Distinctive Pressure Effects on the Association Equilibrium in Cyclodextrin Group-Inclusion Complex as Studied with Electron Paramagnetic Resonance

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The cyclic oligosaccharide cyclodextrin (CD) has been shown to form isomeric guest−host complexes in which each functional group in the guest molecule is individually included by the cyclodextrin cavity. Spectroscopic separation of these isomer complexes or group-in complexes has been demonstrated only when free radical probes were used as guest molecules, followed by the detection with electron paramagnetic resonance (EPR) spectroscopy. In the present study, using a high-pressure EPR system, external static pressure up to 700 bar (70 MPa) was applied to the sample solution in a pressure-proof EPR sample tube, and the changes in the equilibrium constants of group-in complexes were monitored. The external pressure either increased or decreased the equilibrium constant, depending on the sizes of the included group relative to that of the cyclodextrin cavity. For instance, when the free radical probe α-phenyl-2,4,6-trimethoxybenzyl *tert*-butyl nitroxide ($(CH_3O)_3$Ph-CH(Ph)-N(O•)-*t*-Bu) was mixed with γ-CD, both phenyl-in and *tert*-butyl-in complexes were identified. With an increasing external pressure, the equilibrium between *tert*-butyl-in and phenyl-in complexes shifted to the phenyl-in complex side. In contrast, when β-CD was used the equilibrium shifted to the *tert*-butyl-in complex side. The analysis of the pressure effect in various group-in complexes with γ-CD or β-CD has revealed that there is a pressure-dependent competing complexation between the included group and water molecules.

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides that possess hydrophobic cavities capable of forming guest−host or inclusion complexes with a variety of organic molecules in aqueous solution. The diameter of the CD cavity is a function of the number of glucose residues that conform hydrophobic cavity inner-wall, and the addition of functional groups to the glucose residues is known to alter the inclusion characteristics.[1−3] Therefore, CDs have attracted widespread interest as models to study molecular recognition and enzyme−substrate interaction.[4,5]

A rapid in-and-out equilibrium of the guest molecule exists in CD complexes in solution, which made it difficult to determine the static structure of the complex. Spectroscopic discrimination of free guest molecules in solution from the complexed ones is not always possible because magnitudes of linewidth and time constant vary in each spectroscopy. For example, UV−visible spectroscopy has a fast spectroscopic time-constant but a wide linewidth, while NMR spectroscopy has a narrow linewidth, but the time constant is not as fast as to discriminate multiple species in the equilibrium. Electron paramagnetic resonance (EPR) spectrum has a relatively narrow linewidth and the time constant in the order of 10^6 s^{-1}, thus separated EPR spectra may be obtained from free and complexed molecules. However, it requires the use of free radical probes.[6,7] When the size of the probe molecule is larger than the diameter of the CD cavity opening, each functional group in the probe may be able to form a distinctive inclusion complex. Such a notion had not been proven until an appropriate paramagnetic probe was synthesized. Kotake and Janzen were the first to show that a nitroxide radical with more than one relatively bulky functional group forms two group-in complexes (Figure 1), where either a phenyl or a *tert*-butyl group is included in the CD cavity to produce an EPR-spectroscopically separable complex.[8−12] The combination of EPR spectroscopy and free radical probes is so far the sole system that can identify group-in CD complexes.

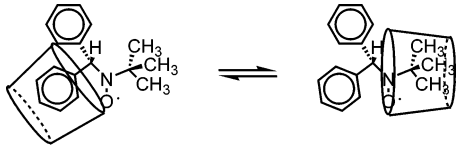

Figure 1. Schematic illustration of inclusion equilibrium between *tert*-butyl-in and phenyl-in complexes of cyclodextrin.

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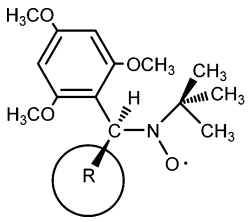

Probe	*R*
1	Cyclohexyl
2	Cyclopentyl
3	Phenyl
4	*n*-Hexyl
5	*sec*-Butyl
6	*n*-Butyl
7	Ethyl

Figure 2. Structure of nitroxide-radical probes (**1**−**7**) used in this study.

