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Characterization of Bile Salt/Cyclodextrin Interactions Using Isothermal Titration Calorimetry

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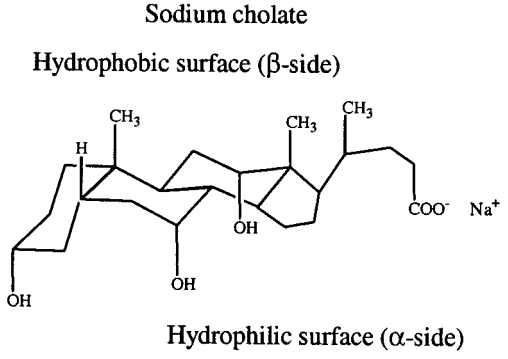
The interactions of cholate, deoxycholate, glycocholate, and taurocholate with methyl-β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin were studied by means of isothermal titration calorimetry and molecular modeling. The binding constants, standard molar enthalpy, Gibbs free energy, and entropy changes were determined for the formation of bile salt/cyclodextrin inclusion complexes. We observed a 1:1 stoichiometry for all inclusion complexes and could demonstrate marked differences in binding affinity between the different bile salt and cyclodextrin molecules. The dihydroxy bile salt deoxycholate showed significantly higher affinity toward methyl-β-cyclodextrin ($K = 6276 \pm 164$ M^{-1}) and 2-hydroxypropyl-β-cyclodextrin ($K = 4429 \pm 34$ M^{-1}) compared to the trihydroxy bile salt cholate ($K = 2693 \pm 25$ M^{-1} and $K = 2510 \pm 98$ M^{-1}, respectively). The conjugation of cholate with glycine or taurine lowered its affinity markedly toward methyl-β-cyclodextrin ($K = 1958 \pm 178$ M^{-1} and $K = 2148 \pm 33$ M^{-1}, respectively). Our molecular modeling and docking data suggest that the most probable mode of binding would be by insertion of the bile salt A-ring into the rim of the cyclodextrin containing the secondary alcohol moieties. Our results show that bile salt binding to cyclodextrin is influenced both by the degree of bile salt hydroxylation and by bile salt conjugation.

Introduction

Cyclodextrins (CyDs) are naturally occurring cyclic oligosaccharides built up from 6, 7, or 8 glucopyranose units and are thus called α-, β-, or γ-cyclodextrins. They have a central hydrophobic cavity, with which they can bind hydrophobic or amphipathic molecules. They have been shown to be useful tools to manipulate the lipid composition in model and biological membranes.[1−4]

Bile salts are important biological amphiphiles that have a steroid structure (Chart 1). They are synthesized from cholesterol by hepatocytes.[5] The polar moieties that bile salts possess are located on one surface of the steroid structure. This gives them an amphipathic character with a hydrophobic and hydrophilic side, which increases their water solubility compared to cholesterol from which they are synthesized.[6] Earlier studies have shown that β-CyDs are efficient sterol acceptor molecules, apparently because their hydrophobic cavity, with a diameter of 6 Å and a height of 7.9 Å, matches the size of sterol molecules such as cholesterol and bile salts.[3,7−9] Thermodynamic and NMR studies have shown that inclusion complexes are formed between bile salt molecules and β-CyD and that the most probable mode of ligand binding involves the insertion of the more hydrophobic part of the ligand into the CyD cavity.[10−17] The more polar part of the ligand prefers the hydrophilic environment on the outside of the hydrophobic core of CyD. The principal forces involved in binding are believed to be primarily hydrophobic,[13,17−25] although

Chart 1. Structure and Hydrophilic−Hydrophobic Balance of Cholate



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hydrogen bonding and steric effects have been shown to play a role.[16,17]

