Molecular Recognition Thermodynamics of Bile Salts by β-Cyclodextrin Dimers: Factors Governing the Cooperative Binding of Cyclodextrin Dimers

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Received: October 13, 2004

The complex stability constants (K_S), standard molar enthalpy ($\Delta H°$), and entropy changes ($\Delta S°$) for the inclusion complexation of two families of β-cyclodextrin (β-CD) dimers, i.e. β-CD dimers **Se1**−**Se4** bearing 2,2′-diselenobis(benzoyl) tether (**Se-dimers**) and β-CD dimers **Py1**−**Py4** bearing 2,2′-bipyridine-4,4′-dicarboxy tether (**Py-dimers**), with four bile salt guests, i.e. sodium cholate (**CA**), sodium deoxycholate (**DCA**), sodium glycocholate (**GCA**), and sodium taurocholate (**TCA**), were determined at 25 °C in Tris buffer solution (pH 7.4) at 298.15 K by means of isothermal titration microcalorimetry. The thermodynamic parameters obtained, together with the ROESY spectra of interactions between β-CD dimers and bile salts, consistently suggest that the length, flexibility, and structure of spacers linking the two β-CD cavities not only determine the binding modes but also significantly alter the molecular selectivity of β-CD dimers.

Introduction

Possessing the attractive ability of binding a wide variety of organic molecules into their hydrophobic cavities forming host−guest inclusion complexes, native and modified cyclodextrins (CDs) have been extensively applied in many areas of science and technology to serve as artificial enzymes for biological mimicking.[1−3] It is well-known that allosteric effect is an important characteristic of the interactions between enzyme and substrate; that is to say, the enzyme possesses the alterable conformation and will change it to achieve the excellent enzyme−substrate interactions when induced by the substrate. Recently, the modification of native CD by introducing different alterable functional units to mimic the active site of catalytic groups in enzyme becomes the pivotal topic in enzyme mimicking research of CD. Then the following problems have been brought forward in designing the novel CD derivatives with special functions. (1) What kinds of structures are favorable and logical? (2) How can the conformational change of CDs be controlled to improve the interaction with guest (substrate)? Simultaneously, possessing two hydrophobic cavities and a functional linker in one molecule, CD dimer not only displays the dramatically strong binding abilities toward certain guest molecules[4−10] but also can mimic the allosteric effect of enzyme to a large extent. Therefore, it is of great importance to discover the correlations between the structures and binding behaviors of CD dimers. More recently, Nolte et al. have reported the combinative research involving the conformation and binding behavior of CD dimers linked from the secondary side, showing that the inclusion of spacers can affect the binding affinities of CD dimers for ditopic guest molecules.[8]

On the other hand, since thermodynamic investigations on CDs can provide us with deeper understanding of the factors governing the supramolecular complexation through cooperative multiple intermolecular interactions, it has been widely performed to study the complexation of native and/or some monomodified CDs with different guests.[11−16] Inoue et al. revealed the relationship between binding mode and chiral recognition of modified CD with amino acid derivatives by thermodynamic investigations.[17] Recently, we also investigated the correlations between thermodynamic behaviors and conformations of some monomodified β-CDs and CD dimers.[18,19] Unfortunately, the reported thermodynamic data of CD dimers is still too limited so far to exhibit the detailed host−guest interactions in the complexation process for understanding the binding behavior of CD dimers. Many investigations have demonstrated that substituent effect is a key factor for the complexation of monomodified CDs with guests. These promote us to investigate the control action of the structure, flexibility, and/or length of the spacer toward the binding behavior of CD dimers. However, previous studies on CD dimers are mainly focused on the synthesis of novel molecules[20] and investigation of their binding ability [6,7,10,21−23] or catalytical behaviors,[1−3] and there are few reports[24] concerning the spacer effects in the complexation of CD dimers, especially from the thermodynamic viewpoint, though it is of great importance for elucidating the underlying correlations between structure and binding behavior of CD dimers.

