Inclusion Complexes of PBN-Type Nitrone Spin Traps and Their Superoxide Spin Adducts with Cyclodextrin Derivatives: Parallel Determination of the Association Constants by NMR Titrations and 2D-EPR Simulations

David Bardelang,[†] Antal Rockenbauer,[‡] Hakim Karoui,[†] Jean-Pierre Finet,*[,†] and Paul Tordo*[,†]

Laboratoire SREP, UMR 6517 CNRS et Universités d'Aix-Marseille 1, 2 et 3, Avenue Escadrille Normandie Niemen, Marseille 13397, France, and Chemical Research Center, Institute for Structural Chemistry, H-1525 Budapest, P.O. Box 17 Hungary

Received: February 23, 2005; In Final Form: March 24, 2005

^{1}H NMR and electron paramagnetic resonance (EPR) titrations were used to determine the association constants of the complexes of α-phenyl-*N*-*tert*-butylnitrone (PBN) analogues and their superoxide spin adducts, respectively, with methylated *β*-cyclodextrins. A 1:1 stoichiometry for the nitrones with randomly methylated *β*-cyclodextrin and 2,6-di-*O*-methyl-*β*-cyclodextrin and 1:1 and 1:2 stoichiometries for the corresponding cyclodextrin−nitroxide complexes were observed. After the superoxide radical spin trapping reaction, EPR titrations afforded the association constants of the corresponding cyclodextrin−nitroxide complexes. Two-dimensional EPR simulations indicated a bimodal inclusion of the nitroxide free radical spin adducts into the cyclodextrins. For all the nitrone−cyclodextrin and nitroxide−cyclodextrin complexes, the association constants were always higher for the nitroxide complexes than for the nitrone complexes. A cooperative system concerning the complexation of the nitroxide spin adduct with a cyclodextrin was evidenced by EPR titrations. The efficiency of the cyclodextrin inclusion technique to trap superoxide and to resist bioreduction by sodium L-ascorbate was also investigated.

Introduction

Cyclodextrins are versatile biocompatible macrocycles based on 1,4-glucopyranose units (Figure 1), with a truncated cone shape, which present outside hydrophilic sites and an inside hydrophobic cavity allowing the complexation of a wide variety of compounds in aqueous solution.[1−3]

The capacity of cyclodextrin to form inclusion complexes has been used to protect persistent nitroxides against bioreducing agents such as ascorbic acid.[4] The first example of a persistent nitroxide−cyclodextrin complex was reported in 1975 by Rassat and colleagues.[5] Then, the interactions between stable nitroxides and different cyclodextrins have been studied for molecular[6] or chiral[7] recognition. Electron paramagnetic resonance (EPR) is a technique of choice for studying inclusion complexes of stable free radicals with cyclodextrins.[4a,6a,8] Furthermore, it allows the characterization of multimodal inclusion complexes[6a,8b,9] in terms of stoichiometry and association constants. However, spin trapping in the presence of cyclodextrins has been barely investigated. Trapping of carbon-centered radicals with nitroso compounds stabilized by cyclodextrins has been studied by Chen and coll.[10] However, nitrones are more efficient in trapping free radicals, especially for oxygen-centered radicals in a biological milieu.[11] We recently reported that the addition of randomly methylated *β*-cyclodextrin (RM-*β*-CD) during the trapping of superoxide in the presence of 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO)[12] or α-phenyl-*N*-*tert*-butylnitrone (PBN)[13] leads to an increase of the intensity of the EPR spectrum of the spin adducts as well as to a longer half-life time of these spin adducts. Moreover, the action of ascorbate monoanion as a reducing agent is impeded when nitrones are in the presence of cyclodextrins before generation and trapping of superoxide. Thus, the use of cyclodextrins in spin trapping could be interesting, especially for ex vivo and in vivo experiments, as the presence of several bioreducing agents prevents the spin adducts from being detected by EPR because of their instantaneous reduction into diamagnetic species. In a previous paper,[14] we studied the correlations between the structure of linear nitrones and their complexation with natural *β*-cyclodextrin. However, a number of points need to be resolved before trapping of free radicals can be performed efficiently in a biological milieu in the presence of a cyclodextrin. To increase the solubility of the nitrone−cyclodextrin complexes, methylated *β*-cyclodextrins were used to study by NMR techniques the association properties of their complexes with PBN and the three PBN analogues that gave the most significant results with natural *β*-cyclodextrin[14] (Figure 1). These complexes were subsequently used for the spin trapping of superoxide that was studied by EPR titration techniques. Finally, the influence of cyclodextrin inclusion on the resistance to bioreduction by sodium L-ascorbate was investigated.

Experimental Section

General. PBN was purchased from Avocado Research Chemicals. Xanthine oxidase (XOD), diethylenetriaminepentaacetic acid (DTPA), randomly methylated *β*-cyclodextrin (RM-*β*-CD), and other chemicals were from Sigma Chemical Co.

* To whom correspondence should be addressed. E-mail: finet@srepir1.univ-mrs.fr (J.-P.F.); tordo@srepir1.univ-mrs.fr (P.T.). Phone: + 33 491 28 85 62. Fax: + 33 491 28 87 58.

[†] UMR 6517 CNRS et Universités d'Aix-Marseille 1, 2 et 3.

[‡] Institute for Structural Chemistry.

10.1021/jp0509414 CCC: $30.25
Published on Web 04/26/2005

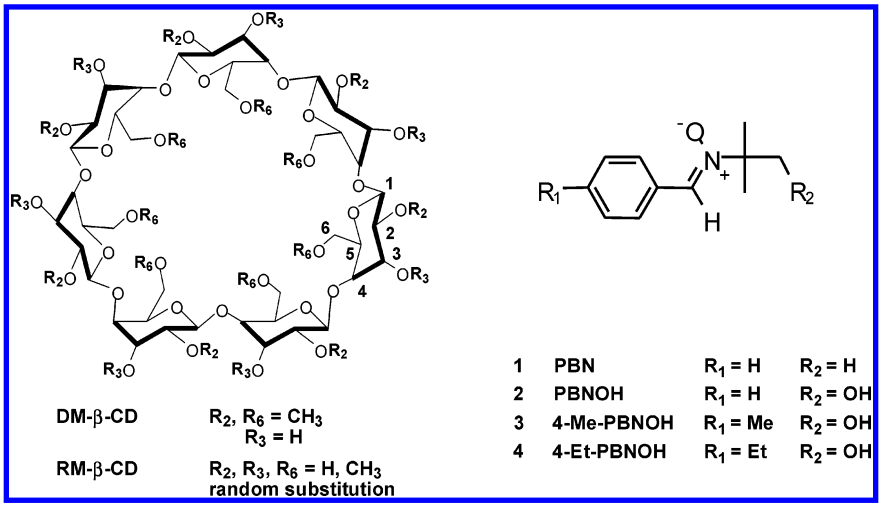


Figure 1. Chemical structures of the studied cyclodextrins, PBN, and selected analogues.

2,6-Di-*O*-methyl-β-cyclodextrin (DM-β-CD) was purchased from Acros. PBN analogues **2**−**4** were prepared as previously reported.[14]

NMR Measurements. The ^{1}H NMR titration spectra were recorded with a Bruker DPX 300 spectrometer at 300.13 MHz equipped with a QNP probe. All spectra were recorded at 300 K in D_2O (Euriso-Top, CEA Saclay, 99.90%), with the residual HOD signal (δ 4.79 ppm) being used as the internal reference.[15]

Continuous Variation Method (Job's Plot). This technique requires the total product concentration to be kept constant [20 mM except for titrations with 4-Et-PBNOH, (10 mM) because of its lower water solubility]. For the eight studied supramolecular systems (four nitrones and two methylated β-cyclodextrins), NMR samples were prepared by mixing equimolar amounts of freshly made stock solutions at 20 mM (except 10 mM for 4-Et-PBNOH) in different volumes so that the molar fractions of both components varied from 0 to 1 by steps of 0.1 (0−500 μL by steps of 50 μL for one of the components, V_{tot} = 500 μL). The complexation induced shifts (CIS) were determined by comparison of ^{1}H NMR spectra of the nitrone alone and of the mixture containing the nitrone and the highest cyclodextrin concentration.

