Electron Nuclear Double Resonance Detection of Distinct Multimodal Inclusion Complexes of β-Cyclodextrin with a Nitroxide Spin Adduct Probe

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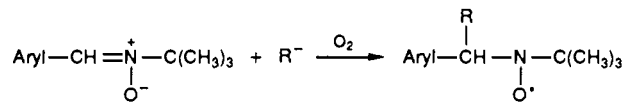
Abstract: Observation and identification of multimodal inclusion complexes of β-cyclodextrin with a nitroxide spin adduct probe are reported. When a nitroxide radical having three different functional groups attached to the nitroxyl function is dissolved in an aqueous solution of β-cyclodextrin, electron nuclear double resonance (ENDOR) signals from three distinct complexes are detected. These are assigned to multimodal inclusion complexes in which each functional group of the nitroxide occupies the void space of β-cyclodextrin. Since both the nitroxide probe and cyclodextrin are chiral, the complexation results in the formation of diastereomers. In some cases the ENDOR spectrum from a diastereomer is also observed.

The possibility that cyclodextrin, a cyclic oligomer of α-D-glucopyranose with molecular void space, could form distinct inclusion complexes with each functional group within the same molecule has long been suggested.[3] Although rapid equilibria of formation and dissociation of the complex exist in solution, electron paramagnetic resonance (EPR) spectroscopy using a paramagnetic substrate is capable of detecting both free and complexed probes at the same time,[4] whereas simultaneous NMR detection of both species separately is rare.[5] Recently bimodal inclusion complexes of cyclodextrin in which two functional groups in a nitroxide free radical form different complexes were identified by selecting a suitable probe and by using the EPR/ENDOR technique[6] (ENDOR = electron nuclear double resonance). Spectra were assigned to the complex where each functional group resides in the void space of cyclodextrin. "Identification" of the inclusion complexes is understood only in the framework of the time scale of EPR spectroscopy, i.e., within a time interval longer than 10^{-6} s. Thus there is no contradiction with the recent lifetime distribution model of a complex.[7] The actual situation is, presumably, that the fastest equilibrium is present within slightly different structures in the same group-in complex and a slower equilibrium exists between each complex and the free species. The time scale of NMR is usually slower than the latter equilibrium. The question arises whether the number of distinct complexes are the same as the number of different functional groups in the probe capable of fitting into the available space. The advantage of ENDOR spectroscopy is utilized in this work to resolve the spectrum from each complex, and the detection of three distinct β-cyclodextrin complexes of a nitroxide radical probe having three different functional groups is reported.

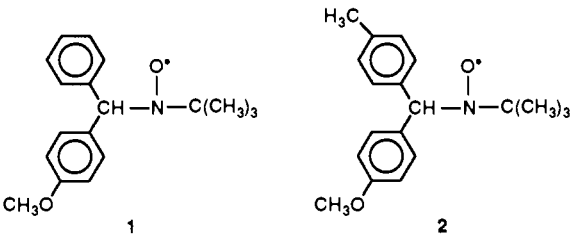
Selection of Probes. The probe substrate has to fulfill certain conditions in order to reveal spectroscopically different properties for each functional group-in complex. The probe must have more than two different functional groups, having enough volume to fill the cyclodextrin cavity on its own. Also each group-in complex needs to have a similar association constant so that the spectral lines from the other complex will not mask the one being observed.

The reaction of nitrone spin traps with Grignard reagents offers a convenient and systematic way of synthesizing nitroxides having various functional groups. α-Aryl-*N*-*tert*-butylnitrones are selected as the starting nitrone spin trap in this study and aryl or alkyl groups are introduced to produce the resulting nitroxide:



Thus the present probe has an arrangement of bulky functional groups such as aryl, *tert*-butyl, and R around the nitroxide.

