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Investigation of the complexation of (+)-catechin by β-cyclodextrin by a combination of NMR, microcalorimetry and molecular modeling techniques †

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Received 13th March 2003, Accepted 27th May 2003
First published as an Advance Article on the web 10th June 2003

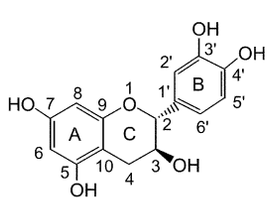
(+)-Catechin is a polyphenolic compound of natural origin that presents anti-oxidant properties of interest for therapeutics or cosmetics uses. Preliminary studies on inclusion into cyclodextrin cavities yielded contradictory results both for the quantitative (affinity constant) and qualitative description of the interaction. By a combination of several experimental and theoretical methods, the present study resolved the previous ambiguities about the interaction between (+)-catechin and β-cyclodextrin. Thermodynamic data measured by isothermal titration calorimetry demonstrate that the binding is enthalpy driven. Excellent agreement has been obtained for the measurement of the association constant by NMR and microcalorimetry. The several docking modes obtained by systematic docking studies have been compared to intermolecular contacts measured by NMR and the overall geometry of the complex can be proposed.

Introduction

Naturally occurring cyclodextrins (CDs) are cyclic oligosaccharides made of (α-1,4)-linked D-glucopyranose units which are able to include various small organic compounds in their hydrophobic cavity.[1,2] Such inclusion complexes induce modification of the physicochemical properties of the 'guest' molecules, particularly in terms of water solubility and solution stability. For this reason, cyclodextrins are widely used in various industries. The most common cyclodextrins are α-, β- and γ-cyclodextrins which consist of 6, 7 and 8 glucopyranose units, respectively. Nevertheless, β-cyclodextrin is by far the most widely used compound owing to the optimal size of its internal cavity (8 Å) for the encapsulation of molecules.[3–5]

Catechins are phenolic compounds extracted from plants and present in natural food and drinks, such as green tea[6] or red wine.[7] The role of such molecules in the prevention of cancer and cardiovascular disease has received a great deal of attention.[8,9] Catechins are scavengers of reactive oxygen species, and their resulting anti-oxidant properties are of great interest in dietetics and cosmetology. Furthermore, their antiviral and cancer inhibiting properties could have pharmaceutical applications.[10] Inclusion in cyclodextrins is envisaged in order to mask the nasty aspects (taste, colour. . .) of such phenolic compounds. Due to their potential applications, and also to their optimal size, these molecules have been widely used as model compounds for inclusion studies with β-cyclodextrins.[11–13]

The flavan-3-ol compound (+)-catechin (**1**) consists of two benzene rings (A- and B-rings) and a pyran ring (called the C-ring). Several detailed studies on nonenzymatic and enzymatic oxidations of (+)-catechin have been performed.[14,15] Although the oxidation products depend on the oxidant system, the anti-oxidant activity of (+)-catechin appears to be related to both the A-ring and the B-ring. Conformational flexibility includes the orientation of the linkage between ring B and ring C, and the puckering of the pyran ring. The conformational interchange that occurs within the C-ring brings the B-ring into a pseudoequatorial (**E**-conformer) or a pseudoaxial (**A**-conformer) position (Fig. 1). Molecular modeling calculations combined with conformational NMR studies[16] confirmed that (+)-catechin adopts a mixture of **A**- and **E**-conformers in aqueous solution and their conformational equilibrium has been evaluated to be 33 : 67. Preliminary NMR studies on the inclusion of (+)-catechin by β-cyclodextrin yielded contradictory results for both the evaluation of the affinity constant and the proposed geometry of the complex.[11–13] The aim of the present work is to re-investigate the thermodynamics and conformational requirements of the complex of (+)-catechin with β-cyclodextrin using a combination of experimental and theoretical approaches.



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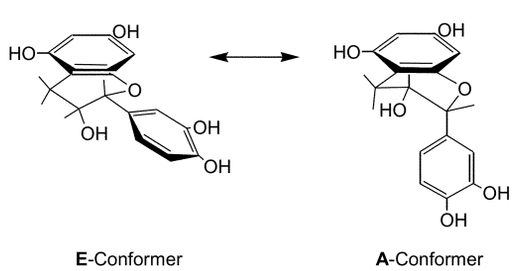

E-Conformer **A**-Conformer

Fig. 1 Schematic representation of **E**- and **A**-conformations of (+)-catechin.