In the present study, we have determined the themodynamic parameters of the complexation of eight CD dimers with four bile salts, i.e. sodium cholate (**CA**), sodium deoxycholate (**DCA**), sodium glycocholate (**GCA**), and sodium taurocholate (**TCA**) (Chart 1) by means of microcalorimetric titration and the 2D ROESY experiments. Our interest is not only to emphasize the enhanced binding ability and/or the molecular selectivity but also to investigate the spacer effects in the complexation of CD dimers with guests thermodynamically. It is of our special interest to reveal the factors governing the cooperative binding of cyclodextrin dimers, not only the general size/shape fitting between the cavities of CD dimers and guest molecules but also the structure, length, and flexible of spacer from a general viewpoint, for further understanding and controlling of the binding mode and the molecular recognition behavior of CD dimers toward guests.

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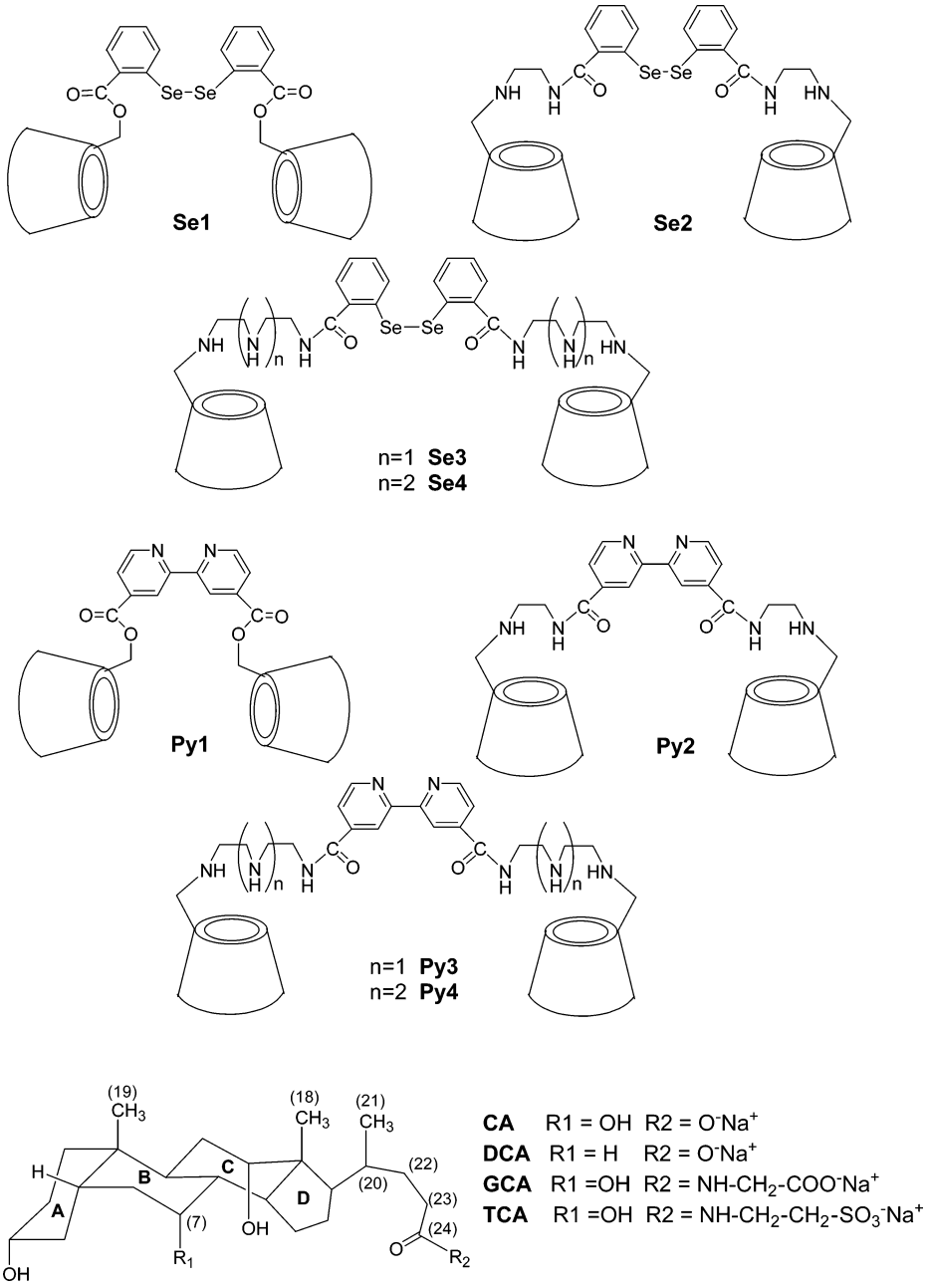
10.1021/jp045332v CCC: $30.25
Published on Web 00/00/0000 PAGE EST: 5.3