Binding Constant Calculations. NMR titrations were made with a constant nitrone concentration (3 mM) and by progressively increasing the amount of cyclodextrin derivative. The relatively large solubility of RM-β-CD and DM-β-CD in water (>300 mM) allowed the exploration of a broad range of concentrations for monitoring the possible complexation induced shifts (CIS). CIS are interpreted as the mean weight of the shifts assigned to the complexed and the noncomplexed nitrones, since the rate of association is supposed to be fast on the NMR time scale. For titrations involving RM-β-CD, the cyclodextrin concentration was used up to 225 mM to check the presence of 1:2 complexes susceptible to change the CIS. The same method was used for the titrations involving 4-Et-PBNOH and DM-β-CD. The results helped in choosing the work range of cyclodextrin concentrations (from 0.5 to 141 mM). The NMR samples were prepared by mixing adequately defined volumes of freshly made stock solutions of cyclodextrin derivative and nitrone and completing with D_2O if necessary to reach the desired final concentrations. For 4-Me-PBNOH as guest and the two methylated cyclodextrins as host, the titrations were repeated twice and afforded similar results.

EPR Measurements. EPR spectra were recorded at room temperature using a Bruker ESP 300 EPR spectrometer at 9.5 GHz (X-band) employing 100 kHz field modulation. Reaction mixtures were prepared in a chelex-treated phosphate buffer (0.1 M, pH 7.4). EPR spectra were simulated with the 2D-EPR software developed by A. Rockenbauer.[16]

Superoxide Trapping: Hypoxanthine−Xanthine Oxidase System. Xanthine oxidase (XOD, 0.05 U·mL^{-1}) was added to an oxygenated solution (bubbled for 1 min 30 s) of PBN (**1**) or PBNOH analogues **2**−**4** (25 mM unless otherwise specified), diethylenetriaminepentaacetic acid (DTPA, 0.5 mM), and hypoxanthine (HX, 0.2 mM) in phosphate buffer (0.1 M, pH 7.4) in the presence of the desired concentrations of RM-β-CD or DM-β-CD.

Binding Constant Calculations. EPR titrations were performed at a constant nitrone concentration (25 mM, except 12.5 mM for nitrone **4**) and with a progressive increase of the amount of cyclodextrin (from 3 to 170 mM, and from 3 to 120 mM for nitrone **4**). The samples were prepared by the above-described procedure, changing progressively the cyclodextrin concentrations and completing with phosphate buffer, if necessary, to reach the desired final concentrations. The EPR spectra were recorded at a constant time within 5 min after the addition of XOD. The rate of association was considered as slow on the EPR time scale, and thus, all spectra were computed as a superimposition of complexed and noncomplexed nitroxide radicals.

l-Ascorbate Reduction of Spin Adducts. XOD (0.05 U·mL^{-1}) was added to a solution of the appropriate PBN analogue (15 mM), DTPA (0.5 mM), HX (0.2 mM), and RM-β-CD or DM-β-CD (50 mM) in oxygenated phosphate buffer (pH 7.4). After 5 min of incubation, superoxide dismutase (SOD, 50 U·mL^{-1}) and sodium L-ascorbate (0.1 mM) were added. The decrease of the EPR signal intensity was monitored until complete disappearance.

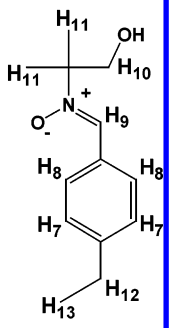
Results and Discussion

NMR Study. The characteristic features of the inclusion complexes between PBN-type nitrones and methylated cyclodextrins were determined by ^{1}H NMR studies. As the ^{1}H NMR spectra of the methylated cyclodextrins are not well defined, precise attribution of H_{CD3} and H_{CD5} (protons susceptible to shift in the presence of a guest) was not possible, especially in the case of RM-β-CD, a statistical mixture of randomly methylated β-cyclodextrins. Indeed, depending on the position of methylation of the hydroxy groups, the chemical shifts of common protons between the cyclodextrins exhibit different values. The symmetry of natural β-cyclodextrin is disturbed, providing an average NMR spectrum.

TABLE 1: ^{1}H NMR CIS for PBN and Analogues in the Presence of Methylated β-Cyclodextrins

RM-β-CD	H7	H8	H9	H11	H12	H13
PBN	+0.008	+0.014	-0.162	+0.048	-	-
PBNOH	+0.007	+0.019	-0.103	+0.038	-	-
4-Me-PBNOH	-0.020	+0.039	-0.111	+0.042	+0.014	-
4-Et-PBNOH	-0.078	+0.057	-0.080	+0.027	-0.002	+0.029

DM-β-CD	H7	H8	H9	H11	H12	H13
PBN	+0.008	-0.004	-0.239	+0.040	-	-
PBNOH	+0.012	0.000	-0.170	+0.052	-	-
4-Me-PBNOH	-0.019	+0.015	-0.180	+0.059	+0.013	-
4-Et-PBNOH	-0.090	+0.031	-0.130	+0.048	-0.015	+0.019



Continuous Variation Method. The continuous variation method,[17] which gave reliable information in the case of linear nitrones in the presence of natural β-cyclodextrin,[14] was used to determine the formation and stoichiometry of the inclusion complexes with methylated β-cyclodextrins. The mean values of the affected protons between the free and included guests were monitored as a function of cyclodextrin concentration. The absence of new peaks assignable to the pure complex suggested a fast exchange between the free and bound states on the NMR time scale. The main ^{1}H NMR complexation induced shifts (CIS) are reported in Table 1.

The nitronyl protons H9 exhibited the highest $\Delta\delta$ values, in good agreement with the values obtained for the same nitrones in the presence of natural β-cyclodextrin.[14] The trend $|\Delta\delta(\text{PBN})| > |\Delta\delta(\text{PBNOH})| > |\Delta\delta(\text{4-Me-PBNOH})| > |\Delta\delta(\text{4-Et-PBNOH})|$ was deduced from the evolution of the nitronyl proton H9 signal of the four nitrones in the presence of the two methylated cyclodextrins. In the case of PBNOH (**2**), the hydroxy group on the *tert*-butyl moiety is responsible for the decrease in $\Delta\delta$(H9), probably because of a change in the geometry of the inclusion complex, with the nitrone function being less inserted in the cone of the cyclodextrin. This interpretation is comforted by the further decrease of $\Delta\delta$(H9) in the case of 4-Me-PBNOH (**3**) and 4-Et-PBNOH (**4**). With these two nitrones, the bulkier *para*-substituted aryl group is likely to occupy more and more the cavity space, thus progressively shifting the nitrone center outside of the cyclodextrin cavity. Similarly, the corresponding $\Delta\delta$ values for H7 and H8 are increasing when going from PBN (**1**) to 4-Et-PBNOH (**4**). These results suggest that the nitronyl and *tert*-butyl parts of PBN (**1**) are located inside the cavity of the cyclodextrin. When the *tert*-butyl is substituted with a hydrophilic group and the phenyl part with hydrophobic groups in the *para* position, the center of gravity of the PBN structure that interacts with the inner cavity protons of cyclodextrins is shifted from the nitronyl-*tert*-butyl part (nitrone **1**) to the phenyl-nitronyl part (nitrone **4**). Therefore, the geometry of the inclusion complexes depends on the substitution pattern of the PBN skeleton.[14] In the case of the selectively methylated DM-β-CD, the CIS for H9 are more important than that for the randomly methylated RM-β-CD (mean difference +0.070 ppm), while the difference in $\Delta\delta$ for the other protons between the two cyclodextrins is less important (<0.027 ppm). That could result from a better structural complementarity for the selectively substituted DM-β-CD compared to the statistical mixture of the RM-β-CD. However, the highest $\Delta\delta$ values are observed for H9 and H11 (except in the case of nitrone **4**). The hypothesis that there is not only one precisely defined inclusion complex but that two inclusion complexes centered on each part of the nitrone function are present in solution cannot be excluded because of the average nature of the CIS parameters deduced from the NMR titrations.

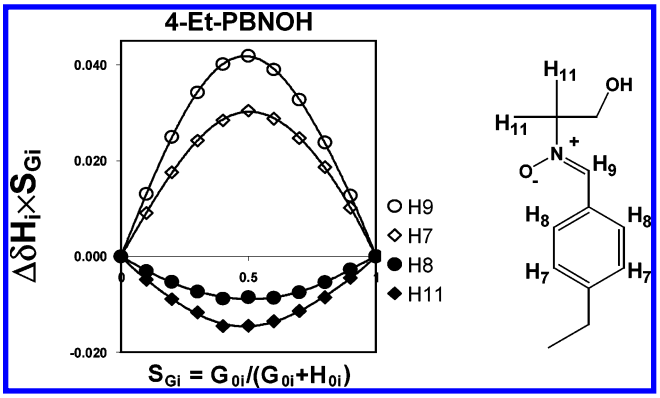


Figure 2. Job's diagram for the 4-Et-PBNOH/DM-β-CD equilibrium.