Results and discussion

NMR investigation of the association parameters

^{1}H NMR data for (+)-catechin dissolved in D_2O are given in Table 1. Assignment of the ^{1}H NMR spectrum was achieved by means of heteronuclear ^{1}H–^{13}C correlation methods (HMQC, HMBC) and literature data.[17] The $^3J_{HH}$ coupling constants for

† Electronic supplementary information (ESI) available: NMR spectra. See http://www.rsc.org/suppdata/ob/b3/b302935m/

DOI: 10.1039/b302935m

Table 1 Chemical shifts (δ, ppm) and coupling constants (J, Hz) for the heterocyclic ring of (+)-catechin in aqueous solution at 298 K. The coupling constants are time-averaged values from rapid flexing of the heterocycle between **A**- and **E**-conformations

Atom	δ	J	J^a
H-2	4.80	7.5	7.6
H-3	4.26	(m)	(m)
H-4a	2.91	5.3, 16.1	5.3, 16.1
H-4b	2.58	8.1, 16.1	8.0, 16.1

[a] Literature values obtained in D_2O containing about 20% MeOH-d_4.[16]

the pyran ring of (+)-catechin are similar to those reported previously by Hemingway *et al.*[16,18] who proposed that the observed coupling constants can be considered as time-averaged values from rapid puckering of the pyran ring between the **E**- and **A**-conformational isomers.

Inclusion of (+)-catechin in β-cyclodextrin is evidenced by modifications of the ^{1}H NMR spectra of both the host and guest molecules.[19–21] The NMR spectra of mixtures of β-CD and (+)-catechin strongly differ from those of the pure compounds. As only shifts are observed (no new peaks appear on the spectra of the mixtures), it can be concluded that complexation occurs under fast exchange conditions (relative to the NMR timescale). The differences observed between the NMR spectra of free molecules and complexed ones are similar to those reported previously,[13] and will therefore not be lengthily described here. In the case of β-CD, the largest shifts are observed for the cavity protons (H-3 and H-5) (see Fig. 2). These upfield shifts are due to ring current effects of the included phenyl moiety. Upfield shifts can also be observed for the H-6 protons located on the smaller rim. Concerning (+)-catechin, upfield shifts are observed for all the aromatic protons of the B-ring (H-2′, H-5′ and H-6′) whereas only one of the two aromatic protons of the A-ring (H-8) experiences perturbation (see the supplementary data †). Moreover, all proton signals, except the H-6 one, undergo a line broadening in the presence of β-CD. This effect is particularly significant for the proton signals of the pyran ring with the two H-4 protons becoming chemically equivalent as the concentration of cyclodextrin increases. The line broadening observed for the proton signals of (+)-catechin may reflect modifications in the conformational interchange of the heterocyclic C-ring induced by the insertion of the B-ring in β-CD and a restriction of the motions. Variable temperature experiments in the range 298–353 K were carried out in order to examine the temperature effect on line broadening. Raising the temperature yields a line sharpening for catechin proton signals (see the supplementary data †). This behaviour may be a consequence of a temperature-induced increase of the motions and exchange rates, but also of the dependence of the complex concentration on temperature. Indeed, the origin of complex formation is enthalpic ($\Delta H < 0$, according to the microcalorimetry experiment described below), and hence, the association constant decreases when the temperature increases. Consequently, the spectra measured at higher temperatures are similar to those obtained with an excess of catechin. This is particularly evidenced by the chemical shift variations of the H-6 CD protons as a function of the temperature.