Isothermal titration calorimetry (ITC) is a valuable tool for studying ligand−macromolecule interactions, because it yields thermodynamic information such as the heat of binding (ΔH), the association constant (K-value) of the ligand−macromolecule complex, and the stoichiometry of binding.[26] From this information, the free energy of binding (ΔG) and the change in entropy (ΔS) can be calculated.[27] Calorimetry is the only direct method to determine the enthalpy of binding.[28] An understanding of inclusion complex formation and the association energetics of this process is of particular interest due to the potential use of CyD as a vehicle to administer pharmaceuticals and to increase the bioavailability of poorly soluble drugs.[29−36] Several studies have been published on the complexation of bile salts with CyDs.[7−9,37]

In this study, we have investigated the thermodynamics of bile salt binding to methyl-β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin by means of isothermal titration calorimetry. We have also employed a molecular modeling approach to study the binding mechanism(s) in detail. The aim of the present work was to study the differences in binding thermodynamics and the mode of binding of dihydroxy versus trihydroxy bile salts to these two CyDs. Furthermore, we were interested in how conjugation of the bile salts would affect binding.

Experimental Procedures

Material. The sodium salts of cholate (3α,7α,12α-trihydroxy-5β-cholanoic acid), deoxycholate (3α,12α-dihydroxy-5β-cholanoic acid), glycocholate (*N*-[3α,7α,12α-trihydroxy-24-oxocholan-24-yl] glycine) and taurocholate (2-[(3α,7α,12α-trihydroxy-24-oxo-5β-cholan-24-yl) amino] ethanesulfonic acid) were all obtained from Sigma Chemicals (USA) and used as provided. Methyl-β-cyclodextrin (mβCyD) and 2-hydroxypropyl-β-cyclodextrin (2OHpβCyD) were both purchased from Sigma Chemicals. All chemicals were used without further purification. The water used for the ITC experiments was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system, yielding a product with a resistivity of 18.2 MΩ cm. If not otherwise specifically denoted, the buffer composition used was 10 mM Tris, 140 mM NaCl, pH 7.4.

Isothermal Titration Calorimetry. Isothermal titration calorimetry was performed using a high-sensitivity isothermal titration calorimeter 4200 from Calorimetry Sciences Corp. (USA). The isothermal titration calorimeter was calibrated electrically. The data were acquired by computer software provided by Calorimetry Sciences Corp. Bile salt/CyD binding experiments were performed by injecting 10 μL aliquots of a CyD solution (15 mM) into the sample cell containing a bile salt solution (1 mM). All experiments were performed with constant stirring (200 rpm) driven by a stepping motor coupled to the isothermal titration calorimeter. The sample cell volume was 1335 μL in all experiments. Bile salt solutions were applied at a concentration of 1 mM, which is below their critical micelle concentration (cmc). In control experiments, 10 μL aliquots of a CyD solution (15 mM) were injected into the sample cell containing buffer without detergent. The dilution heat was corrected for during the data analysis.

Determination of the cmc for the Bile Salts. The cmc's for the bile salts were determined because it is critical to use bile salt concentrations below the cmc in experiments. 8-Anilinonaphthalene-1-sulfonate (ANS) was used as a fluorescence probe to determine the cmc for cholate, deoxycholate, glycocholate, and taurocholate. The cmc of the surfactant was determined by subsequently adding 2 μL of bile salt from a 1 M stock solution to a 10 μM solution of ANS. As the cmc of the surfactant was approached, one could observe a gradual shift to a higher fluorescence intensity due to the partitioning of ANS into the hydrophobic core of the micelles. Excitation was carried out at 370 nm, and emission was recorded at 430 nm, at room temperature, using a Hitachi F-2000 spectrofluorimeter (Hitachi Ltd., Tokyo, Japan). The acquired cmc values are as follows: cholate, 9.0 ± 1 mM; deoxycholate, 1.8 ± 0.2 mM; glycocholate, 7.5 ± 0.1 mM; and taurocholate, 7.0 ± 0.5 mM. These results are in good agreement with earlier findings.[38]