However, whatever the mode of complexation for the eight studied complexes (four nitrones with two cyclodextrin derivatives), all the protons exhibiting sufficient CIS gave a Job's plot in line with a 1:1 stoichiometry (inflection points close to 0.5, Figure 2). When higher concentrations of PBN and DM-β-CD (100 mM each) were used, the same results were found without any deviation from the symmetric curve.

Association Constant Calculations.[18] The results of the continuous variation method indicated that the formation of 1:1 complexes occurs in an extended range of concentrations of methylated cyclodextrin. Macomber's model[14,19] was used to evaluate the binding constants supposing the following equilibrium:

$$H + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HG$$

Experimentally, the guest concentration was kept constant and 12 or more NMR spectra were recorded, allowing the chemical shifts to be monitored as a function of increasing concentration of the cyclodextrin (Figure 3).

Although, for each system, several protons were suitable for computation of the binding constants, the nitronyl proton H9 was the only one to be selected because of (i) the largest $\Delta\delta$ values for this proton and (ii) its strategic location on the PBN skeleton (Figure 2). Indeed, this choice was motivated by the proximity between H9 in the nitrone structure (used for the determination of K_{nitrone}) and the single electron carried by the oxygen atom of the nitroxide function after spin trapping (allowing determination of $K_{\text{nitroxide}}$ by EPR titrations), to

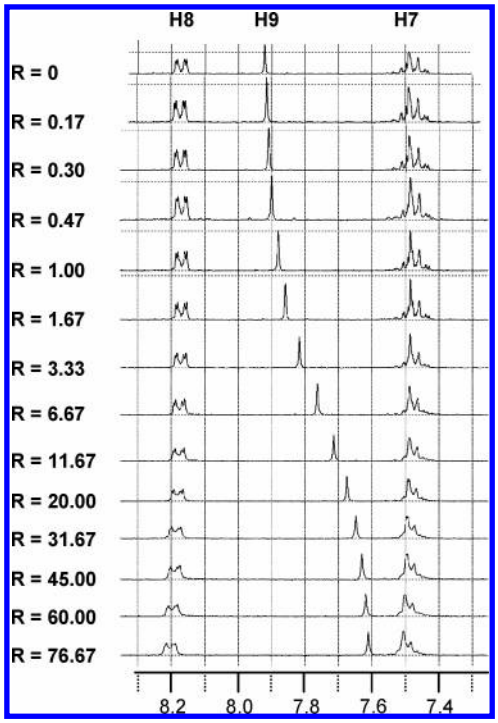


Figure 3. Chemical shift variations of selected guest protons as a function of increasing cyclodextrin concentration (R = [CD]/[nitrone]) for the ^{1}H NMR titration of PBN/RM-β-CD.

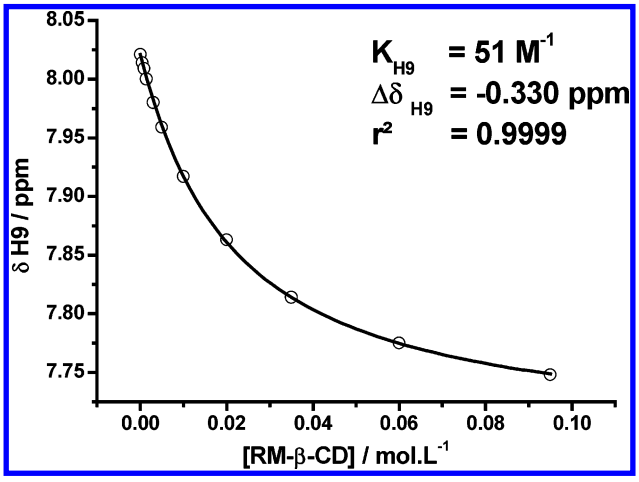


Figure 4. Nonlinear curve fitting procedure for the ^{1}H NMR titration based on the variation of the values of the H9 proton of the PBN/RM-β-CD system.

compare the binding constants derived from the two different techniques. Moreover the values of the CIS for all the guest protons obtained by this method of titration were higher than those obtained by the continuous variation method (higher values of cyclodextrin concentration). Thus, all of these protons should have been taken into account for the calculations. However, they were not deemed suitable for the calculation of the binding constants, because of the limited CIS range and the less good fit between experimental values and the computed curves for these other protons in comparison with H9.

In this way, the nonlinear curve fitting process between experimental points and calculated curves was very good, confirming the assumed 1:1 stoichiometry (Figure 4). However, it must be noted that, instead of an excellent fit, the points corresponding to high cyclodextrin concentrations were spread out of the curves, more or less significantly, depending on the nature of the considered nitrone−cyclodextrin couple.

TABLE 2: Calculated Values of the Binding Constants and Maximum ^{1}H NMR CIS for Proton H9 Depending on the Nitrone and Cyclodextrin

CD	nitrone	$\lvert\Delta\delta\rvert$ (ppm)	r^2	K (M^{-1})	CD range (mM)
RM-β-CD	**1**	0.330 (±0.010)	>0.999	51 (±4)	0.5−95
	2	0.255 (±0.005)	>0.998	30 (±2)	0.2−230
	3	0.205 (±0.005)	>0.999	64 (±4)	0.5−60
	4	0.119 (±0.003)	>0.999	206 (±15)	0.6−66
DM-β-CD	**1**	0.406 (±0.013)	>0.999	80 (±7)	0.5−60
	2	0.346 (±0.013)	>0.999	57 (±5)	0.5−60
	3	0.283 (±0.008)	>0.999	118 (±10)	0.5−60
	4	0.187 (±0.002)	>0.998	271 (±12)	0.5−60

A more precise analysis of the CIS, depending on the considered domain of cyclodextrin concentration, revealed a change in the behavior of the CIS at high levels of cyclodextrin concentration. Indeed, whereas H7, H9, and H11 are affected by the cyclodextrin concentrations until around 60 mM, H7 and H9 are no longer changing, while CIS appeared for H8 and H12 at concentrations greater than 60 mM. These changes are probably due to the formation of 1:2 complexes at these higher concentrations of cyclodextrin. However, the formation of aggregates or modification of the viscosity cannot be excluded.

For this reason, optimal ranges for the cyclodextrin concentrations have to be chosen to allow more accurate determination of the binding constants with a mathematical model elaborated for 1:1 complexes.[7c] The choice of the domain of cyclodextrin concentration retained for the analysis was made considering the best values of the correlation coefficient (r^2) obtained after the fitting procedure by variation of the number of points considered. The errors were estimated as recommended in a previous work.[14] The results are reported in Table 2.

The trends in calculated CIS are the same as those experimentally obtained with the continuous variation method, but the values of the association constants are not directly correlated with the magnitude of the CIS. Indeed, the CIS for a specific proton depends on the geometry of the inclusion complexes, changing with the nitrones for one cyclodextrin. Thus, although the 4-Et-PBNOH nitrone (**4**) gave the most important values for binding constants, the corresponding CIS are the smallest.

The binding constants obtained with the two methylated cyclodextrins are quite similar, although somewhat more important for DM-β-CD, because of the small differences in chemical structures compared to natural β-cyclodextrin. These results are in good agreement with those obtained by Lucarini et al.[20] for the binding constants of *tert*-butyl benzylamine (PBN precursor) with DM-β-CD in deuterated water. The value calculated from ^{13}C NMR titrations (295 ± 50 M^{-1}) is greater than that of PBN (80 ± 7 M^{-1}). Interestingly, Lucarini et al. measured a value of 1079 M^{-1} for the binding constant of benzyl *tert*-butyl nitroxide, which is the H$^\bullet$ spin adduct of PBN, thus indicating a stronger association of DM-β-CD with the linear nitroxide than with the amine.