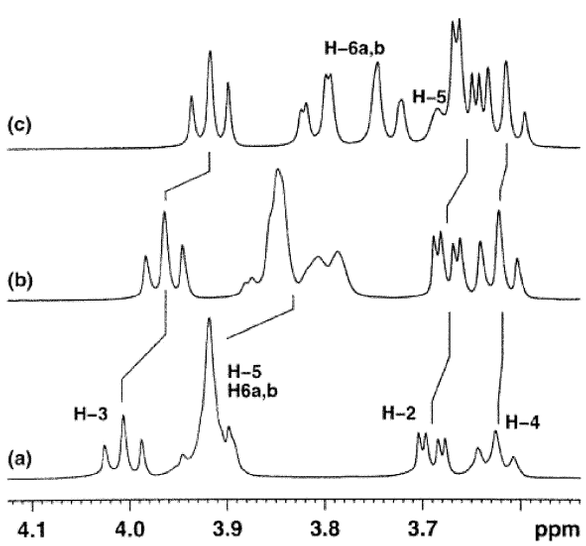

Fig. 2 Partial ^{1}H NMR spectra (500 MHz, 298 K) of β-CD–(+)-catechin mixtures (region of the CD protons). The following concentrations were used: (a) 7 mM β-CD; (b) 4.9 mM β-CD and 2.1 mM (+)-catechin; (c) 2.1 mM β-CD and 4.9 mM (+)-catechin.

A more detailed analysis of the inclusion process was performed by measuring the apparent association constant K_a. The precise determination of the association constant implies that the stoichiometry of the interaction process is unambiguously determined. Both the stoichiometry and the binding constant can be derived by observing the changes in chemical shifts of selected protons from the host and guest molecules. Determination of the stoichiometry of the complex was performed using the continuous variation method (Job's method).[22] In this procedure, the total concentration of the host and guest molecules is kept constant, the molar ratio r of each component being varied from 0 to 1. A low total concentration (7 mM) was used in order to prevent self-association of catechin.[23] The continuous variation analysis was applied using the H-3 proton signal of β-CD. A CD proton was selected for this experiment because cyclodextrin proton signals are less affected by line broadening than the catechin ones, and their chemical shift variations can be therefore more precisely estimated. Moreover, the H-3 CD protons are expected to be the most prone to variations of chemical shifts upon complexation since they are located in the hydrophobic cavity. The stoichiometry of the complex was found to be 1 : 1 ($n = 1$) as described previously. The average value for the apparent association constant K_a derived from a numerical simulation of the experimental data[21,24] (using the variations in chemical shifts of the H-3 CD protons for the same reasons as given above) was found to be 9800 M^{-1} at 25 °C.

Thermodynamic parameters by microcalorimetry approach

The thermodynamic parameters of the complexation of (+)-catechin by β-CD could be derived from temperature variable NMR experiments, using the van't Hoff equation. However the uncertainty is rather large in such analysis since the heat capacity is considered as invariant over the temperature range. Titration calorimetry, in which the association constant and reaction enthalpy are directly and simultaneously determined by using the data of a single constant temperature, is the method of choice to determine thermodynamic parameters. Fig. 3 shows the data obtained for the microcalorimetry titration of catechin with β-CD in water at 25 °C. As shown in Fig. 3A, exothermic heat is produced after each injection of β-CD. The magnitude of the released heat decreases progressively with each injection until complete complexation of (+)-catechin is achieved. Fig. 3B shows the experimental data and the calculated best fit binding curve which passes very closely through the experimental points ($n = 1.18$, $K_a = 8860$ M^{-1}, $\Delta H^0 = -35.1$ kJ mol^{-1}). Thermodynamic binding parameters are listed in Table 2. Association constants determined by NMR and microcalorimetry are therefore in good agreement in our study.

Table 2 also lists K_a values previously determined by means of circular dichroism (CD) or NMR methods. The association constant determined by Smith *et al.*[12] by CD ($K_a = 8700$ M^{-1} at 25 °C) is in good agreement with the data reported here. However, this value is approximately three to four times higher than

Table 2 Apparent association constants (determined by the use of different experimental methods) and thermodynamic parameters (obtained from microcalorimetry titration) for the interaction of (+)-catechin with β-CD

K_a/M^{-1}	Temp./K	Method (and reference)	$-\Delta G^0$/kJ mol^{-1}	$-\Delta H^0$/kJ mol^{-1}	$T\Delta S°$/kJ mol^{-1}
8860 ± 270	298	ITC (this work)	22.5 ± 0.1	35.1 ± 0.5	−12.6 ± 0.1
9800 ± 295	298	NMR (this work)			
8700	298	CD [12]			
2908 ± 87	318	NMR [11]			
2209	308	NMR [13]			