The equilibrium in CD guest−host complexes is a thermodynamic process; therefore, it should be manipulated by changing external thermodynamic parameters such as temperature and pressure. We have previously shown that the effect of pressure on the equilibrium between free probes and CD complexes can be monitored using a high-pressure EPR technique[13] and determined equilibrium states in group-in complexes of CD.[14] In our recent report, we demonstrated differential pressure effects on phenyl-in and *tert*-butyl-in complexes with β-CD, where the external pressure shifted the equilibrium to the *tert*-butyl-in complex side.[14] In the present study, the use of new free radical probes allowed us to obtain well-separated EPR spectra from two group-in complexes, thus we were able to show that under high pressure, the equilibrium between the *tert*-butyl-in complex and the other group-in complex is dependent on the diameter of the CD cavity. For example, external pressure drives the cyclopentyl group into the γ-CD cavity but expels it out of the β-CD cavity. The analyses revealed that the number of water molecules expelled upon inclusion of the functional group plays an important role in the differing effects. We conducted a pressure-dependence study on the inclusion equilibria in β-CD and γ-CD complexes of cyclohexyl, cyclopentyl, phenyl, *n*-hexyl, *sec*-butyl, and *n*-butyl groups and the role of water molecules coincluded in the CD cavity.

Experimental Section

Materials. Seven nitroxide probes (α-substituted-2,4,6-trimethoxybenzyl *tert*-butyl nitroxide) were synthesized by the reaction of 2,4,6-trimethoxyphenyl-*tert*-butylnitrone (MO_3PBN) with appropriate Grignard reagent or organolithium (Figure 2).[11] Grignard reagent, organolithium, and MO_3PBN were purchased from Aldrich Chemical Co. β- and γ-CDs were also obtained from Aldrich and used as received. Water was distilled before use.

Measurement. The high-pressure EPR cell and procedure of the observation of EPR signals at high pressure were identical with those described elsewhere.[15] The diagram of a high-pressure EPR system is shown in Figure 3. The probe solution was loaded into a thick wall quartz capillary tubing (i.d. 1 mm, o.d. 6 mm) which was connected to a copper−beryllium high-pressure line using epoxy resin. After applying pressure, the stop valve was closed, and the cell was disconnected from the high-pressure pump system and set into the EPR cavity. EPR measurements were performed using a JEOL FE3XG spectrometer at room temperature. CD concentration (2×10^{-2} mol dm^{-3}) was higher than spin probes concentration (about 2×10^{-4} mol dm^{-3}) in order to avoid line broadening from intermolecular spin exchange. The spectrometer setting for EPR measurement was as follows: microwave power 1 mw; field modulation amplitude 0.063 mT at 100 kHz; time constant 0.1 s; field scan rate 1.25 mT/min. Computer spectral simulation was conducted using WIN−RAD program (Radical Research Inc., Hino Japan).

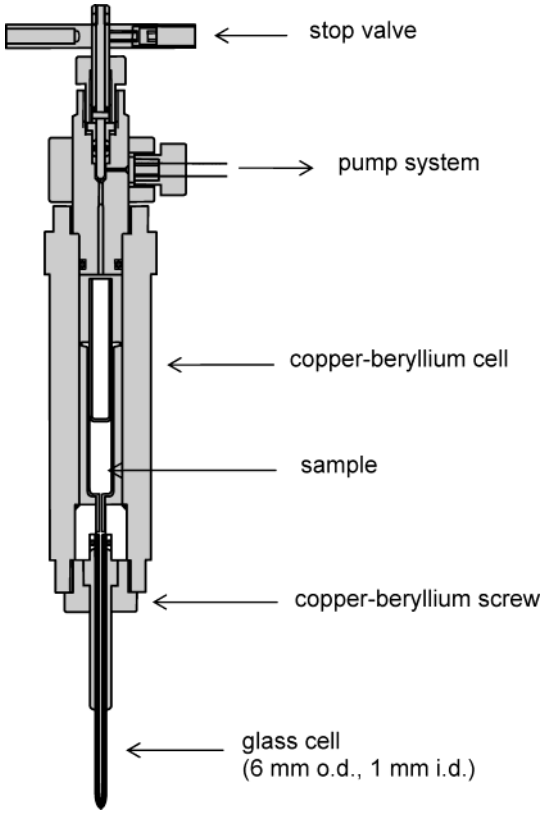

Figure 3. High-pressure cell for EPR measurement. The pressure was applied using a plunger pump through the joint to the solution in the pressure-proof tube.