EPR Spectrum. When α-anisylbenzyl *tert*-butyl nitroxide (**1**) is dissolved in water, its EPR spectrum shows three groups of two lines because of the presence of one nitrogen and one β-hydrogen nucleus (Figure 1a). When β-cyclodextrin (0.015 M) is added



to this solution, the EPR spectrum is indicative of the presence of more than one species each of which is different from the free probe in water (Figure 1b). The decrease in the probe's nitrogen hyperfine splitting (hfs) as well as its *g*-value as compared to the free probe in water reveals that the probe is experiencing a slightly

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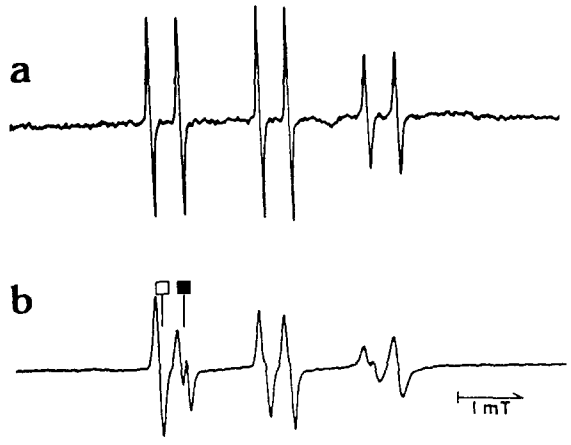

Figure 1. (a) EPR spectrum of α-anisylbenzyl *tert*-butyl nitroxide (**1**) in water. (b) EPR spectrum of **1** in the presence of 0.015 M β-cyclodextrin. Marks in the spectrum show the field position where ENDOR was observed.

more hydrophobic environment. In addition, the higher field wing of the EPR spectrum shows considerably weaker amplitude (broader line width) than the other manifold, indicating that the probe is under motional constraint. We have assigned these EPR peaks to the inclusion complex of β-cyclodextrin and the probe.[6] The reason for the observation of more than one complex has been ascribed to the slightly different EPR spectrum for the functional group-in complex, where each functional group (not a whole molecule) resides inside the cyclodextrin cavity. The difference in β-hydrogen hfs occurs because the inclusion of each group causes a slightly different amount of torsion at the N–α-C bond. Impure or decomposed probes cannot be the reason for the complicated spectrum because extraction of the probe from the complex solution by benzene followed by the re-dissolution in water reveals an EPR signal of a single species with the same parameters as that of the free probe.[8]

ENDOR Spectrum. Although it is not clear from the EPR spectrum shown in Figure 1b how many different types of complexes are present, the proton-ENDOR spectrum obtained at 290 K by setting the magnetic field at the most low-field line shows three pairs of ENDOR lines whose hfs values range from 0.2 to 0.5 mT (Figure 2a). In addition, a small hfs derived from protons remote from the unpaired electron was observed closer to the nuclear Zeeman frequency. Slowing down the motion of the probe by complexation must have raised the ENDOR optimum temperature since it is difficult to observe the proton-ENDOR spectrum of the free probe in water at 290 K.[9] The complexes detected are all assigned to a one-to-one complex, where a single probe is located in a cyclodextrin cavity because the association constant estimated from the relative EPR intensity of each component at different β-cyclodextrin concentrations gives consistent values for a one-to-one complex. The complex with more than one cyclodextrin per one substrate may exist; however, the EPR spectrum is expected to be too broad to be detected because the volume of the complex is large.

Three pairs of proton-ENDOR peaks in Figure 2a originate from three different radical species since each line shows a different intensity, depending on the ESR line position selected when ENDOR spectra are obtained (Figure 2b). The EPR spectrum can be reproduced by computer simulation using the proton hfs obtained from the proton-ENDOR spectrum, an appropriate nitrogen hfs, and the relative concentration of each complex.