Molecular Modeling of the Inclusion Complexes. The three-dimensional structures of βCyD (CSD code, BEDZOZ)[39] and mβCyD (CSD code, CEQCUW) [40] were obtained from the Cambridge Structural Database (CSD).[41] The 2OHpβCyD molecule was built by modifying βCyD and then energy minimizing 2OHpβCyD in SYBYL 6.6 (Tripos Associates, St. Louis, MO) using their conjugate gradient method. Bile salts were energy minimized in SYBYL 6.6 and docked to mβCyD and 2OHpβCyD with AutoDock 3.0.[42] AutoDock 3.0 is a semirigid docking program; AutoDock 3.0 considers the whole ligand molecule in the conformational search at the binding site, but it is possible to choose the torsion angles of the ligand that are allowed to rotate (AutoTors), while the bond angles and bond lengths are kept fixed. AutoDock 3.0 uses a Lamarckian genetic algorithm minimization scheme combined with a rapid, atomic-resolution, grid-based method of energy evaluation utilizing the AMBER force field[43,44] in order to find low-energy conformations of ligands in the receptor-binding site. The overall interaction energy between chemical species is estimated by considering both Lennard-Jones atom−atom potentials and electrostatic effects, summed for the individual interactions between atoms. Partial charges for CyDs and ligands were calculated using the MMFF94 force field.[45] The interaction of a probe group (corresponding to each type of atom in the ligand) with a receptor model was calculated at grid positions 0.25 Å apart in an ($15 \times 15 \times 15$) Å box centered at the binding site using the program AutoGrid in the AutoDock package. For each ligand, 20 separate docking simulations were performed.

Results and Discussion

This study presents a thermodynamic description of the binding of four bile salts (cholate, deoxycholate, glycocholate, and taurocholate) to mβCyD and 2OHpβCyD. The number of hydrophobic residues and the conjugation

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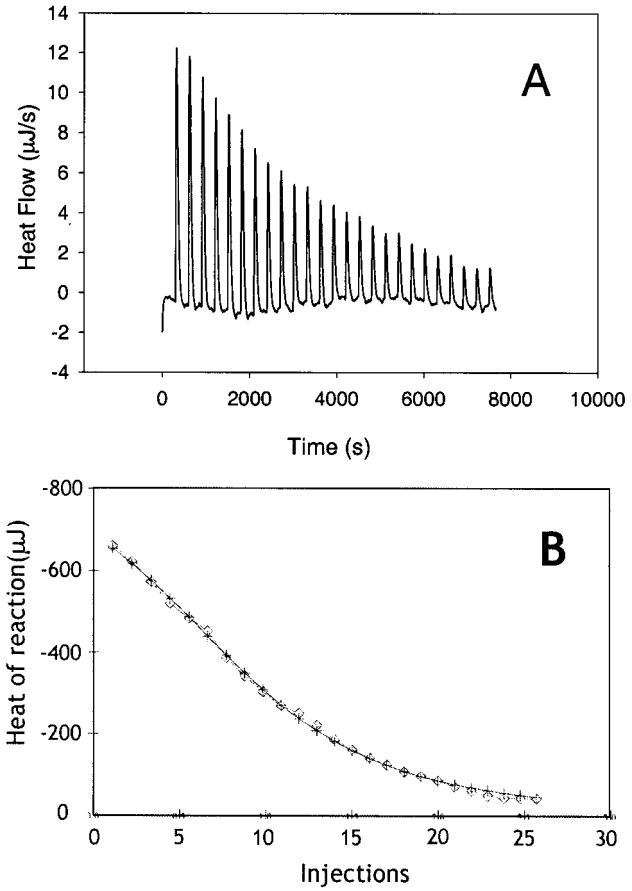

Figure 1. (A) Calorimetric traces (heat flow) observed upon injecting 10 μL aliquots of CyD solution (15 mM) into the sample cell (V = 1.335 mL) containing bile salt solution (1 mM). Experiments were performed at 25 °C. (B) Heats of reaction as obtained from the integration of the calorimetric traces (◊). The solid line resembles the theoretical fit of the binding isotherm. The acquired fit parameters were $K = 2711$ M^{-1}, $\Delta H = -6.1$ kJ mol^{-1}, and 1:1 stoichiometry.

of bile salts with glycine or taurine was shown to markedly affect binding thermodynamics. Our results also show that the four bile salts had a slightly higher affinity toward mβCyD compared to 2OHpβCyD. The formation of bile salt/CyD inclusion complexes was modeled for the cases of cholate/mβCyD and deoxycholate/mβCyD.