CHART 1: Host and Guest Structures



Experimental Section

M**aterials.** Host cyclodextrins were synthesized as reported previously.[25] Commercially available sodium cholate and deoxycholate (Acros) and sodium taurocholate and glycocholate (Sigma) were used without further purification. A 10 mM Tris buffer water solution (140 mM NaCl, pH 7.4) was used as solvent throughout the measurement.

Microcalorimetric Titration. The isothermal titration calorimeter (VP-ITC, Microcal Co.) was used for all microcalorimetric experiments. The instrument was calibrated chemically by performing the complexation reaction of β-CD with cyclohexanol, which gave thermodynamic parameters in good agreement with the literature data.[26,27]

The microcalorimetric titrations were performed at atmospheric pressure and 25 °C in aqueous Tris buffer solution (pH 7.4). All solutions were degassed and thermostated using a ThermoVac accessory before titration experiment. In each run, a buffer solution of CD (2.03−4.18 mM) in the 0.250 mL syringe was sequentially injected with stirring at 300 rpm into a solution of bile salt (0.15−0.28 mM; below the critical micelle concentration (cmc) for the bile salts examined) in the sample cell (1.4227 mL volume). Each titration experiment was composed of 25−29 successive injections (5 or 10 μL/injection). A typical titration curve is shown in Figure 1.

A control experiment was performed to determine the heat of dilution by injecting a CD buffer solution into a pure buffer solution, containing no bile salt. The dilution enthalpy was subtracted from the apparent enthalpy obtained in each titration run, and the net reaction enthalpy was analyzed by using the one set of binding sites model (see Supporting Information).

The ORIGIN software (Microcal) allowed us to simultaneously determine the binding constant (K_S) and reaction enthalpy ($\Delta H°$) with the standard derivation based on the scatter of data points from a single titration experiment. All thermodynamic parameters reported in this work were obtained by using the "one set of binding sites model". This model will work

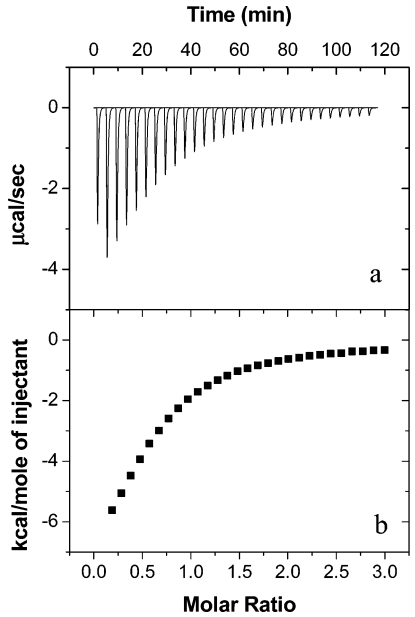


Figure 1. Calorimetric titration of host **Py1** with **DCA** in Tris buffer (pH 7.4) at 25 °C. (a) Raw data for sequential 10 μL injections of CD solution (2.67 mM) into bile salt solution (0.2 mM). (b) Heats of reaction as obtained from the integration of the calorimetric traces.