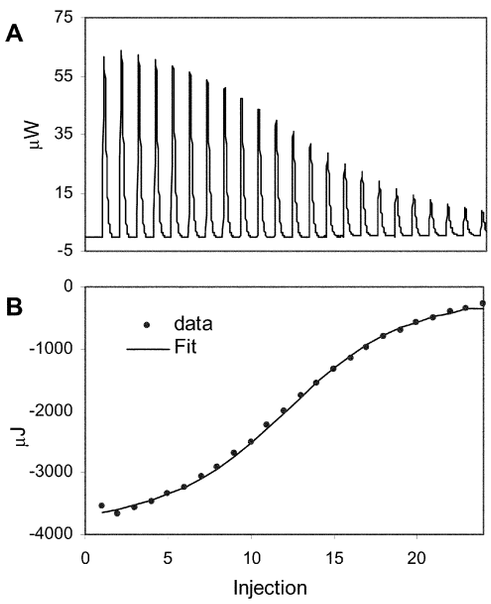

Fig. 3 Calorimetric titration of (+)-catechin (1 mM in pure water) with β-CD (11.4 mM in pure water) at 298 K: (A) raw data obtained for 24 automatic injections, each of 10 μL, of β-CD; (B) the integrated curve showing experimental points and the best fit.

those obtained by NMR experiments at higher temperatures (35 and 45 °C, see Table 2).[11,13] These differences might be partly explained by the fact that the apparent association constant of CD complexes decreases with increasing temperature. From the thermodynamic parameters obtained from our titration microcalorimetry, *i.e.* ΔG^0, ΔH^0 and ΔS^0, the theoretical values for the association constant of the (+)-catechin–β-CD complex can be calculated at different temperatures. The K_a at 45 °C has a calculated value of 3666 M^{-1} which is in good agreement with the experimentally determined one.[11] The agreement is not so good between calculated (5614 M^{-1}) and experimental (2209 M^{-1}) values for the association constant at 35 °C. The difference may be related to some imprecision errors in the NMR measurements of the variations in chemical shifts of the H-8 proton signal of (+)-catechin.[13] Indeed, complexation by β-CD induces line broadening of the H-8 proton signal (see supplementary material †).

NMR investigation of the complex geometry

Some information about the inclusion mode can be directly inferred from the 1D NMR spectra. The induced shielding of all the B-ring protons of (+)-catechin suggests that this ring is inserted in the β-CD cavity. The fact that only the H-8 proton of the A-ring is strongly affected by the presence of β-CD suggests that only one part of the A-ring is involved in the inclusion process.

More direct indications concerning the geometry of the inclusion complex can be derived from the evidence of spatial proximities between protons of β-CD and catechin. For this purpose, 2D T-ROESY experiments dedicated to evidence dipolar interactions (nuclear Overhauser effects) were performed. Strong cross-peaks between the protons of the B-ring and the H-3, H-5 protons of β-CD on the one hand, and between some protons of the C-ring and the H-3, H-5 protons of β-CD on the other hand, can be observed (Table 3). However, no cross-peak appears between the protons of the A-ring and the protons of β-CD. This supports our previous conclusions suggesting that the B-ring is preferentially inserted in the cavity of β-CD. Furthermore, the presence of a cross-peak between the H-6′ and H-3 protons of (+)-catechin is indicative of the existence of the **E**-conformation although it does not exclude totally the existence of the **A**-conformation also in solution.

Molecular modeling

Problems related to molecular modeling of CD were extensively discussed in the literature.[25,26] They include both energy calculation methods[27] and sampling problems.[28] Our goal in the modeling part of this paper is not to describe detailed thermodynamics in solvent but to explore the conformational space in order to determine conformational families. The aim is to combine theoretical and experimental methods, which is, so far, not very common in the literature. It is likely that, as described in the literature,[28] detailed thermodynamic description would require several quite long molecular dynamics trajectories to run with no guarantee that all conformational space is explored.