Results and Discussion

Effects of External Pressure on Group-in Complexes. EPR spectra for various α-substituted-2,4,6-trimethoxybenzyl *tert*-butyl nitroxides were obtained in water at room temperature. Because the trimethoxyphenyl group in the probes has a larger diameter than the wider end of the γ-CD or β-CD cavity,[12] inclusion of probes **1**−**6** should occur by way of either the α-substituent (*R*) or *tert*-butyl group. Figure 4(a) shows EPR spectra of Probes **1**−**6** in aqueous solution in the presence of excess β- or γ-CD, exhibiting well-separated EPR peaks from each group-in inclusion complex, i.e. *tert*-butyl-in and *R*-in complex. Two sets of six lines in Figure 4(a) can be assigned to the hyperfine splitting (hfs) due to one nitrogen and one hydrogen nucleus in each complex.[11] The spectrum changed into Figure 4(b) when the external pressure (637 bar or 63.7 MPa) was applied to the solution. The EPR spectra can be reproduced by computer simulation by superimposing two sets of triple-doublet spectra such as illustrated in Figure 5, and the hyperfine coupling constants (hfsc's) are listed in Table 1. Hfsc's are pressure-independent molecular constants; therefore, the equilibrium constant $K = [t\text{-butyl-in}]/[R\text{-in}]$ was calculated by adjusting the relative concentration of each complex in EPR spectral simulation. The equilibrium constants (K) for Probes **1**−**6** at various pressures are listed in Table 1.

Equilibrium constants are functions of the magnitude of hydrophobic interaction between the cavity wall and the included group as well as the amount of residual space in the CD cavity. The magnitudes of K values for *R*-in complexes with β-CD are in the order of **1**(cyclohexyl) < **5**(*sec*-butyl) < **2**(cyclopentyl) < **4**(*n*-hexyl) < **3**(phenyl) < **6**(*n*-butyl) (Table 1), indicating that the inclusion of the cyclohexyl group with β-CD is the tightest within Probes **1**−**6**. Selkti et al.[16] carried out an inclusion study using a β-CD derivative, 6^A-boc-L-phenylalanylamino-

TABLE 1: EPR Hyperfine Coupling Constants[a] (HFSCs), Equilibrium Constants[b] under Various External Pressures, and Reaction Volumes for Group-in Complexes of 1−6 in Water at 298 K

		tert-butyl-in		*R*-in		*K*				ΔV	$-\Delta V_R$	$\Delta\Delta V_{repel}$ ($m-n$)
CD	probe[c]	A_H	A_N	A_H	A_N (mT)	1	98.1	343	637 (bar)	(cm^3 mol^{-1})	(cm^3 mol^{-1})	(cm^3 mol^{-1})
β-CD	**1**	0.958	1.540	0.480	1.572 (cyclohexyl-in)	0.301	0.294	0.264	0.249	7.6 ± 0.6	113 ± 1	0
β-CD	**2**	1.210	1.625	0.825	1.650 (cyclopentyl-in)	0.660	0.675	0.705	0.740	-4.0 ± 0.1	101 ± 1	0
β-CD	**3**	1.160	1.540	0.580	1.510 (phenyl-in)	4.50	4.75	5.00	6.50	-14.0 ± 1.8	91.0 ± 1.8	0
β-CD	**4**	1.170	1.645	0.550	1.635 (*n*-hexyl-in)	0.780	0.800	0.980	1.16	-16.7 ± 0.8	88.3 ± 0.8	0
β-CD	**5**	0.992	1.522	0.710	1.601 (*sec*-butyl-in)	0.522	0.528	0.537	0.554	-2.2 ± 0.1	103 ± 1	0
β-CD	**6**	1.295	1.582	0.682	1.598 (*n*-butyl-in)	4.67	5.24	6.22	7.46	-17.2 ± 0.9	87.8 ± 0.9	0
γ-CD	**1**	0.850	1.615	0.280	1.604 (cyclohexyl-in)	0.430	0.400	0.360	0.250	20.0 ± 2.2	113 ± 1	$12.0 \pm 2.3(0.7)$
γ-CD	**2**	1.200	1.630	0.600	1.670 (cyclopentyl-in)	1.85	1.75	1.55	1.30	13.7 ± 0.3	101 ± 1	$17.7 \pm 0.3(1.0)$
γ-CD	**3**	1.200	1.550	0.590	1.540 (phenyl-in)	0.980	0.963	0.927	0.870	4.4 ± 0.2	91.0 ± 1.8	$18.4 \pm 1.8(1.0)$
γ-CD	**4**	1.504	1.653	0.450	1.643 (*n*-hexyl-in)	4.80	4.71	4.29	4.01	6.9 ± 0.4	88.3 ± 0.8	$23.6 \pm 0.9(1.3)$
γ-CD	**5**	1.156	1.601	0.493	1.599 (*sec*-butyl-in)	2.47	2.34	2.09	1.74	8.4 ± 0.4	103 ± 1	$10.4 \pm 0.4(0.6)$