Assignment of the ENDOR Peaks. One of the three complexes of probe **1** reveals a proton-ENDOR peak at 0.442 mT (outermost pair in Figure 1) that is close to the β-H hfs of the free probe (A_H = 0.43 mT) but has a different nitrogen hfs (a_N = 1.54 mT, free probe a_N = 1.59 mT). These hfs values indicate that the site of

(8) The probe purified by HPLC gave the same results; see: ref 6a.
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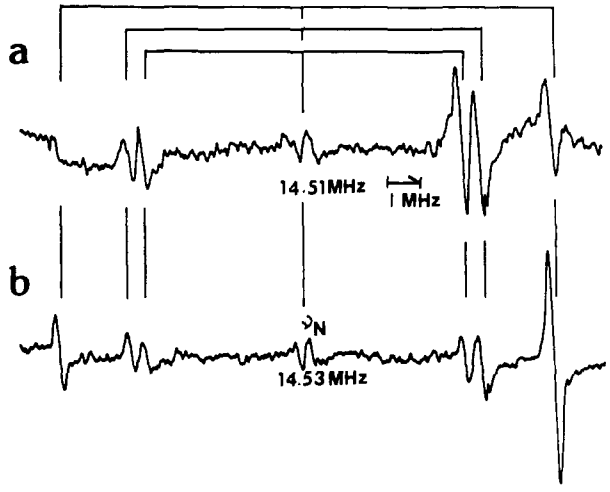
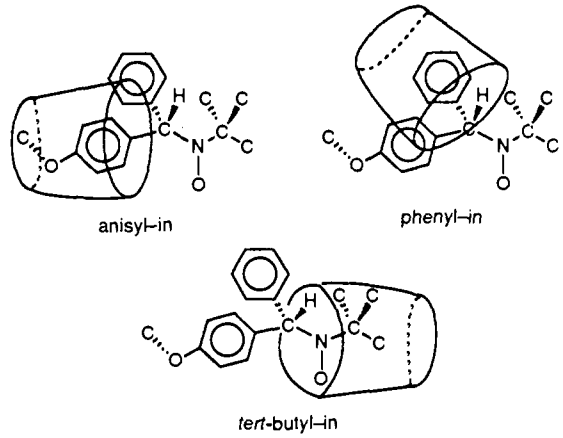


Figure 2. ENDOR spectrum of **1** in an aqueous solution of β-cyclodextrin (0.015 M) (a) Proton–ENDOR at 290 K by setting the magnetic field at the position marked by □ in the EPR spectrum in Figure 1b. Three pairs of lines from β-protons centered at ν_N nuclear Zeeman frequency) are shown with stick spectra. Spectrometer settings were as follows: microwave power, 100 mW; RF power, 150 W; sweep rate, 1 MHz/s; time constant, 0.1 s; number of accumulations, 200. A field frequency lock was used. (b) Same as a except the fixed field position is at ■ in Figure 1.

inclusion is remote from the β-hydrogen because the inclusion does not have much influence on the relative disposition between the substituted benzyl group and the nitroxyl group. Thus this species is assigned to the *tert*-butyl-in complex in which the *tert*-butyl group resides in the cyclodextrin cavity. The other two species are assigned to either the phenyl-in or anisyl-in complex. A slight rotation of the CH–N(O) bond occurs upon inclusion of aryl groups in the direction so that the diheedral angle of CH–N(O) is decreased. Inclusion characteristics of the two aryl-in complexes is so close that assignment is not possible. A schematic structure of these three group-in complexes is shown as follows:



Diasteromeric Inclusion Complexes. In the ENDOR spectrum shown in Figure 3a for the β-cyclodextrin complex of α-anisyl-4-methylbenzyl *tert*-butyl nitroxide (**2**), four pairs of lines, i.e., four different complexes, are found in the frequency range of β-hydrogen. However probe **2** has only three functional groups capable of occupying space in the cavity to form stable inclusion complexes. Each nitroxide probe used in this study has an asymmetric carbon at the α-carbon position and thus can form enantiomeric mixtures. In addition, the available cyclodextrins are all from a natural source and thus are D enantiomers. Since the resulting complex has two chiral centers, there should be diastereoisomerism; thus inclusion of three different groups in the probe could generate a total of six isomers. In the ENDOR spectrum of Figure 3a, four of these are resolved. The identification of a diastereomeric pair can be recognized by the close line position and similar intensity of two lines. EPR and ENDOR