On the other hand, whatever the nature of the cyclodextrin, the presence of a hydroxyl substituent on the *tert*-butyl group decreases the value of the binding constant in comparison with that of the nonsubstituted PBN. As in the case of natural β-cyclodextrin,[14] introduction of a methyl group in the *para* position restores the initial binding constant value (PBN), although the geometry of the inclusion complex is probably modified. Even higher values are obtained in the case of 4-Et-PBNOH due to the steric bulk and the lipophilic character, leading to a better attraction for the cyclodextrin cavity. Thus, the differences in binding constant values occurring upon PBN substitution suggest that the phenyl moiety is directly involved

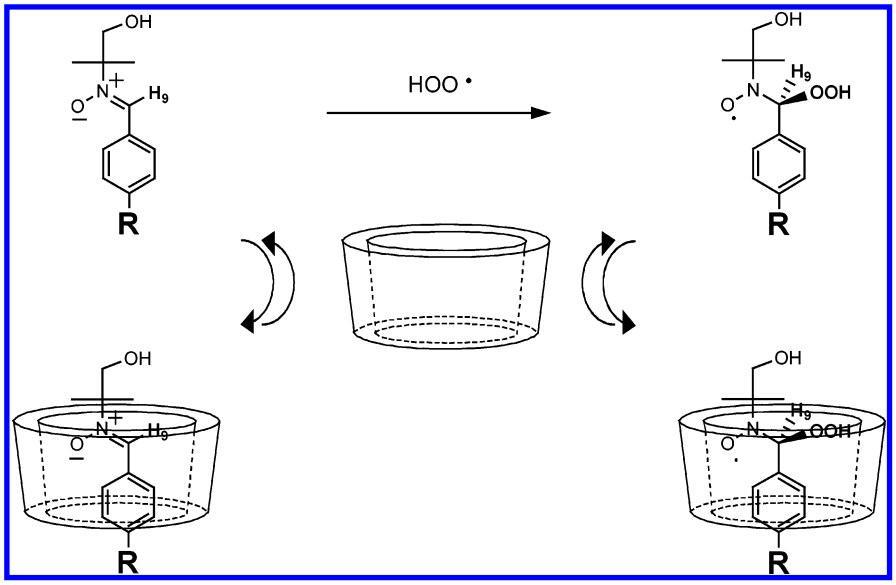


Figure 5. Schematic representation of the equilibriums occurring during spin trapping of superoxide by PBN analogues in the presence of cyclodextrins.

in the complexation equilibrium, since the *tert*-butyl part remains identical between the three PBNOH analogues. Because of the excellent fitting procedure obtained, the assumed 1:1 stoichiometry was confirmed in the domain of cyclodextrin concentration. The results are in good agreement with those obtained with the continuous variation method as well as for the CIS and for binding constant calculations, suggesting an inclusion process in the cyclodextrin cavity by the phenyl side of the nitrones (except for PBN). Finally, it must be mentioned, as suggested by Rekharsky and Inoue,[21] that binding constants calculated from experiments performed in D_2O are probably slightly overestimated in comparison with experiments performed in water.

EPR Study. In the absence of cyclodextrin, spin trapping of superoxide by PBN or its analogues gave only a low intensity signal. Moreover, in the case of PBNOH derivatives, a second signal, again of low intensity, was superimposed. This signal is linked to the equilibrium occurring between the open form of the nitrone and a cyclic hydroxylamine form (addition of the OH function on the nitrone).[22] Although this equilibrium is very displaced in favor of the open form, oxidation of the cyclic hydroxylamine will lead to this second nitroxide species. Indeed, addition of potassium ferricyanide (oxidant 0.5 mM) to a solution of the PBNOH (25 mM) followed by DM-β-CD (50 mM) resulted in the facile observation of this cyclic nitroxide. The stability of this nitroxide was improved by inclusion in the cyclodextrin, and the intensity of the EPR signal increased with time up to 35 min. However, when superoxide was trapped by the PBNOH analogues in the presence of cyclodextrin, this signal was no longer detected.

Spin Trapping of Superoxide in the Presence of Cyclodextrins. During the spin trapping experiments, the nitrone enters into an equilibrium with the cyclodextrin complexed form and the superoxide spin adduct can also exist as free species and as complexed species. The equilibriums depicted in Figure 5 can be suggested, with the total pool of cyclodextrin being shared between each product susceptible to being complexed.

To study the influence of the cyclodextrin concentration on the EPR spectra, the spin trapping experiments were performed at different cyclodextrin concentrations. Increasing concentrations of methylated β-cyclodextrins in spin trapping experiments led to a better detection of the spin adducts with EPR spectra exhibiting better signal-to-noise ratios (Figure 6).

The improvement resulting from the presence of the cyclodextrin is remarkable up to 100 mM, and then, a plateau is reached. Beyond the 100 mM critical concentration, the nitroxides can all be considered to be complexed whatever the association. In the case of PBN and as already observed with RM-β-CD,[13] DM-β-CD stabilizes a product issued from decomposition of the superoxide spin adduct, detected as a three-line signal on the spectra and assigned to an acyl-type nitroxide ($a_N = 0.83$ mT). Although its formation is not governed by a simple equilibrium process (the relative intensity of this signal increases with time), a remarkably good fit could be obtained in the whole cyclodextrin concentration domain, if a 1:2 complexation is assumed for this radical species. In the case of DM-β-CD, the acyl radical is stabilized through inclusion in the cyclodextrin cavity.[13] Moreover, the intensity of the signal increased with the concentration of methylated β-CD. Interestingly, this species has a significantly reduced intensity in the reactions performed with the PBNOH analogues. The intensity of the EPR spectra is also a function of the nitrone considered for a fixed cyclodextrin concentration. Indeed, PBN and PBNOH exhibited the most intense EPR spectra, whereas, when going from PBN (**1**) to 4-Et-PBNOH (**4**), the intensity of the EPR spectrum decreased. That can be correlated with the increase of the binding constant for the nitrones toward the cyclodextrins. In the OH-substituted *tert*-butyl series (nitrones **2**−**4**), the intensities of the EPR spectra decreased with the increasing bulk and hydrophobicity of the aryl group of the nitrone. Thus, higher association constants between the nitrone and the cyclodextrin derivatives are correlated with a lower efficiency of the trapping reaction. A similar phenomenon was observed in the case of carotenoids used as scavengers of the HOO• radical.[23] Generally, a lower water solubility of the guest is correlated with a stronger association with cyclodextrins (except for amphiphilicity, charge,[24] or size effects). Indeed, the lower solubility of 4-Et-PBNOH (**4**) in water is associated with a relatively greater binding constant toward methylated β-cyclodextrins.

Nitroxide−Cyclodextrin Association Constant Calculations. The classical methods of evaluation of the binding constants in the case of stable nitroxide−cyclodextrin complexes use simplified mathematical models, that are valid only if two species are present, the nonassociated and the associated radicals. However, many examples exist where the association is more complex. Multiple types of association can exist when the

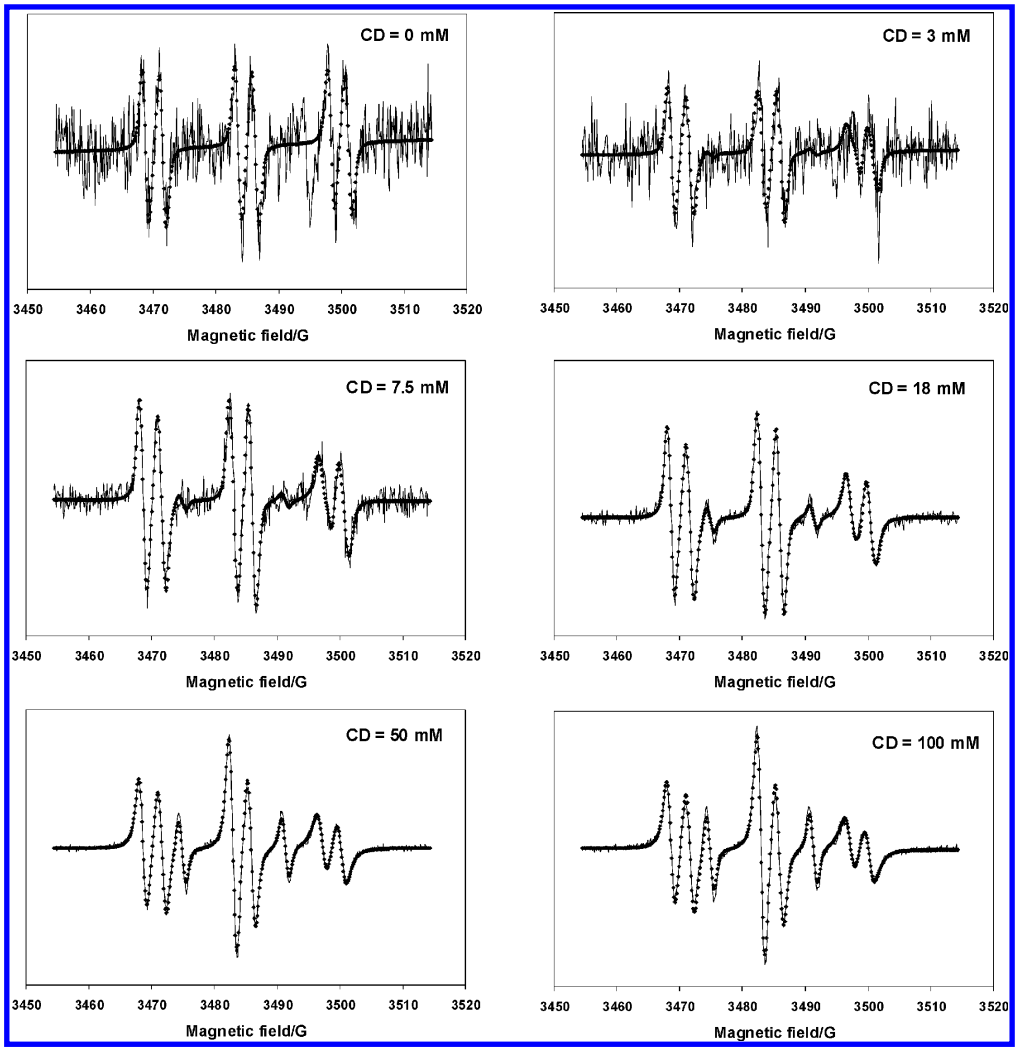


Figure 6. Influence of the DM-β-CD concentration on the EPR spectra of superoxide trapped by PBN (25 mM). Solid line, experimental; dotted line, calculated. Spectrometer settings: microwave power, 20 mW; modulation amplitude, 0.1 mT; time constant, 0.128 s; gain, 1×10^5; scan range, 6 mT; and scan time, 42 s.