[a] Error is ±0.005 mT. [b] Precision within 5% error. [c] The well-separated EPR spectra could not be obtained for the isomer inclusion complexes of probe **6** with γ-CD.

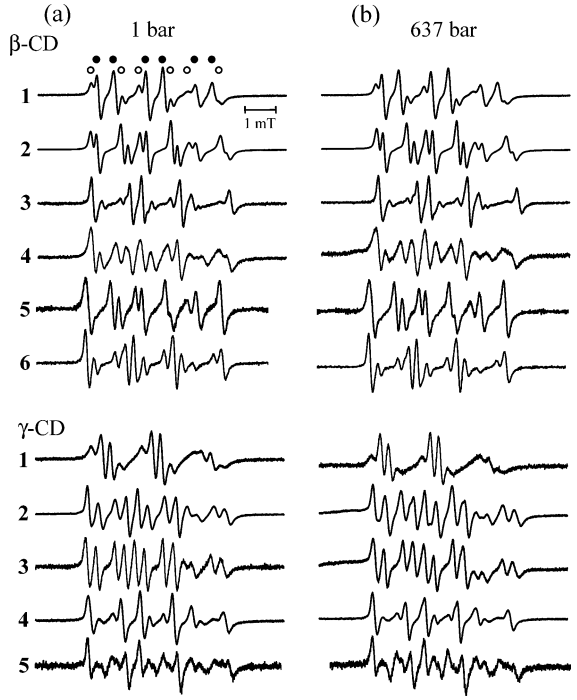

Figure 4. EPR spectra of Probes **1**−**6** in the presence of excess β- or γ-CD in solution under external static pressure that was applied using a sample tube shown in Figure 3: (a) at 1 bar and (b) at 637 bar. The probe and CD concentrations were approximately 1.5×10^{-4} mol dm^{-3} and 5.0×10^{-2} mol dm^{-3}, respectively. In the EPR spectrum (**1**)-a, the peaks marked with open circles (○) and closed circles (●) are assigned to the *tert*-butyl-in and *R*-in complexes, respectively. In all other spectra, the assignment of the peaks are similar, i.e. 1st, 4th, 5th, 8th, 9th, and 12th peaks from the lower field (left) are to the *tert*-butyl-in complex and 2nd, 3rd, 6th, 7th, 10th, and 11th peaks are to the *R*-in complex (**1**: cyclohexyl, **2**: cyclopentyl, **3**: phenyl, **4**: *n*-hexyl, **5**: *sec*-butyl, and **6**: *n*-butyl).

6^A-deoxyl-β-CD with two bulky terminal side chains (the aromatic moiety and the *tert*-butyl group), demonstrating that a hydrophobic group (like *tert*-butyl group) is preferentially included in the β-CD cavity. This is in agreement with our observation of the relatively large *K* value for **3**(phenyl). Graphs in Figure 6 show the relationship of ln*K* vs external pressure (*P*) for the equilibrium between *tert*-butyl-in and *R*-in complexes for Probes **1**−**6** with β- and γ-CDs. The *K* values for β-CD became larger with increasing external pressure except probe **1**, conversely those for γ-CD decreased with increasing pressure. Overall, the equilibrium in the β-CD complexes for most probes shifted to the *tert*-butyl-in side with increasing pressure, while it shifted to the *R*-in side in γ-CD complexes.