To achieve the above goals, the CICADA program[29] was used to explore a conformational space that includes docking geometry together with flexibility of both ligands. Calculation was stopped after 16291 points were generated on the energy surface, comprising 2560 energy minima and 3160 transition states. 821 conformers were found in an energy window of 5.0 kcal mol^{-1} above the energy minimum. These conformers could be clustered into families, according to both rms and geometrical parameters describing the orientation of the ligand into the CD cavity. The characteristics of the five most populated families, according to Boltzmann population calculations, are listed in Table 4. For geometrical description (Fig. 4), λ defines the "deepness" of the binding mode, Θ the verticality of the B-ring in the CD (90° for vertical orientation) and Ω the orientation of the A-ring above the CD (0° for fully folded on the CD).

Fig. 5 displays the different families of binding mode predicted to occur. Short proton–proton distances (< 3 Å) and hydrogen bonds are listed in Table 3 and Table 4, respectively. In the given energy window, all docking modes present the B-ring of the (+)-catechin inserted in the CD cavity. No inverse binding mode with the A-ring in the cavity is predicted to occur. Ring-A is located above the large, hydrophilic face of the CD, and a strong hydrogen bond between O-3 of CD and O-7 of the A-ring is predicted to occur in most binding modes. As for the ligand conformation, the flexible C-ring is predicted to adopt the **A**-conformer most of the time but occurrence of the **E**-conformer is also observed (Mode_3).

The two most populated families (Mode_1 and Mode_2) differ mainly in the orientation of the hydroxyl hydrogen atoms of the B-ring (not shown) that create a different network of hydrogen bonds with O-6 of CDs and thereby, a slightly different orientation of the B-ring in the CD cavity. In binding Mode_4, the (+)-catechin ligand is not very deeply included in the CD but the A-ring folds back on the top of the host, creating additional hydrogen bonds. In Mode_5, the ligand is completely

Table 3 Comparison of theoretically and experimentally determined intermolecular short distances between protons. M: medium contact (2.8–3.5 Å), S: strong contact (< 2.8 Å)

H–H distance		Mode_1	Mode_2	Mode_3	Mode_4	Mode_5	Exp.
CD	Ligand						
H-3	H-2′	M	M	S	S		S[a]
	H-5′	M					S[a]
	H-6′	M	M	S	S	M	S
	H-2	M	M	M	M		
	H-3	S	S	M	S		S
	H-4a	M	M		S	S	
	H-4b	M	M			S	M
	H-8	M	M			S	
H-5	H-2′	S	S	M	S	M	S[a]
	H-5′	S	S	M	S	M	S[a]
	H-6′	M	M	M		S	S
	H-3	M				S	
	H-4a	S	M			M	M
	H-4b						M
H-6a	H-5′				M	S	M[b]
H-6b	H-5′					S	M[b]

[a] H-2′ and H-5′ cannot be distinguished. [b] H-5′ and/or H-2′.

Table 4 Description of the five lowest energy families of docking mode between (+)-catechin and β-cyclodextrin

Docking mode	Number of solution	Boltzmann population	Ring included in CD	Conformation of ligand	E_{rel}	λ/Å	Θ/°	Ω/°	Intermolecular H-bonds
Mode_1	147	27.4	B	A	0.00	−1.4	86	40	$O3_{CD}\longrightarrow O7$ $O6_{CD+1}\longrightarrow O4'$ $O6_{CD+2}\longleftarrow O3'$
Mode_2	432	54.4	B	A	0.36	−1.0	77	26	$O3_{CD}\longrightarrow O7$ $O6_{CD-1}\longrightarrow O4'$ $O6_{CD-2}\longrightarrow O3'$
Mode_3	18	3.4	B	E	1.56	−1.2	90	76	$O2_{CD}\longrightarrow O3$ $O6_{CD+1}\longrightarrow O4'$ $O6_{CD+1}\longrightarrow O3'$
Mode_4	8	1.6	B	A	1.88	−0.7	57	30	$O3_{CD}\longrightarrow O7$ $O2_{CD-2}\longrightarrow O5$ $O3_{CD-2}\longrightarrow O5$ $O6_{CD-1}\longrightarrow O4'$ $O6_{CD-3}\longrightarrow O3'$
Mode_5	3	0.5	B	A	2.32	−2.7	57	84	$O3_{CD}\longrightarrow O7$ $O3_{CD-1}\longrightarrow O5$ $O6_{CD-2}\longrightarrow O3'$

included in the host and the B-ring protrudes from the narrow site. Mode_3 is the only one where the C-ring adopts the pseudo-equatorial (**E**) shape, generating a vertical orientation of the ligand in the host tore. In this case no contact is observed between the A-ring and the CD and the hydrogen bond between O-3_{CD} and O-7 is not conserved.