for any number of sites N if all sites have the same K_S and $\Delta H°$. In this case, the total heat Q was fitted via a nolinear least-squares minimization method to the total CD concentration in the cell (M_t) using the following equation:

$$Q = \frac{NX_t\Delta HVo}{2}\left[1 + \frac{M_t}{NX_t} + \frac{1}{NK_SX_t} - \sqrt{\left(1 + \frac{M_t}{NX_t} + \frac{1}{NK_SX_t}\right)^2 - \frac{4M_t}{NX_t}}\right] \quad (1)$$

where N is the number of binding sites of CD, X_t is the total concentration of bile salts in the cell, and V_o is the cell volume. The value of Q above can be calculated (for any designated values of N, K, and ΔH) at the end of the ith injection and designated $Q(i)$. Then the correct expression for the heat released, $\Delta Q(i)$, from the ith injection is

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_o}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1) \quad (2)$$

where dV_i is the volume of titrant added to the solution. Along with obtaining K_S and $\Delta H°$ in this fitting program, the N value in eq 1 can also be obtained, which represents the numbers of bile salts bound to one CD molecule. Table 1 lists the complex stability constant, standard enthalpy, and entropy changes for all of the 1:1 inclusion complexation of CD dimers with bile salts. In contrast, other CD dimer/bile salt complexations exhibit 1:2 binding stoichiometry. For these host−guest complexations, the "two sets of binding sites" or "sequential binding sites" model should be used to calculate the complex stability constant and thermodynamic parameters. However, the parameters calculated by these two binding models may be further verified by other experimental results in the present case. Therefore, the corresponding data for the 1:2 binding mode are not discussed herein.

Results and Discussion

Binding Mode of Bile Salts Complexes. As important biological amphiphiles, the bile salts possessing a steroid structure have been extensively used as guest molecules to examine the inclusion complexation behavior of CDs. We can see that all bile salts examined possess a similar framework containing four rings (A−D) and a side chain (Chart 1). The bile salts **CA** and **DCA** only show a small difference in the structure of the C-7 substituent (R_1), that is, a hydroxyl group for **CA** and a hydrogen atom for **DCA**. However, this slight difference will lead to the great distinction in their hydrophobic nature and binding abilities with CDs. On the other hand, different from **CA** and **DCA**, guests **GCA** and **TCA** possess the more polar side chain (R_2),[28] which will not only affect their binding abilities but also sometimes change their binding mode with CD dimers.

TABLE 1: Complex Stability Constant (K_S/M^{-1}), Standard Enthalpy ($\Delta H°$/(kJ·mol^{-1})), and Entropy Changes (T$\Delta S°$/(kJ·mol^{-1})) for Inclusion Complexation of Bile Salts with β-CD Dimers in Tris Buffer Solution (pH 7.4) at 298.15 K

host[a]	guest[b]	N[c]	K_S	$\Delta H°$	T$\Delta S°$
β-CD	CA	1.14	3830	−21.9	−1.4
	DCA	1.06	4740	−29.4	−8.4
	GCA	1.03	2590	−21.8	−2.3
	TCA	1.08	2270	−21.6	−2.5
Se3	CA	1.0	4100 ± 200	−24.9 ± 0.5	−4.3 ± 0.6
	DCA	1.0	5400 ± 200	−35.0 ± 0.4	−13.7 ± 0.5
Se4	CA	1.0	5030 ± 20	−29.1 ± 0.2	−8.0 ± 0.2
	DCA	1.0	6100 ± 20	−40.2 ± 0.2	−18.6 ± 0.2
Py2	CA	1.2	12700 ± 200	−32.4 ± 0.1	−9.0 ± 0.1
	DCA	1.2	12400 ± 200	−45.4 ± 0.1	−22.0 ± 0.1
Py3	CA	1.2	12400 ± 300	−25.5 ± 0.1	−2.2 ± 0.1
	DCA	1.2	13100 ± 400	−31.9 ± 0.1	−8.3 ± 0.1
Py4	CA	1.2	6800 ± 200	−25.4 ± 0.6	−3.5 ± 0.6
	DCA	1.2	7500 ± 200	−35.2 ± 0.4	−13.1 ± 0.5