Isothermal Titration Calorimetry. The binding of cholate, deoxycholate, glycocholate, and taurocholate to mβCyD and 2OHpβCyD was studied at 25 °C with isothermal titration calorimetry. A typical titration pattern of an experiment is shown in Figure 1 (panel A). Each titration of CyD into the sample cell gave rise to a heat of reaction, caused by the formation of inclusion complexes between bile salt molecules and CyD. The heats of reaction decrease after each injection of CyD because less and less bile salt molecules are available to form inclusion complexes. The binding experiments served to measure the K-value of inclusion complex formation, the stoichiometry, and the change in binding enthalpy (ΔH). The binding stoichiometry, ΔH, and K-value were given as parameters when fitting the binding isotherm (panel B in Figure 1). The analysis was performed with software provided by Calorimetry Science Corp. Knowledge of the K-value and ΔH enabled calculation of the free energy of binding (ΔG) and the change in entropy (ΔS), according to

$$\Delta G = -RT \ln K = \Delta H - T\Delta S \qquad (1)$$

Table 1. Binding Constant (K-Value), ΔH, ΔG, and ΔS for 1:1 Inclusion Complex Formation of Bile Salts with mβCyD and 2OHpβCyD[a]

guest	K (M^{-1})	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J K^{-1} mol^{-1})
		mβCyD		
CA	2693 ± 25	−5.7 ± 0.4	−19.6	46.6
DCA	6276 ± 164	−6.8 + 0.2	−21.7	49.9
		2OHpβCyD		
CA	2510 ± 98	−7.9 ± 0.1	−19.4	38.6
DCA	4429 + 34	−10.65 + 0.15	−20.8	34.0

[a] Aliquots of CyD (15 mM) in buffer were injected into the sample cell containing the bile salt solution (1 mM) at 25 °C. Values are given as the average value ± range from two different experiments.

Table 2. Binding Constant (K-Value), ΔH, ΔG, and ΔS for 1:1 Inclusion Complex Formation of Bile Salts with mβCyD and 2OHpβCyD[a]

guest	K (M^{-1})	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J K^{-1} mol^{-1})
		mβCyD		
GCA	1958 ± 178	−7.9 ± 0.1	−18.8	36.6
TCA	2148 ± 33	−7.2 ± 0.2	−19.0	39.6
		2OHpβCyD		
GCA	1764 ± 115	−8.2 ± 0.1	−18.5	34.5
TCA	1399 + 196	−8.75 + 0.3	−18.0	31.0

[a] Aliquots of CyD (15 mM) in buffer were injected into the sample cell containing the bile salt solution (1 mM) at 25 °C. Values are given as the average value ± range from two different experiments.

where R is the gas constant and T is the absolute temperature.[46]