nitroxide molecules have different functional groups that can be recognized by the host molecule. In these complex cases, the simple mathematical approaches are no longer relevant. We developed a 2D-EPR simulation approach, where the simultaneous analyses for the total spectrum set were carried out, instead of the separate decomposition of each EPR spectrum into the superimposed components. In this procedure, the relative concentrations of the different species are given by the solution of mass−balance equation and the best fit is searched by using combined iteration techniques to adjust the nonlinear parameters including both the formation constants of different species and the EPR parameters of the component spectra.[25]

If we consider that EPR spectra are detected at N different ligand concentrations and with S different species in the system, the ith spectrum can be described as a function of magnetic field (H) as a superimposition:

$$F_i(H) = c_{i1}f_1(H) + c_{i2}f_2(H) + \ldots c_{iS}f_S(H) \qquad (1)$$

where $f_j(H)$ describes the EPR spectrum of the jth species and c_{ij} is the weight factor of the jth species in the ith spectrum. Each $f_j(H)$ spectrum can be described as a hyperfine multiplet characterized by its respective g-factor, hyperfine constants, and line width parameters. In the present case, all equilibriums are considered slow in the EPR time scale, when the lifetime of the species is long enough to describe the overall spectra as a superimposition. In the case of fast equilibrium, the $F_i(H)$ spectrum can be described by the weight averaged g-factor, hyperfine constants, and line widths. The difference between the one-by-one spectrum analysis and the two-dimensional approach is that separate adjustment of all the c_{ij} coefficients (there, the number is N*S) is not needed; only the K_κ formation constants (their number is S) in the mass balance equations must be computed:

$$[R_nL_k] = K_\kappa[R]^n[L]^k \qquad (2)$$

$$[R_n'L_k] = K_\kappa'[R']^n[L]^k \qquad (3)$$

where R and R' stand respectively for the trapped nitroxide radicals and for the nitrone trap. Equation 2 describes the distribution of EPR active R_nL_k species, while equation 3 gives the distribution of EPR silent nitrones. Since, in most cases, $[R']$ is much larger than $[R]$, the knowledge of K_κ' is crucial in the calculation of free $[L]$ concentration. However, as its determination is very difficult purely from EPR data, we preferred to determine its value separately by using NMR technique and to keep K_κ' as a constant in the EPR analysis. When NMR values are not available for the association constant of the nitrone, we can consider K_κ' as an adjustable parameter.

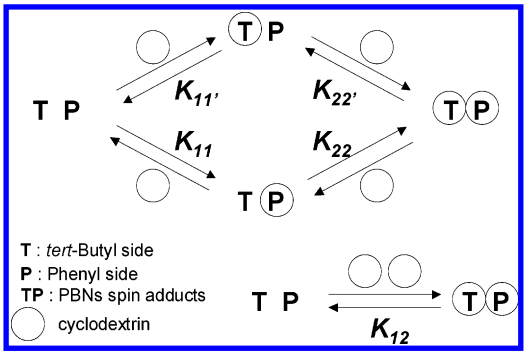


Figure 7. Proposed equilibriums occurring during complexation of spin adducts with cyclodextrins.[18]

The *i*th spectra can be characterized by the overall cyclodextrin and nitrone concentration of the sample, which are denoted by $[L_{i0}]$ and $[R_{i0}']$, respectively:

$$[L_{i0}] = \Sigma_{\kappa} k[R_n L_k] + \Sigma_{\kappa'} k'[R_n' L_{k'}] \quad (4)$$

$$[R_{i0}'] = [R_{i0}] + \Sigma_{\kappa'} n'[R_n' L_{k'}] \quad (5)$$

where $[R_{i0}]$ is the overall concentration of the nitroxide radical. The latter quantity can be estimated from the intensity of the EPR spectra determined either by double integration or by the individual simulation of the respective spectrum. In an EPR spin trapping experiment, a part of the nitroxide radical can decay, when the spectra are recorded; however, this loss is not included in eq 5. Since $[R_{i0}]$ is usually small compared to $[R_{i0}']$, the decrease in the nitrone concentration cannot be large and thus this omission cannot produce significant error. The 2D-EPR simulation method could also handle speciation, where more than one nitroxide is associated with one cyclodextrin molecule, but in the following discussion, we discard this case, since (i) two nitrones (or parts of the nitrone structure) cannot enter the cavity of the cyclodextrin, because of steric hindrance, and (ii) we never observed such a type of association. The program and the statistical parameters describing the regression of fit and the significance criteria of various models were given in detail in an earlier paper.[25] The only essential modification in the present calculations was the inclusion of the term $[R_{i0}]$ in eq 5. To select the best model, we compared the overall regression parameter characterizing the quality of fit for the whole set of spectra. In this analysis, we considered speciation with different types and numbers of associations. We could not find any significant improvement when the 2:1-type association (two radicals to one cyclodextrin) was added to the model, but inclusion of two different 1:1-type associations and occasionally also one 1:2 (one radical to two cyclodextrins)-type association helped to obtain a good fit in the whole cyclodextrin concentration domain (Figure 7). The superoxide spin adducts with the various PBN derivatives form open-chain nitroxide radicals, and their association could be different on the two sides of the molecule. This property is obvious from the best model, which requires two different types of single association and also one double association.

EPR techniques have already proved effective in separating the contributions from the *tert*-butyl and the phenyl part of the deuterated phenyl adduct of PBN in the presence of α-cyclodextrin and β-cyclodextrin.[26] However, the presence of 1:2 complexes is revealed with K_{12} values 100−500 times greater than K_{11} or $K_{11'}$ values. This type of complexes appears only at high cyclodextrin concentrations, when a just formed 1:1 inclusion complex can meet another cyclodextrin unit to produce the 1:2 complex (Figure 8).

In the case of methylated β-cyclodextrins, the solubility allowed the association to be monitored up to 100 mM. The spectra could be recorded even at higher cyclodextrin concentra-