[a] [Guest] = 0.15−0.20 mM. [b] [Host] = 2.03−3.15 mM. [c] Stoichiometry given by fitting program.

Investigations have revealed that different binding modes exist for the complexation of CD with bile salts.[28−30] It should be noted that both CDs and bile salts possess two sides, that is, the primary and secondary hydroxyl side for CDs, as well as the A-ring side and the carboxylate group side for bile salts. The A-ring and the carboxylate group of bile salt can enter the CD cavity from either the primary or secondary side, which will result in dramatically different binding modes between host and guest. In most of the investigations reported for mono-CDs, the bile salt molecule is usually included into the CD cavity from the secondary side. However, in the case of the complexation of primary-linked CD dimers, there exist two possibilities. The CD dimer can either include two guests from the secondary face to give 1:2 (Figure 2a1,[19]a2), or cooperatively associate one guest to yield a 1:1 sandwich complex (Figure 2b), and the thermodynamic parameters should be a combination of the binding modes that may exist.

Calorimetry. To perform the ITC experiments below the critical micelle concentration of bile salts,[28] CD solutions were added into the bile salts solution in each titration. All K_S and $\Delta H°$ reported in this paper were calculated by using the fitting procedure of the Origin software. The thermodynamic parameters for the 1:1 complexation of host CDs with **CA** and **DCA** were obtained directly by using the one set of binding sites model (Table 1). Unexpectedly, the data treating process for **GCA** and **TCA** can only give 1:2 stoichiometry. Considering the polar side chain of **GCA** and **TCA** molecules, they seem unable to be cooperatively included by two CD cavities but only can be included from the A-ring moiety into one CD cavity to form 2:1 complexes with CD dimer (Figure 2a2).[28]

Two-Dimensional NMR Experiment. To obtain further information about the binding modes between bile salts and CD

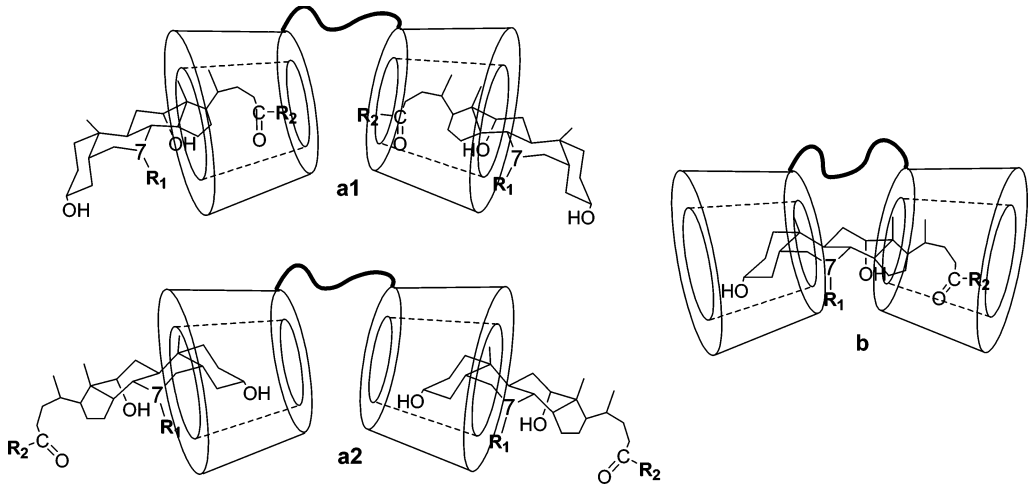


Figure 2. Possible binding modes of β-CD dimer−bile salt complexes.