Comparison between NMR data and docking simulations

There is a good agreement between the experimental and the theoretical approach about the overall orientation of the (+)-catechin ligand in the cyclodextrin cavity: the B-ring is the one that is included, and the A-ring only creates some additional contact with the top of the cyclodextrin. This agreement is reflected in Table 3. It is however more difficult to select only one of the docking modes that have been depicted in Fig. 5. It seems that the ligand may fluctuate in the binding site and adopts more or less deeply buried conformations: the less buried ones (Mode_1 to Mode_4) yield strong NOEs between the protons of the B-ring and H-3 and H-5 at the top of the CD cavity, while the most buried one (Mode_3) displays short contacts between the CD hydroxymethyl protons (H-6) and H-5 of the B-ring. Interestingly, Mode_1 to Mode_5 are less or more buried, but they all display a conserved hydrogen bond between O-3 of CD and O-7 of the A-ring.

There are still some uncertainties about the pucker of the C-ring. Modeling studies predict that both can form complexes with the CDs, albeit with a more favorable energy for the pseudo-axial (**A**) shape. The more extended **E**-shape may be favored in aqueous solution due to interactions between the hydroxyls of the A-ring and water molecules. From the NMR studies, the **E**-shape is indeed present in the complex, with a NOE signature between the H-3 proton of the C-ring and the H-6′ proton of the B-ring. The **A**-form is characterized by a short contact between H-3 and H-2′ that cannot be unambiguously confirmed or rejected from the ROESY spectrum because of overlapping.

The role of the O-3 oxygen of the catechin also depends on the pucker of the C-ring. It is known that the stereochemistry of the hydroxyl group plays an important role since (−)-epicatechin, which displays the opposite configuration at C-3, is bound by cyclodextrin with lower affinity.[11] This could be in favor of the **E**-shape of (+)-catechin in the complex since only Mode_3 displays an hydrogen bond involving O-3. However, it also appears that in the **A**-shape (Mode_1 to Mode_5), O-3 points towards the external part of the cavity, and epimerization would lead to a steric conflict with the CD, and therefore would not favor the interaction either.

In conclusion, with the use of a combination of several experimental and theoretical methods, the present work

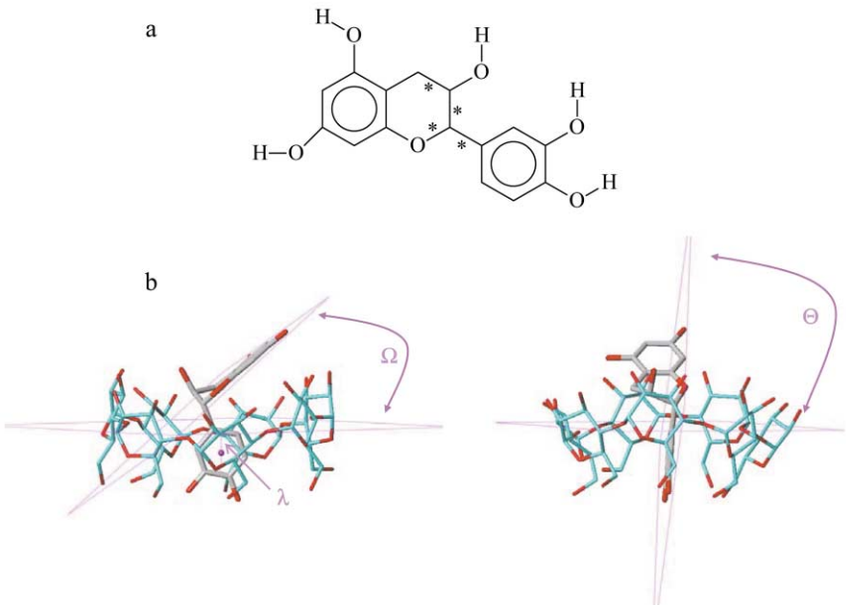

Fig. 4 Representation of the geometrical parameters that allow for the description of inclusion mode for (+)-catechin in β-cyclodextrin (two orthogonal views).

unambiguously determined the characteristics of the interactions between (+)-catechin and β-cyclodextrin. Both NMR and microcalorimetry experiments revealed an association constant which was slightly lower than 1×10^5 M^{-1} at 298 K, the latter methods allowing us to determine that the binding is enthalpy driven. Combination of NMR and molecular modeling converged on inclusion of the B-ring in the cyclodextrin cavity, albeit with some residual flexibility of the ligand.