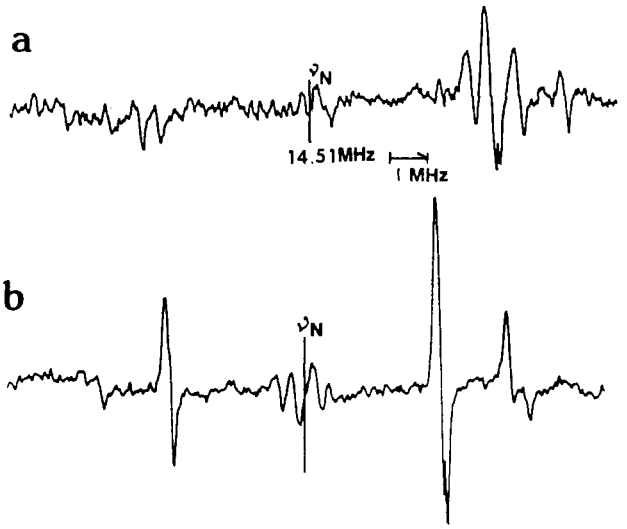


Figure 3. Proton-ENDOR spectrum of (a) α-anisyl-4-methylbenzyl *tert*-butyl nitroxide (**2**) and (b) α-cyclopentylbenzyl *tert*-butyl nitroxide (**3**) in water at 290 K. Spectrometer settings are the same as those in Figure 2.

detections of diasteromeric radicals have been reported before for nitroxides and quinones.[10]

The difference in hfs in the diastereomer depends on the distance between the two asymmetric carbons as well as the bulkiness of the group having the chiral center. However, the intensities of corresponding diastereomers are always very similar. It is noted that the outermost peak which is assigned to the *tert*-butyl-in complex does not show the presence of a diastereomeric pair. The fact that the distance between the two chiral centers is relatively large in the case of the *tert*-butyl-in complex compared to the other two complexes may be the reason why two ENDOR peaks for the *tert*-butyl-in complex are not detected. However, since the diastereomer of the complex of **1** is not resolved, the distance between two chiral centers may not be the only cause of the resolution.

ENDOR spectra of multimodal inclusion complexes were also found when α-cyclopentyl-4-methoxybenzyl *tert*-butyl nitroxide (Figure 3b) and α-*n*-hexylbenzyl *tert*-butyl nitroxide (**4**) were used as probes. The presence of diastereomers formed by the inclusion

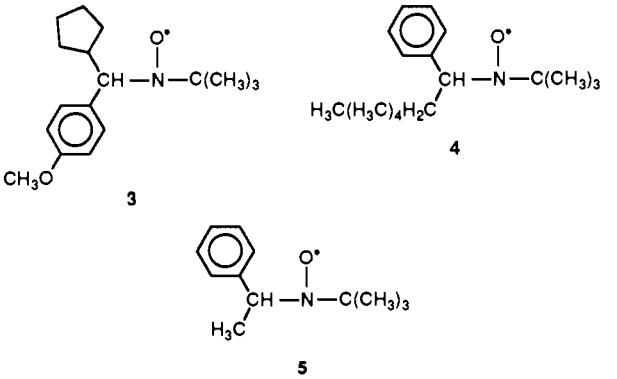


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Table I. Hyperfine Splitting Constants of β-Cyclodextrin Inclusion Complexes of Nitroxide Probes in Water at 290 K