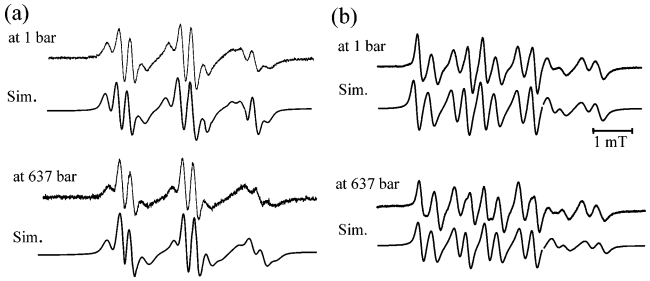

Figure 5. EPR spectra and simulated spectra of **1** (a) and **2** (b) in the presence of γ-CD at 1 and 637 bar: [Probe] = 1.5×10^{-4} mol dm^{-3} and [γ-CD] = 5.0×10^{-2} mol dm^{-3}.

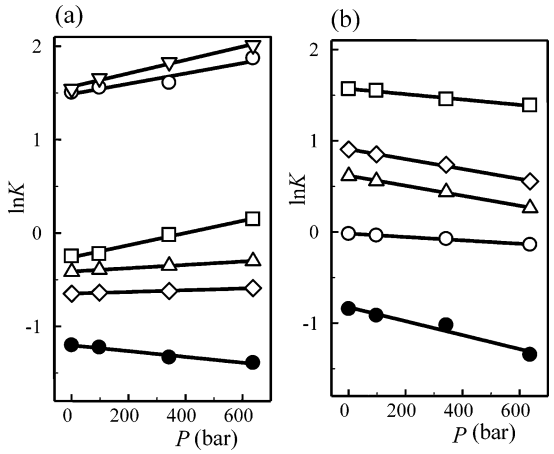

Figure 6. Dependence of association constants (*K*'s) on the applied external pressure (*P*). ln*K*'s obtained from EPR spectrum-simulation analyses are plotted against external pressure for the group-in complexes of Probes **1**−**6** with β-CD (a) and γ-CD (b). ●: cyclohexyl, △: cyclopentyl, ○: phenyl, □: *n*-hexyl, ◇: *sec*-butyl, and ▽: *n*-butyl.

Using *K*-values shown in Table 1, the reaction volume ΔV at 1 bar can be evaluated according to the following thermodynamic equations, and results are also listed in Table 1.

$$\ln K = aP^2 + bP + c \quad (1)$$

$$\Delta V = -RT\,(\partial \ln K/\partial P)_T \quad (2)$$

In β-CD complexes all but probe **1** showed negative ΔV values, but in γ-CD complexes, ΔV 's were all positive.

Evaluations of Components in Reaction Volume ΔV. Previously, using di-*tert*-butyl nitroxide[13] and spiropyran[17] as guest molecules we demonstrated that CD inclusion equilibrium was pressure dependent. We also showed that the reaction

TABLE 2: EPR Hyperfine Coupling Constants[a] (HFSCs) and Equilibrium Constants[b] K_2 for γ-CD *tert*-Butyl-in Complex of Probe 7 under Various External Pressures in Water at 298 K[d]

free		*tert*-butyl-in[c]			K_2 (mol^{-1} dm^3)					
A_H	A_N	A_H	A_N	(mT)	1	98.1	343	637	(bar)	ΔV_2 (cm^3 mol^{-1})[d]
1.122	1.658	1.334	1.610		84.0	81.7	75.1	68.8		6.8 ± 0.1

[a] Error is $\pm$0.005 mT. [b] Precision within 5% error. [c] Probe **7** plus γ-CD produces *tert*-butyl-in complex alone (no ethyl-in complex, see Figure 8). [d] ΔV_2 denotes the reaction volume of the complexation.

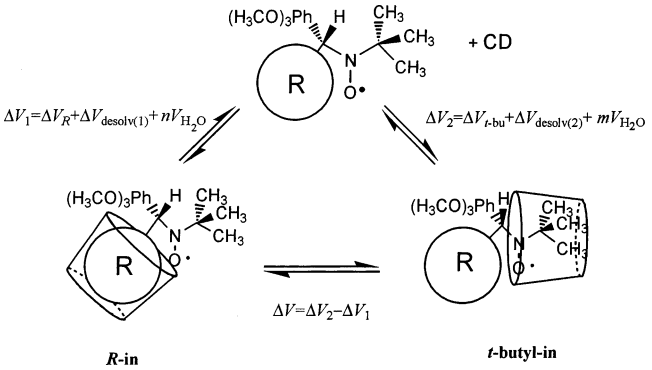

Figure 7. The scheme that defines reaction volumes, ΔV, ΔV_1, and ΔV_2, where ΔV_R, ΔV_{t-bu}, ΔV_{desolv}, and V_{H_2O} denote the changes in volume accompanying inclusion from *R* and *tert*-butyl sides, desolvation around guest, and molar volume of one water molecule, respectively.