dimers, 2D ROESY spectra for typical host−guest pairs are determined (see Supporting Information, Figure S2). In the spectrum for dimer **Se2** and **CA** (Figure S2a), the methyl protons on C-18 of **CA** show strong correlations with the H-3 protons but no correlation with the H-5/H-6 protons of CD. This suggests two possible binding modes; that is, the carboxylate side chain and D-ring of **CA** may penetrate into the CD cavity from the primary side deeply or from the secondary side shallowly. On the other hand, no correlation between the interior protons (H-3, H-5, and H-6) of CD and the methyl protons on C-19 of **CA** can be observed in Figure S2a, which excludes the former possibility. Therefore, we can confirm that the secondary-side inclusion (Figure 2a1) is the actual mode for the complexation of **Se2** with **CA**. Furthermore, there is no correlation between the spacer of **Se2** and **CA** in the ROESY spectrum, indicating that two **CA** molecules are bound separately into two cavities of **Se2** from the secondary side, which is consistent with the 1:2 binding stoichiometry deduced from microcalorimetric titration ($N = 2.0$).

Interestingly, the **Py2**−**DCA** pair displays an entirely different ROESY spectrum from that of the **Se2**−**CA** pair, showing obvious correlations between the protons both on C-18 and C-19 of **DCA** and the H-5/H-6 protons of **Py2**, which is well consistent with the 1:1 cooperative binding mode, as shown in Figure 2b. In addition, the thermodynamic parameters also indicated a 1:1 binding stiochiometry for the **Py2**−**DCA** pair ($N = 1.2$). Moreover, the methyl protons on C-19 of **DCA** also show appreciable correlations with H-3 protons of **Py2**. This indicates that the A-ring of **DCA** penetrates deeply into one CD cavity of **Py2**, attributing to the less steric hindrance and higher hydrophobicity of the substituent group (−H for DCA vs −OH vs other guests) on the C-7 position of **DCA**.

Another interesting point is that, under the same experiment using **DCA** as guest, host **Py1** adopts a different binding mode (Figure S2c) from **Py2**. On the other hand, though, binding with different guests, **Se2** (Figure S2a, with **CA**) and **Py1** (Figure S2c, with **DCA**), takes a similar binding mode; that is, two bile salt molecules are separately included into two CD cavities from the secondary side. These results suggest that the main factor governing the host−guest binding mode may come from CD dimers but not bile salt guests (**CA** and **DCA**). However, the inclusion compactness of **Py1**−**DCA** and **Se2**−**CA** pairs is inequitable. The correlations between the C-21 protons of **DCA** and the H-5 of **Py1** indicate that the carboxylate side chain of **DCA** deeply penetrates into the CD cavity of **Py1** from the secondary side, whereas the corresponding signals for the **Se2**−**CA** pair cannot be observed, suggesting that **CA** is shallowly included from the secondary side of **Se2**.

Although only a part of the host−guest pairs are examined by ROESY spectra, they all show consistent results with those obtained from ITC experiments. Therefore, in the following discussion about thermodynamics, different binding modes are assumed respectively for different host−guest pairs by the N values obtained in ITC experiments; that is, $N = 1$ represents the binding mode shown in Figure 2b, and $N = 2$ represents the binding mode shown in Figure 2a1,2a2.

Cooperative Binding of CD Dimers toward Guests. Parameters in Table 1 suggest that, the binding constant of every host−guest pair is strictly controlled by the structure and length of the spacer of CD dimer, showing a complicated but ordered sequence from both host and guest viewpoint.