Experimental

Starting materials

The (+)-catechin was obtained from Sigma. The β-cyclodextrin was kindly supplied by Roquette Frères (Lestrem, France).

NMR spectroscopy

^{1}H NMR experiments were performed using Bruker DRX500 and DRX400 spectrometers operating at 500 and 400 MHz, respectively. Samples were prepared in D_2O obtained from SDS (Vitry, France) to give solutions with a pH of 6. 1D NMR spectra were collected using 16K data points. 2D T-ROESY results were acquired using 2K data points and 256 time increments. The phase sensitive time proportional phase incrementation method (TPPI method) was used and processing resulted in a 1K x 1K (real–real) matrix. Chemical shifts were relative to the DOH signal positioned at 4.83 ppm at 298 K. The variable temperature spectra were measured in the temperature range 298–353 K using a second capillary tube containing DMSO-d_6 as an external reference. Details concerning experimental conditions are given in the figure captions. Association constants for the formation of the 1 : 1 complex were determined using eqn. (1),[24]

$$[G]_t = \Delta\delta_{Hobs}/\Delta\delta_{Hc}([H]_t + (K(1 - \Delta\delta_{Hobs}/\Delta\delta_{Hc})) - 1) \quad (1)$$

where $[H]_t$ and $[G]_t$ are the total concentrations of the host and guest molecules, respectively. $\Delta\delta_{Hobs}$ represents the chemical shift difference (for a given proton) between free H (obtained in the absence of G) and the observed value in the presence of G, whereas $\Delta\delta_{Hc}$ represents the chemical shift difference between free H and the pure complex. The experimental data (corresponding to $[H]_t$, $[G]_t$ and $\Delta\delta_{Hobs}$) were processed using a multiparameter iterative fitting procedure contained in the SIMPLEX algorithm.[24] A low concentration of catechin (≤ 7 mM in D_2O) was used to prevent self-association of catechin.[23]

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry (ITC) was performed using a Model 4200 Microcalorimeter from Calorimetry Sciences Corporation (Utah, USA). Injections of 10 μL of natural β-cyclodextrin in pure water were added from the computer-controlled 250-μL microsyringe at an interval of 5 min into the (+)-catechin solution in pure water (cell volume = 1.3 mL), while stirring at 297 rpm at 298 K. Identical injections of β-CD into a cell containing only water produced heat signals corresponding to heat of dilution. The raw experimental data were presented as the amount of heat produced per second following each injection of β-CD as a function of time. The amount of heat produced per injection was calculated by integration of the area under individual peaks by the instrument software, after taking into account heat of dilution. The experimental data were fitted to a theoretical titration curve using the instrument software, with ΔH^0 (the enthalpy change in kJ mol^{-1}), K_a (the association constant in M^{-1}), and n (complex stoichiometry) as adjustable parameters.

Molecular modeling

The single-coordinate-driving (SCD) method implemented in computer program CICADA[29] was used in modeling of catechin inclusion in the β-cyclodextrin molecule. This program has been recently interfaced with the program TINKER[30] in order to allow for application of a flexible docking approach on both ligand and host molecules.[31] The MM3 force field[32] was used for energy calculation. Three translation and three space rotation variables were driven during the procedure together with four dihedral angles of catechin: one corresponding to rotation of the bond between the C-ring and the B-ring, and three driving the pucker of the C-ring. The following steps were used for the driving: 20° and 4° for exocyclic and endocyclic torsions, respectively, 2° for space rotations and 0.3 Å for translations. The search was performed within a box of 20 × 22 × 17 Å surrounding the cyclodextrin molecule. The relative permittivity was set to 4. Another search was performed with relative permittivity of 78.5 to mimic a water environment. Three different orientations of the catechin molecule in the cyclodextrin were used as starting points for the searches.