probe	R_1[a]	R_2[a]	free probe in water[b]/mT	complex in water/mT a_N[b]	complex in water/mT $a_{\beta\text{-H}}$	assignment
1	phenyl	anisyl	$a_{\beta\text{-H}}$ = 0.43	1.54	0.442	*tert*-butyl-in
			a_N = 1.59	1.60	0.313	phenyl-in
				1.60	0.285	anisyl-in
2	tolyl	anisyl	$a_{\beta\text{-H}}$ = 0.43	1.57	0.441	*tert*-butyl-in
			a_N = 1.59	1.60	0.364	tolyl-in
				1.60	0.313	anisyl-in
				1.60	0.280	
3	cyclopentyl	anisyl	$a_{\beta\text{-H}}$ = 0.31	1.60	0.393	anisyl-in
			a_N = 1.60	1.60	0.376	cyclopentyl-in
				1.60	0.368	
				1.58	0.245	*tert*-butyl-in
4	phenyl	*n*-hexyl	$a_{\beta\text{-H}}$ = 0.32	1.58	0.434	phenyl-in
			a_N = 1.58	1.58	0.339	*n*-hexyl-in
				1.57	0.245	*tert*-butyl-in

[a] $R_1R_2CHN(O^\bullet)C(CH_3)_3$. [b] Hfs obtained from EPR spectrum.

is also detected when probe **4** was used. The results of spectral analyses are listed in Table I. In complexes of these probes the assignment of the ENDOR peaks is not always obvious. The assignment to the *tert*-butyl-in complex was made by finding the EPR species that has a smaller nitrogen hfs than that of the free probe and subsequently estimating the ENDOR peak position from its β-H hfs. Also the fact that the association constant of the *tert*-butyl-in complex is about 1800 M^{-1} irrespective of the shape of the probe[6] helps to confirm the assignment. Some group-in complexes such as cyclopentyl-in or *n*-hexyl-in have larger association constants than that of *tert*-butyl-in complex. Interestingly, when α-methylbenzyl *tert*-butyl nitroxide (**5**) was used with β-cyclodextrin, only two complexes were detected by ENDOR. We have reported that a functional group needs to have a certain volume in order to show a detectable association constant as a cyclodextrin complex.[6] The methyl group appears to be too small to form the methyl-in complex; thus only two complexes, anisyl-in and *tert*-butyl-in, are detected.

Experimental Section

All nitroxide probes are prepared by the reaction of nitrone spin traps and the appropriate Grignard reagent. The nitrone spin traps, α-phenyl-*N*-*tert*-butylnitrone and α-(4-methoxyphenyl)-*N*-*tert*-butylnitrone were synthesized in these laboratories. Phenyl-, tolyl-, methyl-, *n*-hexyl-, and cyclopentylmagnesium bromides were purchased from Aldrich Chemical Co. as solutions in ether. A typical procedure to prepare the nitroxide probe is described as follows: to 2 mL of a 1×10^{-3} M benzene solution of spin trap was added 0.1 mL of a 0.2 M ether solution of Grignard reagent under nitrogen bubbling followed by the introduction of air until heat generation ceased. The solution was washed with an equal volume of saturated aqueous sodium bicarbonate twice, and the solvent was removed by the nitrogen gas flow. Dry residue was dissolved in 1 mL of ethanol and was used as a stock solution after the concentration of the nitroxide was determined by EPR. For ENDOR measurements small volumes of the stock solution were placed in a test tube, and ethanol was purged by nitrogen gas followed by the addition of the cyclodextrin solution. The solution was loaded in a 1-mm-i.d. Pyrex tube, and one end was sealed with Critoseal. EPR and ENDOR spectra were observed with the use of a Bruker 200D spectrometer with an ENDOR accessory. Temperature was controlled by a Bruker ER4111VT temperature controller. The ENDOR signal was not as intense as the EPR signal; thus often a signal accumulation of several hundred times was required. The detailed spectrometer settings are described in the figure legend.

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