Either for **Se-dimers** or for **Py-dimers**, the host−guest stoichiometry (N value) changes in the same order, that is, from 1:2 ($N = 2$) to 1:1 ($N = 1$) with the increase of spacer length. This phenomenon indicates that possessing the long enough spacer might be the precondition for CD dimer to achieve the cooperative binding of two cavities. But there still exists some exception. For **Se-dimers**, only **Se3** and **Se4** adopt the 1:1 binding mode. However, for **Py-dimers**, only **Py1** adopts the 1:2 binding mode; the others all show the 1:1 cooperative binding mode. A possible explanation is that although **Se2** possesses a longer spacer than **Py2**, the flexibility of the Se−Se bond will induce the spacer of **Se2** to take a tortuous conformation, which reduces the actual distance between two CD cavities in **Se2**. In addition, the thermodynamic results reveal that, with the longest spacer, **Se4** gives the largest stability constants ($K_S = 5030$ M^{-1} for **CA**, 6100 M^{-1} for **DCA**) in all **Se-dimers**, while the largest stability constants of **Py-dimers** toward each guest molecule is obtained by the dimers **Py2** ($K_S = 12\,700$ M^{-1} for **CA**) and **Py3** ($K_S = 13\,100$ M^{-1} for **DCA**) with the moderate spacer lengths. This difference not only confirms our hypothesis about the tortuous conformation of the spacers in **Se-dimers** but also suggests that only the CD dimers possessing the proper spacer length can give the perfect cooperative binding toward guests.

It can be found obviously from Table 1 that, for the dimers adopting 1:1 cooperative binding mode, the enthalpy changes are not only the main contribution to the binding process ($-\Delta H^\circ > T\Delta S^\circ$) but also the determining factor for the binding abilities. It is well-known that the large negative enthalpy changes are usually attributed to the remarkable van der Waals interactions induced by the good size/shape fit between host and guest. That

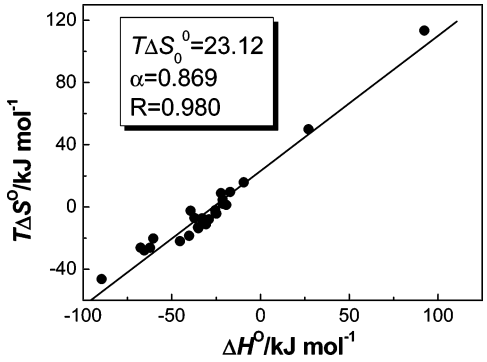


Figure 3. Enthalpy−entropy compensation plots for 1:1 host−guest complexation.

is to say, the cooperative binding ability of two CD cavities is mostly determined by their matching degree with guest molecule. Comparing the **Se-dimers** with **Py-dimers**, we can find that all of the **Py-dimers** display much stronger binding abilities toward bile salts than corresponding **Se-dimers**, which should be attributed to the difference between the flexible 2,2′-diselenobis(benzoyl) center in **Se-dimers** and the relatively rigid 2,2′-bipyridine center in **Py-dimers**. Therefore, we can deduce that the presence of rigid spacer favors formation of a relatively fixed binding mode and results in the close contact between two CD cavities and guest molecule, leading to the stronger binding abilities for **Py-dimers**. Besides the difference in the flexibility of the spacer, the structural differences also affect the binding abilities of CD dimers. Due to the presence of the bipyridine fragment**,** the hydrogen bond between the hydroxyl group of the bile salt and the nitrogen atom of bipyridine might also be taken as a plausible explanation for the strong binding abilities of **Py-dimers** as compared with **Se-dimers**. It should be noted that although fixed binding mode leads to the loss of the conformational freedom, the significant exothermic enthalpy change accompanying the cooperative binding overwhelms the unfavorable entropic loss. Therefore, a strong association of **Py2** with **CA** or **DCA** is still achieved due to the large negative enthalpy changes ($\Delta H° = -32.4$ kJ·mol^{-1} for **CA** and $\Delta H° = -45.4$ kJ·mol^{-1} for **DCA**), although **Py2** displays the most unfavorable entropy changes ($T\Delta S° = -9.0$ kJ·mol^{-1} for **CA** and $T\ \Delta S° = -22.0$ kJ·mol^{-1} for **DCA**) upon complexation with **CA** and **DCA**.