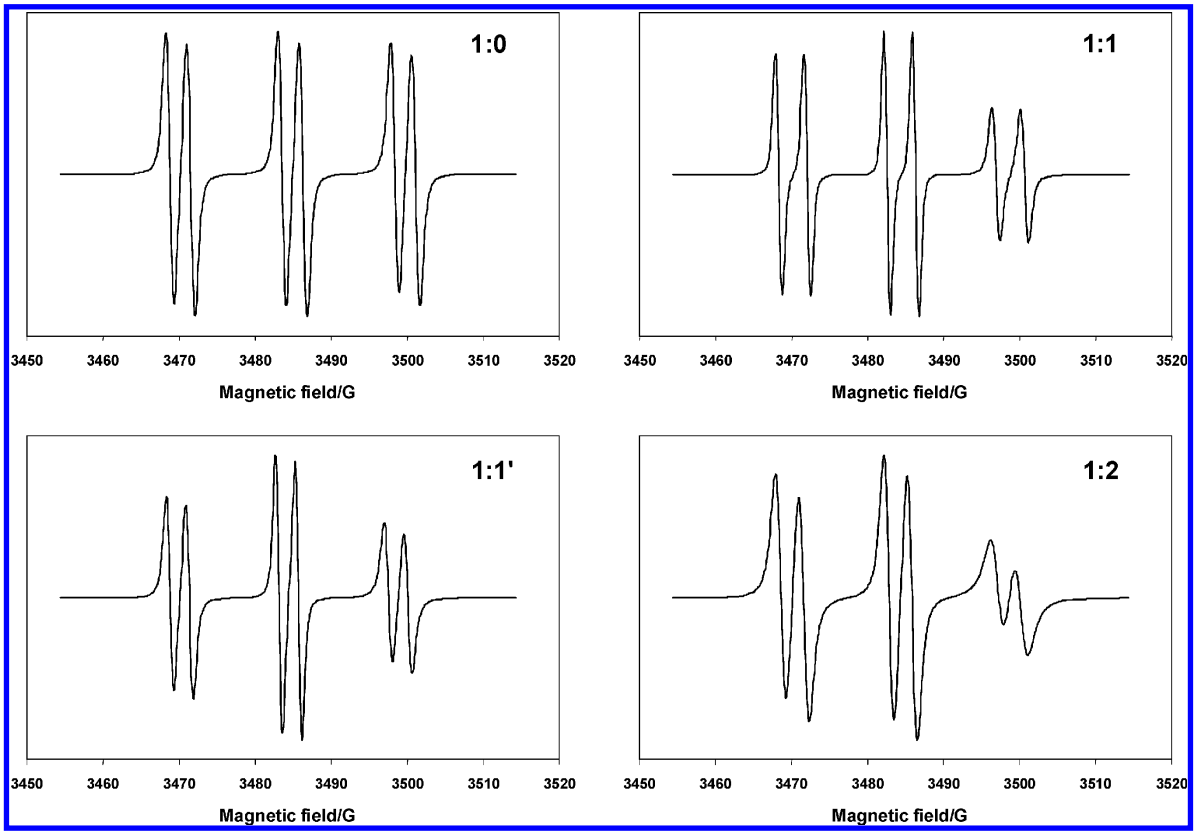


Figure 8. Simulated EPR spectra of the different nitroxide−cyclodextrin inclusion complexes in the trapping of superoxide by PBN/DM-β-CD (1:0 = free nitroxide, 1:1 and 1:1′ = 1:1 complex, and 1:2 = 1:2 complex).

TABLE 3: Calculated Values of Formation Constants, Hyperfine Couplings, and Measure of Cooperativity for the Different Inclusion Complexes Depending on the Considered Nitrone for the Superoxide Spin Trapping in the Presence of Methylated *β*-Cyclodextrin

nitrone	cyclodextrin	parameter	1:0	1:1	1:1′	1:2	$K1^a$	α^b	$a_{TP}{}^c$
1	RM-*β*-CD	a_N (mT)	1.486	1.410	1.439	1.420		**10.7**	**0.26**
		a_H (mT)	0.275	0.283	0.293	0.281			
		K (M^{-1})		**349**	**199**	**17 800**	**548**		
	DM-*β*-CD	a_N (mT)	1.477	1.434	1.426	1.426		**10.5**	**1.10**
		a_H (mT)	0.275	0.255	0.373	0.303			
		K (M^{-1})		**536**	**303**	**178 000**	**839**		
2	RM-*β*-CD	a_N (mT)	1.442	1.414	1.402	1.389		**20.3**	**0.61**
		a_H (mT)	0.262	0.246	0.306	0.271			
		K (M^{-1})		**374**	**234**	**53 400**	**608**		
	DM-*β*-CD	a_N (mT)	1.446	1.416	1.407	1.398		**18.0**	**0.46**
		a_H (mT)	0.258	0.239	0.355	0.282			
		K (M^{-1})		**820**	**205**	**77 000**	**1025**		
3	RM-*β*-CD	a_N (mT)	1.450	1.401	1.401	1.407		**5.1**	**0.20**
		a_H (mT)	0.270	0.323	0.282	0.259			
		K (M^{-1})		**202**	**125**	**4930**	**327**		
	DM-*β*-CD	a_N (mT)	1.452	1.401	*d*	1.413		**3.4**	**0.61**
		a_H (mT)	0.270	0.300	*d*	0.282			
		K (M^{-1})		**404**	*d*	**∼100 000**	**404**		
4	RM-*β*-CD	a_N (mT)	1.451	1.412	1.418	1.401		**3.7**	**0.14**
		a_H (mT)	0.273	0.257	0.365	0.261			
		K (M^{-1})		**658**	**110**	**9800**	**768**		
	DM-*β*-CD	a_N (mT)	1.450	1.412	1.395	1.417		**3.6**	**1.04**
		a_H (mT)	0.273	0.295	0.265	0.281			
		K (M^{-1})		**942**	**40**	**39 000**	**982**		

[a] $K1 = K_{11} + K_{11'}$. [b] $\alpha = K1/K_{nitrone}$. [c] See eq 6. [d] Reliable decomposition of the EPR spectra could not be obtained when two 1:1 species were assumed.

tions, but in this case, the significant increase of viscosity produced broader lines, which could create false results.

There is a basic assumption in our spectrum decomposition process: the signal of the radical species must not change with the cyclodextrin concentration, a condition which cannot be fulfilled with too important viscosity. The determination of microscopic binding constants has been scarcely investigated in the cyclodextrin field.[27] The two-dimensional simulation program was used to compute K_{11}, $K_{11'}$, and K_{12} from the EPR titrations. The stepwise binding constants K_{22} and $K_{22'}$ are easily obtained (K_{12}/K_{11} and $K_{12}/K_{11'}$, respectively).[18] The results are reported in Table 3.

Inclusion of the NO• group inside the cyclodextrin cavity is indicated by the reduced value of the nitrogen splitting (a_N), which is smaller by 0.05−0.1 mT. As it is known[28] from the solvent dependence, the nitrogen splitting of nitroxides is always smaller by 0.1−0.2 mT in nonpolar media than in water. Further evidence for the association is given by the line width. Figure 8 shows the individual spectra of the species. The high field line of the nitrogen triplet has a smaller amplitude than the first two lines in the cases of the 1:1 and 1:2 species, a clear indication of the reduced molecular rotation due to inclusion. Instead of considering K_{11} and $K_{11'}$ independently in the discussion, we preferred to use the $K1$ stoichiometric binding constant ($K1 = K_{11} + K_{11'}$) as defined by Connors.[18] Indeed, this definition is more pertinent for the comparison with the $K_{nitrone}$ value deduced from NMR measurements that could represent the mean value of two coexisting 1:1 complexes (see the NMR section). On the other hand, the K_{11} and $K_{11'}$ constants could be determined only with a large error if $K_{nitrone}$ is not available from NMR measurements. The quality of fit for the EPR spectra only slightly changes when $K_{nitrone}$ is adjusted, and it makes this parameter rather uncertain. If we choose larger $K_{nitrone}$, the adjustment procedure will give proportionally larger K_{11} constants too. This explains why the $K_{nitrone}$ values have been determined independently. However, the relative formation constant ($\alpha = K1/K_{nitrone}$) can be considered as a more appropriate parameter for characterizing the efficiency of cyclodextrin association. It must be noted that this value is probably underestimated because of the overestimation of $K_{nitrone}$ (see the NMR section). In all the investigated examples, the association constants were found to be larger for the spin adducts than for the nitrone ($\alpha > 1$). This trend can be explained by the smaller polarity of the NO• group compared to the N^+O^- group of the parent nitrone, with the less polar group being better included in the nonpolar environment of the cone of the cyclodextrin molecule.

For the two single associations of the PBN/DM-*β*-CD system, the predominant stepwise binding constant ($K_{11} = 536\ M^{-1}$) can be tentatively assigned to inclusion of the phenyl group because of the large variation occurring in changing the substituent in the *para* position of the phenyl moiety. On the other hand, the weaker constant ($K_{11'} = 303\ M^{-1}$) could be attributed to inclusion of the *tert*-butyl moiety, because of minor changes observed with the various PBNOH analogues, since this part remains constant. The value of the macroscopic binding constant for the nitroxide (HOO• adduct of PBN: $839\ M^{-1}$) is larger than that of PBN toward DM-*β*-CD ($\alpha \approx 10.5$). This fact is in good agreement with the results of Lucarini et al.[20] concerning the binding constant of *tert*-butylbenzylnitroxide (the H• adduct of PBN: $1079\ M^{-1}$) and the difference between these species and *tert*-butylbenzylamine (binding constant ratio ≈3.7). The higher value obtained in the case of *tert*-butylbenzylnitroxide compared to our nitroxide (HOO• adduct of PBN) is expected in regard to the less polar molecule resulting from the trapping of H• compared to HOO•. The binding constants of nitroxide toward cyclodextrins are more important with DM-*β*-CD than with RM-*β*-CD, especially in the case of double association. This can be explained by a better structural complementarity in the ternary complex built with two DM-*β*-CD that is less heterogeneous than in the case of the statistical mixture of RM-*β*-CD, a fact which was also found for the nitrone−cyclodextrin equilibriums. The OH substitution in the *tert*-butyl moiety [PBNOH (**2**)] reduces the strength of the