volume (ΔV) for the CD inclusion is not solely due to the change in total (the guest plus CD) volume but consists of three discrete volume changes[13,17,18]

$$\Delta V = \Delta V_{inclu} + \Delta V_{desolv} + \Delta V_{repel} \quad (3)$$

where ΔV_{inclu} denotes a change in total volume, ΔV_{desolv} is the volume change accompanying desolvation around the guest molecule, and ΔV_{repel} denotes the volume change caused by water molecules repelled out from the CD cavity.[19,20] Usually ΔV_{inclu} is a negative value, and ΔV_{desolv} and ΔV_{repel} are positive. The volume-change scheme when the probe is included by CD is illustrated in Figure 7, where ΔV_1 and ΔV_2 represent the volume change accompanying the inclusion of *R* and the *tert*-butyl group, respectively, thus $\Delta V = \Delta V_2 - \Delta V_1$. ΔV_{desolv} can be assumed to be small because probes **1**−**6** are neutral radicals. Therefore ΔV can be expressed as follows

$$\Delta V = \Delta V_2 - \Delta V_1 = \Delta V_{t-bu} - \Delta V_R + (m - n)V_{H_2O} \quad (4)$$

where *m* and *n* are the number of water molecules repelled out from the CD cavity and V_{H_2O} is the molar volume (= 18 cm^3 mol^{-1})[18] of water. ΔV_R was calculated using $\Delta V_{t-bu} = -105$ cm^3 mol^{-1} which was obtained in the previous high pressure study on β-CD inclusion of diphenylmethyl *tert*-butyl nitroxide (DPBN), and we assumed that all water molecules situated in β-CD are repelled out by the *tert*-butyl group, i.e., $m = n$ ($\Delta\Delta V_{repel} = 0$).[14] The calculated results are 113, 101, 91.0, 88.3, 103, and 87.8 cm^3 mol^{-1} for **1** (cyclohexyl), **2** (cyclopentyl), **3** (phenyl), **4** (*n*-hexyl), **5** (*sec*-butyl), and **6** (*n*-butyl), respectively (listed in Table 1 as $-\Delta V_R$).

$-\Delta V_{R(inclu)} = 113$ cm^3 mol^{-1} for cyclohexyl group in **1** is larger than that of the *tert*-butyl group ($-\Delta V_{t-bu(inclu)} = 105$ cm^3 mol^{-1}) in the same probe. Thus, the equilibrium in **1** shifts to the cyclohexyl-in side with increasing external pressure. On the contrary, in probes **2**−**6**, the decrease in volume for the inclusion from the *R* side is small compared to that from the *tert*-butyl side, thus the equilibrium shifts to the *tert*-butyl side with increasing pressure. The ΔV_R for the phenyl group (−91 cm^3 mol^{-1}) obtained from probe **3** is in good agreement with that (−90 cm^3 mol^{-1}) previously calculated by using DPBN,[14] suggesting that possible steric hindrance due to three methoxy groups in probes **1**−**6** has a minor effect on the formation of group-in complexes. By using ΔV_R's calculated (Table 1), the $m-n$ value (the difference in the number of water molecules repelled out from the γ-CD cavity) for γ-CD inclusion can be calculated for probes **1**−**5** (Table 1). Obviously, the *m*-*n* value decreases with an increasing bulkiness of *R*. In comparison, approximately one more water molecule is repelled out by *tert*-butyl group. During inclusion process, the probe could rotate about the symmetry axis of the CD molecule. Thus, a proposed deeper penetrated structure[11,16] of the *tert*-butyl-in complex may be responsible for repelling more water molecules out. The number of water molecules repelled out from the CD cavity plays an important role for pressure behavior of bimodal inclusion equilibrium with γ-CD.