Binding of Cholate and Deoxycholate to mβCyD and 2OHpβCyD. Both cholate and deoxycholate bound to mβCyD and 2OHpβCyD with a 1:1 stoichiometry. Cholate bound to mβCyD with a ΔH of −5.7 ± 0.4 kJ mol^{-1}, while deoxycholate gave a slightly higher ΔH of −6.8 ± 0.2 kJ mol^{-1} (Table 1). The K-value was significantly higher for deoxycholate (6276 ± 164 M^{-1}) binding to mβCyD compared to cholate (2693 ± 25 M^{-1}). This difference in binding affinity is likely explained by the more hydrophobic nature of deoxycholate due to the absence of the C-7 hydroxyl group, which is present in cholate. Cholate bound to 2OHpβCyD with a ΔH of −7.9 ± 0.1 kJ mol^{-1}, which is lower than the corresponding value for deoxycholate (−10.65 ± 0.15 kJ mol^{-1}). The K-value was somewhat lower for cholates binding to 2OHpβCyD (2510 ± 98 M^{-1}) compared to mβCyD (2693 ± 25 M^{-1}), while deoxycholate showed a markedly lower affinity for 2OHpβCyD (4429 ± 34 M^{-1}) compared to mβCyD (6276 ± 164 M^{-1}). When comparing the thermodynamic parameters from experiments with these two CyDs, one can observe that these bile salts show a slightly higher affinity for binding to mβCyD compared to 2OHpβCyD.

Binding of Glycocholate and Taurocholate to mβCyD and 2OHpβCyD. Glyco- and taurocholate were found to have lower affinities to mβCyD and 2OHpβCyD compared to cholate and deoxycholate. Glycocholate and taurocholate bound to mβCyD with K-values of 1958 ± 178 and 2148 ± 33 M^{-1}, respectively (Table 2). The ΔH was slightly higher for glycocholate binding to mβCyD (−7.9 ± 0.1 kJ mol^{-1}) compared to taurocholate (−7.2 ± 0.2 kJ mol^{-1}). Taurocholate bound with lower affinity to 2OHpβCyD (K = 1399 ± 196 M^{-1}) compared to glyco-

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cholate, which gave a K-value of 1764 ± 115 M^{-1}. The ΔH was lower for glycocholate binding to 2OHpβCyD (-8.2 ± 0.1 kJ mol^{-1}) compared to taurocholate (-8.75 ± 0.25 kJ mol^{-1}). Both glyco- and taurocholate gave the same 1:1 stoichiometry for binding to mβCyD and 2OHpβCyD as did cholate and deoxycholate.

Process of Complex Formation. The thermodynamic parameters of the inclusion complex formation are believed to be primarily governed by the partitioning of the hydrophobic molecule into the CyD cavity and by the dehydration of the nonpolar surface of the ligand.[13,16,17] According to the classical hydrophobic interactions between two nonpolar molecules, the removal of water from the nonpolar surfaces of the molecules makes both ΔH and ΔS positive for the process.[47] In such a case, the association of the two molecules can be said to be "entropy driven". But with CyD inclusion complex formation, one is not dealing with a classical hydrophobic interaction as Connors has pointed out.[48] Instead, formation of an inclusion complex has been shown in most cases to be "enthalpy driven", where ΔH and ΔS for the process are both negative. Our results gave a negative ΔH but a positive ΔS for the inclusion complex formation. The cavity of CyD appears to be nonpolar compared to the bulk water, but the cavity is actually semipolar. The binding constant is known to be dependent on the fit of the ligand within the CyD core and on the hydrophobicity of the ligand.[37] Charged groups, such as carboxylate and ammonium,[16] or hydrophilic groups, such as amino, carboxyl, and hydroxyl,[13] remain exposed to the bulk solvent even after the inclusion of the hydrophobic moiety. An exception to this general rule is the aromatic hydroxyl group, which can penetrate deeply into the cyclodextrin cavity where it hydrogen bonds to one of the peripheral hydroxyl groups of cyclodextrin.[16,28] These results clearly show that several different forces simultaneously contribute to binding.