nitrone association but increases the strength of the nitroxide adducts 1:1 association. For the nitrone, the dipolar effects of OH and N^+O^- are summed up and lead to a weaker association. In contrast, for the nitroxides, the major source of polarity of the $NO^\bullet$ group is the polar limiting structure $N^{\bullet+}O^-$, which has a smaller weight due to the OH substitution. This fact is reflected in the nitrogen hyperfine couplings (1.48−1.49 mT for PBN and 1.44−1.45 mT for the PBNOH adducts). The modest polarity of the $NO^\bullet$ group can compensate or even overcompensate the direct effect of the OH substituent, and as a consequence, the association does not change or even could be stronger. These data support the assumption of a predominant inclusion on the phenyl side. There is no clear-cut trend concerning the variation of $K_{11'}$, since the estimation of this minor component is very rough. Concerning the ternary complex in the case of DM-β-CD that can be determined with a better precision, this double association drops significantly for PBNOH (K_{12} = 77 000 M^{-1} for the PBNOH adduct and 178 000 M^{-1} for the PBN adduct). This illustrates the fact that the association with a second cyclodextrin is definitely hindered by the presence of a polar substituant on the guest. In the case of the complexes with RM-β-CD, the values are difficult to interpret probably because of the statistical nature of the cyclodextrin.

The alkyl substitution of the phenyl group affects differently the nitrone−cyclodextrin and nitroxide−cyclodextrin association. While substitution by a 4-methyl slightly increases the nitrone association, both the single and the double association become much weaker in the case of the nitroxides. However, 4-ethyl substitution of the phenyl group strongly enhances the association for both the nitrone and nitroxide complexes. This shows the different role of methyl and ethyl substituents: the methyl only increases the size of the phenyl moiety, but the ethyl group can also act as an independent center in the inclusion process. This role is reflected in the variation of a_N. The decrease between the associated and nonassociated radical is only 0.03−0.04 mT, and this means that the $NO^\bullet$ group is more distant from the inner part of cyclodextrin and thus less affected by complexation. This trend was also observed in the NMR study of complexation of the parent nitrones with cyclodextrins.

Interestingly, not only was the two-dimensional EPR simulation program able to extract all the stepwise binding constants for the spin adduct−cyclodextrin multiple equilibriums but it also contributed to characterizing a slightly cooperative system. Indeed, the Hill analysis was used successfully in the case of nitrone−cyclodextrin equilibriums (h = 1, which agrees well with the NMR Job's analysis, data not shown).[29] However, in EPR experiments, the changes of the g-factor, that were not significant, or in the nitrogen hyperfine splitting (a_N) did not allow the characterization of a cooperativity effect.[18] However, the a_{TP} parameter (a measure of cooperativity for the association of several cyclodextrins with our heteroditopic guests) can be obtained from the calculated stepwise binding constants considering T as the *tert*-butyl side and P as the phenyl side by using the following equation:[18]

$$a_{TP} = \frac{K_{22'}}{K_{11}} = \frac{K_{22}}{K_{11'}} = \frac{K_{12}}{K_{11}K_{11'}} \qquad (6)$$

This parameter represents the extent of interaction (impediment or assistance) occurring between T and P sites in the formation of a 1:2 complex. The cooperativity is negative for all the studied cases ($a_{TP} < 1$) except for the 4-Et-PBNOH/DM-β-CD system (noncooperative, $a_{TP} \approx 1$) and the PBN/DM-β-CD system (slightly positive cooperativity, a_{TP} = 1.1). In this particular case, coordination of the first cyclodextrin with the guest (nitroxide spin adduct) implies an easier complexation of this inclusion complex by a second cyclodextrin molecule.[30] That can be explained by a better fit of the PBN/DM-β-CD 1:1 complex in the second DM-β-CD, since the structural compression is a true effect of positive cooperativity exercized at a binding interface[31] as well as the repositioning effect or the ligand−ligand effect.[18] However, the implication of cooperativity in noncovalent small chemical systems using cyclodextrins has been scarcely investigated.[32−34]

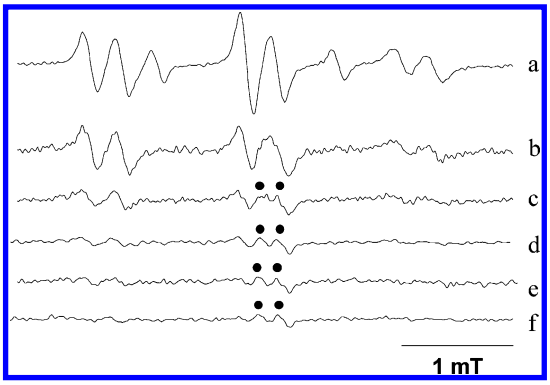


Figure 9. Protection of the PBN−OOH nitroxide spin adduct against L-ascorbate reduction: (a) signal obtained after incubation of a mixture of PBN (15 mM), DTPA (0.5 mM), HX (0.2 mM), and XOD (0.05 $U \cdot mL^{-1}$) in the presence of RM-β-CD (50 mM) in oxygenated phosphate buffer for 5 min; (b) 1 min after the addition of SOD (50 $U \cdot mL^{-1}$) and L-ascorbate monoanion (0.1 mM); (c) after 1 min 50 s; (d) after 2 min 40 s; (e) after 3 min 30 s; (f) after 4 min 20 s. Spectrometer settings: microwave power, 20 mW; modulation amplitude, 0.1 mT; time constant, 0.128 s; gain, 3.2×10^5; scan range, 6 mT; and scan time, 42 s.

Bioreduction Experiments. In the absence of cyclodextrins, when sodium L-ascorbate is added to the mixture resulting from trapping of superoxide by a nitrone, instantaneous disappearance of the EPR signal takes place due to the reduction of the nitroxides into EPR silent hydroxylamines. Addition of RM-β-CD protected efficiently these nitroxides against reduction by sodium L-ascorbate.[12,13]

Bioreduction experiments using L-ascorbate were performed with the eight selected nitrone−cyclodextrin systems. To avoid secondary production of nitroxide species, the minimum amount of SOD necessary to stop the production of superoxide for up to 30 min was determined for PBN/RM-β-CD and for DEPMPO alone. For all the combinations of nitrones **1**−**4** with either of the two methylated cyclodextrins, the signals of the corresponding superoxide spin adducts were observed in the EPR spectra up to 3 min after the addition of sodium L-ascorbate (Figure 9). As in the case of PBN,[13] the three-line signal corresponding to a decomposition product disappeared instantaneously upon the addition of sodium L-ascorbate. Thus, the acyl 1:2 complex is not protected toward reduction by sodium L-ascorbate. Moreover, a small doublet signal (•), attributed to the ascorbyle radical, appeared when the major part of nitroxide had been reduced. On the other hand, after the addition of sodium L-ascorbate, the decrease is primarily due to reduction by sodium L-ascorbate, but the natural decay of the spin adduct is also responsible for a part of the decrease of the EPR signal. This latter cause of decay is not necessarily identical among all the couples of nitrone−cyclodextrin considered, and that makes the interpretation difficult purely in terms of the ascorbate effect. Although significant structural and energetical differences are found between the nitrone−cyclodextrin complexes and the nitroxide−cyclodextrin complexes, only slight differences were

observed between the times during which the superoxide spin adduct signals were observed in the EPR spectra after the addition of L-ascorbate. However, for the relative bioresistance abilities, the two best systems are PBN/DM-β-CD, maybe due to cooperativity, and PBNOH/RM-β-CD.

Conclusion

Linear PBN-type nitrones as well as their superoxide spin adducts form inclusion complexes with methylated β-cyclodextrins with a 1:1 stoichiometry for the parent nitrones, whereas bimodal inclusion of heteroditopic nitroxide spin adducts into cyclodextrins has been evidenced by EPR spectroscopy. All the binding constants were estimated either for single (nitrones−cyclodextrins) or multiple (nitroxides−cyclodextrins) equilibriums, affording values always higher for the nitroxide complexes than for the nitrone complexes. The ability for the nitrones to trap the superoxide radical is inversely proportional to their capacity to be complexed. The OH substitution on the *tert*-butyl group of PBN makes the nitrone−cyclodextrin association weaker without changing significantly the capacity of nitroxide association, while the alkyl substitution of the phenyl group exerts an opposite effect. A slightly positive cooperativity has been observed for the complexation of superoxide spin adducts in the case of PBN by DM-β-CD. Thus, the use of cyclodextrins in superoxide trapping experiments as molecular assistants remarkably enhances the stability of the spin adducts. Bioresistance abilities of PBN superoxide spin adducts due to the presence of RM-β-CD are generalized to other PBN-type nitrones and methylated β-cyclodextrins. The two association constants *K*(nitrone−cyclodextrin) and *K*(nitroxide−cyclodextrin) as well as their ratio are important for evaluating the efficacy of a nitrone−cyclodextrin couple to trap free radical and the nitroxide adduct to be protected. The interaction site parameter is also important in the case of multiple complexation equilibriums, since the cooperativity could play an important role in the encapsulation of the spin adducts by cyclodextrins for easier detection and observation of transient reactive oxygen species in a biological milieu.