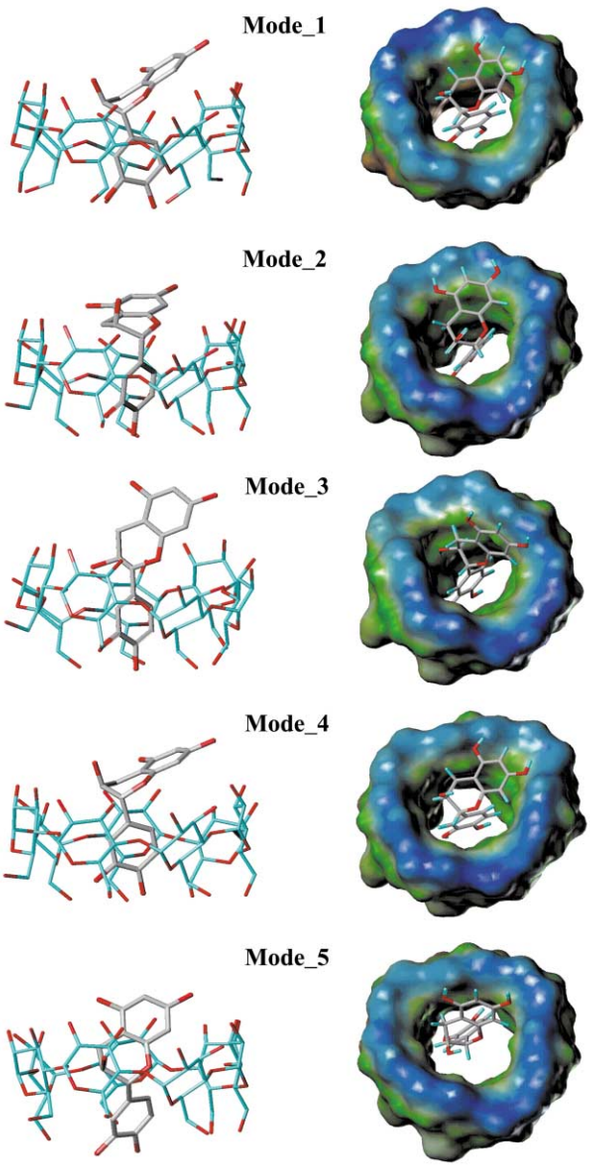

Fig. 5 Five lowest energy modeled docking modes for (+)-catechin in β-cyclodextrin cavity. The accessible surfaces of the β-cyclodextrins have been calculated with the MOLCAD program[36] and color coded according to the hydrophobicity potential (from blue for hydrophilic to brown for hydrophobic).

Two inhouse programs were used to analyze the results. The PANIC program[33] sorts geometry descriptors of single configurations. The FAMILY_RMS program[34] was used for clustering of the configurations into families based on the rms values of a selection of atoms. An rms cutoff of 0.5 Å was selected for the maximum difference allowed in one family. All obtained energy minima within the energy window of 5 kcal mol^{-1} were clustered. A large number of families were obtained because of the 6-fold symmetry of cyclodextrin. In order to cluster conformations independently of this symmetry, additional geometrical criteria were defined (Fig. 4). They describe the shape of the ligand and its overall orientation in the cyclodextrin using the plane defined by the positions of glycosidic oxygen atoms (CD-plane) of the cyclodextrin. λ is the normal distance of the center of gravity of the B-ring to this plane (negative value for the ring under the plane) and defines the "deepness" of the binding mode, Θ is the dihedral between the CD-plane and the plane defined by the B-ring and defines the verticality of the B-ring in the CD (90° for vertical orientation). Ω is the dihedral between the CD-plane and the plane defined by the A-ring, describing the more or less folded orientation of this ring (0° for fully folded on the CD). Definition of planes and measurement of normal distances and plane dihedral angles were performed using the SYBYL graphic software.[35]

Acknowledgements

A.C. is supported by a grant from Ministère de la Recherche. J.K.'s stays in Grenoble were financed by CNRS. The research has also partially been supported (Z.K., J.K.) by the Ministry of Education of the Czech Republic (grant LN00A0016).

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