Binding Stoichiometry. Different stoichiometric ratios between ligands and CyDs, such as 1:2, 2:1, and 2:2, have been presented, but by far the most common ratio has shown to be 1:1.[49−53] The stoichiometric ratios that we observed from the binding patterns fell within the range of 0.9−1.1:1. This clearly indicates that the majority of the inclusion complexes had a 1:1 stoichiometry of bile salts and CyDs. These results are in agreement with earlier findings for bile salt/CyD binding.[49−53]

Binding Thermodynamics. Several studies show that cholate can form inclusion complexes with βCyD.[7−9,37] Yang and Breslow reported that they could not measure the K-value for the binding of cholate to βCyD by titration calorimetry.[37] However, by increasing the molar concentrations of CyD (2 → 15 mM) and cholate (0.1 → 1 mM) and by using mβCyD or 2OHpβCyD instead of βCyD, we were able to obtain the K-value for cholate binding to mβCyD (2693 ± 25 M^{-1}) and 2OHpβCyD (2510 ± 25 M^{-1}). These results are in reasonable agreement with earlier findings where cholate/βCyD complexes have been examined with NMR[8,15] ($K = 1500 \pm 0$ to 3400 ± 400 M^{-1}) and flow microcalorimetry[7] (3150 M^{-1}).

We found that the binding constants for the complexation of the more polar trihydroxy bile salts (cholate, glycocholate, and taurocholate) to the CyDs were lower compared to the less polar dihydroxy bile salt, deoxycholate. This observation is consistent with results published by Tan and Lindenbaum.[7] Our K-values for complexation of mβCyD with glycocholate (1958 ± 178 M^{-1}) and taurocholate (2148 ± 33 M^{-1}) agree closely with their values (1950 ± 1.1 and 2630 ± 1.1 M^{-1}, respectively). The conjugated bile salts differ from the nonconjugated ones only with respect to their glycine or taurine residue. The conjugated residue makes the conjugated bile salt markedly more polar than the nonconjugated counterpart.[5] Hence, it is most likely that it is the polar conjugated residue that lowers the affinity of these bile salts toward mβCyD and 2OHpβCyD.

Molecular Modeling and Mode of Binding. Knowledge of the detailed binding mechanism is unclear since cholate and deoxycholate are able to enter and bind to the asymmetric CyDs from two sides (the primary site refers to the hydroxyl rim with the smaller diameter, and the secondary site is the hydroxyl rim with the larger diameter).[48] It has been suggested that bile salts enter into the cavity of CyDs either with the A-ring of the steroid nucleus[8] or with the carboxylate group (tail).[7−9,15] González-Gaitano and co-workers obtained both types of inclusion complexes using molecular modeling.[8] They acquired binding energies (in a vacuum) of about -37.5 kJ mol^{-1} for the A-ring complex and about -32.5 kJ mol^{-1} for the tail complex. These ΔH values are quite similar and could suggest that both types of bile salt/CyD complexes can form simultaneously. Our results suggest, however, that the most probable mode of binding would be by insertion of the bile salt A-ring into the CyD secondary site cavity (Figure 2). The binding mechanism is easier to explain for mβCyD than for βCD, since the primary site in mβCyD does not contain any OH groups but instead contains methoxy groups, which cannot act as hydrogen bond donors but can function only as hydrogen bond acceptors.

The mechanism of glycocholate and taurocholate binding to CyDs is more straightforward, since glycocholate and taurocholate are much less likely to enter the interior of CyD with their "tail" first, because the side chains are polar. The binding mechanism is likely to be the same for both mβCyD and 2OHpβCyD. The ΔH was higher for 2OHpβCyD compared to mβCyD, which is understandable because the 2-hydroxypropyl group is more flexible and can optimize its orientation in order to interact with the polar tails of glycocholate and taurocholate. This leads to a situation where interaction between the hydrophobic part of the 2-hydroxypropyl group and glycocholate (and taurocholate) is less favorable than with mβCyD and ΔS is lower in comparison to mβCyD. Cholate and deoxycholate most likely bind mβCyD with their A-ring (OH) and not with their tail (COO^-) because mβCyD lacks a hydrogen bond donor. At the primary site of mβCyD, one or two (methoxy) methyl groups may interact with the A-ring and one (methoxy) oxygen would provide a hydrogen bond acceptor for the 3α-OH group. The 12α-OH group (labeled as 12a in Figure 2) can form a strong hydrogen bond with the methoxy group oxygen (02′ in Figure 2) of mβCyD at the secondary site, and the OH group (03′) of mβCyD can maintain the original hydrogen bond with the methoxy group (02′, with only small optimization of the position of the hydrogen) and, in addition, hydrogen bond to the OH group (12a) of deoxycholate. The lack of the 7α-OH group in deoxycholate allows the methoxy group (CH_3 attached to 02′) of mβCyD to rotate toward the ligand and optimize the hydrophobic contacts and surface complementarity with deoxycholate. The resulting structure does not affect the intramolecular hydrogen bonding

