of BR with albumin. Such an assumption has previously been presented.[22] Meanwhile, the resonance Raman spectra of the BR–CDx complexes are almost the same as that of BR in water.[15] As Hsieh and Morris pointed out, the results of the resonance Raman spectroscopy may or may not indicate that intramolecularly hydrogen-bonded BR is included in the CDx cavity. Using the K value obtained in the present study, it is estimated that only 3.4% of the BR molecules is bound to β-CDx under the conditions employed by Hsieh and Morris. Therefore, they mainly measured the resonance Raman spectra of free BR in the BR–CDx systems. In the present study, we revealed that intermolecular hydrogen bonding participates in the enantioselective complexation of BR with β-CDx. Although hydrogen bonding has been assumed to contribute to formation of inclusion complexes of CDxs[23] as well as chiral recognition by CDxs,[24] to the best of our knowledge, evidence for the contribution of hydrogen bonding in complexation with CDx in water has scarcely been presented. The BR–β-CDx complex is an 'outside complex' and the stability of the complex is very low because van der Waals and/or dipole interactions cannot function efficiently between the host and the guest. We know the complexes of ribonucleosides and α-CDx as other examples of outside complexes in solution.[25]

Experimental

β-CDx (Nakalai) was purchased and an antioxidant contained in this material was extracted with THF using a Soxhlet extractor. Commerically-obtained TMe-β-CDx was used without further purification. Heptakis(6-amino-6-deoxy)-β-CDx heptahydrochloride (amino-β-CDx) was prepared from heptakis(6-azido-6-deoxy)-β-CDx according to the procedures described in the literature,[26] yield 74%, $[\alpha]_D^{20}$ +113.0° (c 1.00 in H_2O). Heptakis(6-deoxy)-β-CDx (deoxy-β-CDx) was synthesized from heptakis(6-bromo-6-deoxy)-β-CDx,[27] yield 13%, $[\alpha]_D^{20}$ −111.0° (c 1.00 in pyridine). Reagent grade alkyl alcohols and carboxylic acids (Nakalai) were purchased and used without further purification. 1-Adamantanecarboxylic acid (Nakalai) was recrystallized from methanol, m.p. 174.5–176.5 °C. BR (Sigma) was dissolved in chloroform and washed with aqueous $NaHCO_3$. Chloroform was removed under reduced pressure and the residue was washed with small amounts of methanol.

The absorption spectra were measured using a Shimadzu UV–2100 spectrophotometer. The CD spectra were taken on a Jasco J-500A spectropolarimeter with a data processor. The sample solutions for the CD measurements were bubbled with nitrogen gas for 10 min to prevent oxidation of BR. The 400 MHz ^{1}H NMR spectra in D_2O were measured on a JEOL GX-400 spectrometer at 23 ± 1 °C. The chemical shifts were determined using 3-(trimethylsilyl)propane-1-sulfonate (Merck) as an external standard.

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