On the other hand, upon complexation with **CA** and **DCA**, all hosts adopting a 1:1 binding mode (**Se3**, **Se4**, **Py2**−**4**) show higher binding abilities than native β-CD due to more favorable enthalpy contributions ($-\Delta\Delta H° = 2.5-16.0$ kJ·mol^{-1}), which perfectly confirms the advantage of cooperative binding of guests by two CD cavities.

Enthalpy−Entropy Compensation. It has been demonstrated that the enthalpy−entropy compensation[31,32] effect is a general rule in the complexation of CDs.[33−35] Using the thermodynamic parameters obtained in this work and the reported data,[5,10,21,25a] $T\Delta S°$ for the 1:1 host−guest complexations are plotted against the corresponding $\Delta H°$, giving an excellent straight line with a correlation coefficient of 0.980 (Figure 3).

The comparison of enthalpy−entropy compensation effect for β-CD, monomodified β-CDs, and β-CD dimers is shown in Table 2. The slope (α) and intercept ($T\Delta S_0°$) for CD dimers with 1:1 host−guest stoichiometry are obviously different from those of native and monomodified β-CDs. The moderate α value (0.87) of CD dimers (1:1 stoichiometry) suggests that the entropy loss induced by conformational change cancels the enthalpy gain in a degree higher than native β-CD but lower than monomodified β-CD, and the much larger $T\Delta S_0°$ value (23 kJ·mol^{-1}) reflects the more extensive desolvation effect caused by the cooperative binding of the guest molecule into the two closely located cavities of the CD dimer.

TABLE 2: Enthalpy−Entropy Compensation Analyses of β-CD, Monomodified β-CDs, and β-CD Dimers

host	a	$T\Delta S_0°$/(kJ·mol^{-1})	n[a]	R[b]	ref
native β-CD	0.80	11	488	0.89	c
monomodified β-CDs	0.99	17	128	0.99	c
β-CD dimmers (1:1)	0.87	23	27	0.98	d

[a] Number of data sets used. [b] Correlation coefficient. [c] Reference 35. [d] This work.

Conclusion

The thermodynamics and ROESY results jointly reveal that two different binding modes exist in the binding processes of CD dimers with bile salts; that is, the 1:1 cooperative binding mode and the 1:2 secondary-side binding mode. The spacer of CD dimers is considered to be the crucial factor in determining the binding mode and the binding behavior. By comparison of thermodynamic parameters, we find that the CD dimers possessing the spacer with suitable length and rigidity prefer to achieving the 1:1 cooperative binding toward guests **CA** and **DCA**, while the dimers possessing a short-length spacer tend to include these guests from the secondary side forming 1:2 inclusion complexes. All of the examined dimer−bile salt complexations are enthalpy-driven processes, which means that choosing certain a spacer advantageous for improvement of the van der Waals interaction between host and guest should be first considered in designing the CD dimer to construct the perfect cooperative binding model. Enthalpy−entropy compensation further indicates that there exists the extensive desolvation effect for 1:1 host−guest complexation of CD dimers.

Acknowledgment. This work was supported by NNSFC (Grants No. 90306009 and 20272028) and the Natural Science Fund of Tianjin (Grant 043604411), which are gratefully acknowledged.

Supporting Information Available: Heat effects of dilution and of complexation of the **Se4**−**DCA** pair, ROESY spectra of the **Se2**−**CA, Py2**−**DCA**, and **Py1**−**DCA** pairs, and structures of reference hosts and guests used for enthalpy−entropy compensation analyses (pdf). This material is available free of charge via http://pubs.acs.org.

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