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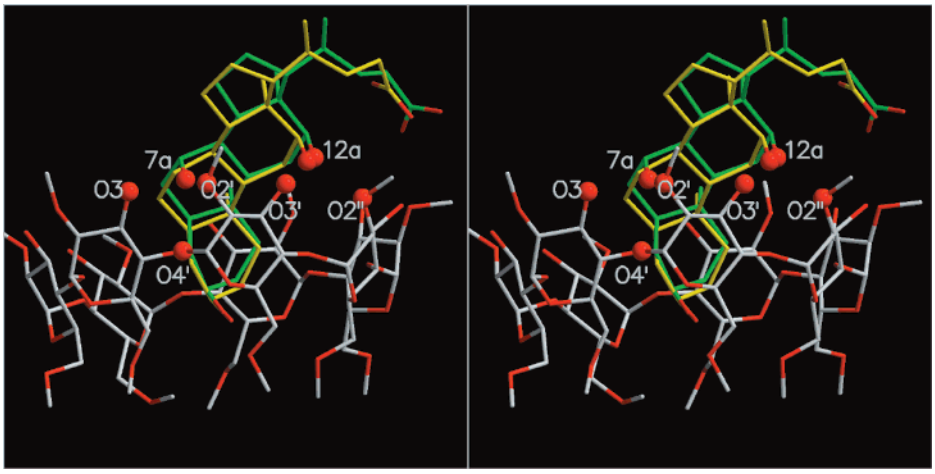

Figure 2. Stereo figure of cholate (green carbon atoms) and deoxycholate (yellow carbon atoms) docked to mβCyD (white carbon atoms). The interacting oxygens at positions 12α (12a) and 7α (7a) of cholate and deoxycholate and the oxygens in the surrounding area of mβCyD are labeled and shown as large red balls.

between the OH group (03) and the methoxy oxygen (02′) of mβCyD. In cholate, glycocholate, and taurocholate, the 7α-OH group (7a) is surrounded by three oxygen atoms and can form a hydrogen bond with the methoxy oxygen (02′, or alternatively with either of the two other oxygen atoms 03 or 04′), but the OH group (03) in mβCyD most likely cannot hydrogen bond to the OH group (7a) of cholate (the angle between the groups is unsuitable for hydrogen bond formation) as was seen for the other OH groups (03′ and 12a). This leads to a situation where the 7α-OH group is hydrogen bonded to only one of three nearby oxygens, which decreases the binding affinity of cholate dramatically compared to deoxycholate. The final conformations of glycocholate and taurocholate, as well as the conformations of ligands docked to 2OHpCyD (data not shown), are similar to the conformations of cholate and deoxycholate in mβCyD (Figure 2).

In conclusion, this study has presented thermodynamic parameters for the binding of four bile salts to two different CyDs. Our modeling efforts also suggest a plausible structure for the inclusion complex formed between cholate or deoxycholate and mβCyD. A detailed insight, at the molecular level, of the formation of ligand/CyD complexes is important for further studies aimed at using modified CyDs to bind small molecular ligands with high selectivity and specificity.[54]

Abbreviations Used

CA	cholate
cmc	critical micelle concentration
DCA	deoxycholate
GCA	glycocholate
ITC	isothermal titration calorimetry
mβCyD	methyl-β-cyclodextrin
TCA	taurocholate
2OHpβCyD	2-hydroxypropyl-β-cyclodextrin

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