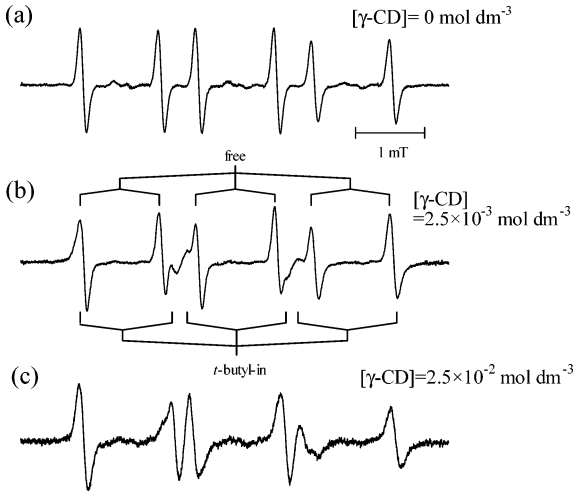

Figure 8. EPR spectra of Probe **7** in water in the presence of γ-CD at atmospheric pressure (1 bar): [**7**] = 1.1 × 10^{-4} mol dm^{-3}.

Probe **7** potentially forms both a *tert*-butyl-in complex and an ethyl-in complex; however, the ethyl-group is too small to form a stable γ-CD complex. In fact, the *tert*-butyl-in complex was the sole complex identified in the EPR spectrum (Figure 8(b)), which made it possible to estimate the reaction volume for the inclusion from the *tert*-butyl side (i.e., ΔV_2 in Figure 7). Therefore in this system the inclusion equilibrium can be simply expressed as follows:

$$\mathbf{7} + \gamma\text{-CD} \rightleftharpoons \textit{tert}\text{-butyl-in}$$

Thus the inclusion equilibrium constant K_2 is given as

$$K_2 = \frac{[\textit{tert}\text{-butyl-in}]}{[\mathbf{7}][\gamma\text{-CD}]} \approx \frac{[\textit{tert}\text{-butyl-in}]}{[\mathbf{7}][\gamma\text{-CD}]_0} \quad (5)$$

Under the condition [γ-CD] $\gg$ [**7**], K_2 can be determined by using the relative concentration [*tert*-butyl-in]/[**7**], which can be quantified through EPR spectral simulation. The calculated K_2 values at high-pressure are listed in Table 2, together with

the EPR parameters of free Probe **7** and *tert*-butyl-in complex. Subsequently, the reaction volume ΔV_2 was calculated according to eqs 1 and 6

$$\Delta V_2 = -RT\left(\frac{\partial \ln K_2}{\partial P}\right)_T - \kappa_T RT \qquad (6)$$

where κ_T is the isothermal compressibility of the solvent, and its magnitude at 1 bar was calculated from available data for water,[21] to be $\kappa_T = 4.425 \times 10^{-5}$ bar^{-1}. According to eq 6, the ΔV_2 value can be estimated to be 6.8 cm^3 mol^{-1} (Table 2).

Based on the solubility data of β-CD in aqueous mixtures, Coleman et al.[22,23] suggested the effect of a water-solution structure on the formation of a CD inclusion complex. High external pressure could cause changes in the water-solution structure including clathlate formation; however, such pressure effect could be negligible under relatively low applied pressure. Thus, in the simplified model of CD inclusion, the content of the volume change upon inclusion at 1 bar is divided into three portions:

$$\Delta V_2 = \Delta V_{t-\mathrm{bu}} + \Delta V_{\mathrm{desolv}(2)} + mV_{\mathrm{H_2O}} \qquad (7)$$

We employed $\Delta V_{t-\mathrm{bu}}(= -105$ cm^3 mol^{-1}) and $\Delta V_{\mathrm{desolv}(2)}(\approx 0$ cm^3 mol^{-1}) to calculate the number of water molecules repelled out from the γ-CD cavity upon inclusion from the *tert*-butyl side. The result was that on the average 6.2 water molecules are repelled out from the γ-CD cavity upon inclusion of the *tert*-butyl group. Based on the data in Table 1, the number of water molecules repelled out from the cavity can be calculated as 5.5 (cyclohexyl), 5.2 (cyclopentyl), 5.2 (phenyl), 4.9 (*n*-hexyl), and 5.6 (*sec*-butyl) for the Probes **1**, **2**, **3**, **4**, and **5**, respectively.

In summary, although the present analysis of high-pressure results utilized a simplified model, the data which show that the number of water molecules repelled out from the CD cavity upon complex formation may be an important aspect of CD inclusion complex formation dynamics. We believe high-pressure EPR studies on group-inclusion CD complexes may be able to provide useful insight into the functional group recognition by hydrophobic void space.

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