Acknowledgment. A.R. thanks the Hungarian Scientific Research Fund for partial financial support (OTKA T-046953). The authors also thank the Conseil Régional Provence Alpes Côte d'Azur and TROPHOS Company (Marseille, France) for partial financial support and Dr Sylvain Marque for fruitful discussions.

References and Notes

(1) Szejtli, J. *Cyclodextrins and Their Inclusion Complexes*; Akadémiai Kiadó: Budapest, 1982.

(2) Duchene, D. *New Trends in Cyclodextrins and Derivatives*; Editions de Santé: Paris, 1991.

(3) Szejtli, J. *Chem. Rev.* **1998**, *98*, 1743−1753.

(4) (a) Ebel, C.; Ingold, K. U.; Michon, J.; Rassat, A. *Nouv. J. Chim.* **1985**, *9*, 479−485. (b) Ebel, C.; Ingold, K. U.; Michon, J.; Rassat, A. *Tetrahedron Lett.* **1985**, *26*, 741−744. (c) Okazaki, M.; Kuwata, K. *J. Phys. Chem.* **1985**, *89*, 4437−4440.

(5) Martinie, J.; Michon, J.; Rassat, A. *J. Am. Chem. Soc.* **1975**, *97*, 1818−1823.

(6) (a) Kotake, Y.; Janzen, E. G. *J. Am. Chem. Soc.* **1989**, *111*, 5138−5140. (b) Kotake, Y.; Janzen, E. G. *Free Radical Res. Comm.* **1990**, *10*, 103−108.

(7) (a) Michon, J.; Rassat, A. *J. Am. Chem. Soc.* **1979**, *101*, 4337−4339. (b) Michon, J.; Rassat, A. *J. Am. Chem. Soc.* **1979**, *101*, 995−996. (c) Franchi, P.; Lucarini, M.; Mezzina, E.; Pedulli, G. F. *J. Am. Chem. Soc.* **2004**, *126*, 4343−4354. (d) Janzen, E. G.; Kotake, Y. *J. Am. Chem. Soc.* **1988**, *110*, 7912−7913.

(8) (a) Lucarini, M.; Luppi, B.; Pedulli, G. F.; Roberts, B. P. *Chem.−Eur. J.* **1999**, *5*, 2048−2054 and references therein. (b) Kotake, Y.; Janzen, E. G. *J. Am. Chem. Soc.* **1992**, *114*, 2872−2874 and references therein.

(9) Sueishi, Y.; Kasahara, M.; Kotake, Y. *Chem. Lett.* **2000**, 792−793.

(10) (a) Luo, L. B.; Han, D.-Y.; Wu, Y.; Song, X. Y.; Chen, H.-L. *J. Chem. Soc., Perkin Trans. 2* **1998**, 1709−1714. (b) Chen, Y.; Chen, H.-L.; Yang, Q.-C.; Song, X.-Y.; Duan, C.-Y.; Mak, T. C. W. *J. Chem. Soc., Dalton Trans.* **1999**, 629−634. (c) Han, D.-Y.; Bai, Z.-P.; Chen, H.-L. *Wuji Huaxue Xuebao* **1999**, *15*, 507−508. (d) Song, X.; Chen, Y.; Chen, H. *New J. Chem.* **2001**, *25*, 985−988.

(11) Davies, M. J.; Timmins, G. S. *Electron Paramagn. Reson.* **2000**, *17*, 1−42.

(12) Karoui, H.; Rockenbauer, A.; Pietri, S.; Tordo, P. *Chem. Commun.* **2002**, *24*, 3030−3031.

(13) Karoui, H.; Tordo, P. *Tetrahedron Lett.* **2004**, *45*, 1043−1045.

(14) Bardelang, D.; Clement, J.-L.; Finet, J.-P.; Karoui, H.; Tordo, P. *J. Phys. Chem. B* **2004**, *108*, 8054−8061.

(15) Schneider, H. J.; Hacket, F.; Rüdiger, V.; Ikeda, H. *Chem. Rev.* **1998**, *98*, 1755−1785.

(16) Rockenbauer, A.; Korecz, L. *Appl. Magn. Reson.* **1996**, *10*, 29−43.

(17) (a) Job, P. *Ann. Chim. (Paris)* **1928**, *10*, 113−199. (b) Djedaïni, F.; Lin, S. Z.; Perly, B.; Wouessidjewe, D. *J. Pharm. Sci.* **1990**, *79*, 643−646.

(18) (a) Connors, K. A. *Binding Constants: the measurement of molecular complex stability*, John Wiley & Sons: New York, 1987. (b) Connors, K. A. *Chem. Rev.* **1997**, *97*, 1325−1357. (c) Fielding, L. *Tetrahedron* **2000**, *56*, 6151−6170.

(19) Macomber, R. S. *J. Chem. Educ.* **1992**, *69*, 375−378.

(20) Lucarini, M.; Mezzina, E.; Pedulli, G. F. *Eur. J. Org. Chem.* **2000**, 3927−3930.

(21) Rekharsky, M. V.; Inoue, Y. *Proceedings−12th International Cyclodextrin Symposium*, Montpellier, May 16−19, 2004; APGI: Chatenay-Malabry, France, 2004; pp 263−266.

(22) (a) Janzen, E. G.; Zawalski, R. C. *J. Org. Chem.* **1978**, *43*, 1900−1903. (b) Kliegel, V. W.; Becker, H. *Chem. Ber.* **1977**, *110*, 2067−2089.

(23) Polyakov, N. E.; Leshina, T. V.; Konovalova, T. A.; Hand, E. O.; Kispert, L. D. *Free Radical Biol. Med.* **2004**, *36*, 872−880.

(24) Rekharsky, M. V.; Inoue, Y. *Chem. Rev.* **1998**, *98*, 1875−1917.

(25) Rockenbauer, A.; Szabó-Plánka, T.; Árkosi, Zs.; Korecz, L. *J. Am. Chem. Soc.* **2001**, *123*, 7646−7654.

(26) (a) Kotake, Y.; Janzen, E. G. *Chem. Phys. Lett.* **1988**, *150*, 199−203. (b) Kotake, Y.; Janzen, E. G. *Chem. Express* **1988**, *3*, 715−718.

(27) (a) Funasaki, N.; Ishikawa, S.; Neya, S. *Langmuir* **2002**, *18*, 1786−1790. (b) Jover, A.; Budal, R. M.; Meijide, F.; Soto, V. H.; Tato, J. V. *J. Phys. Chem. B* **2004**, *108*, 18850−18859. (c) Park, J. W.; Lee, S. Y. *J. Inclusion Phenom. Macrocyclic Chem.* **2003**, *47*, 143−148. (d) Johnson, M. D.; Hoesterey, B. L.; Anderson, B. D. *J. Pharm. Sci.* **1994**, *83*, 1142−1146.

(28) Abdel-Monem, S. A.; Rockenbauer, A.; Tödös, F. *Adv. Mol. Relax. Interact. Processes* **1979**, *15*, 207−212.

(29) (a) Petter, R. C.; Salek, J. S.; Sikorski, C. T.; Kumaravel, G.; Lin, F.-T. *J. Am. Chem. Soc.* **1990**, *112*, 3860−3868. (b) Shinkai, S.; Ikeda, M.; Sugasaki, A.; Takeuchi, M. *Acc. Chem. Res.* **2001**, *34*, 494−503. (c) Dodziuk, H.; Ejchart, A.; Lukin, O.; Vysotsky, M. *J. Org. Chem.* **1999**, *64*, 1503−1507.

(30) Harada, A.; Furue, M.; Nozakura, S.-I. *Polym. J.* **1980**, *12*, 29−33 and references therein.

(31) Calderone, C. T.; Williams, D. H. *J. Am. Chem. Soc.* **2001**, *123*, 6262−6267.

(32) Harada, A.; Nozakura, S.-I. *Polym. Bull.* **1982**, *8*, 141−146.

(33) Giorgi, J. B.; Tee, O. S. *J. Am. Chem. Soc.* **1995**, *117*, 3633−3634.

(34) Dodziuk, H.; Demchuk, O. M.; Bielejewska, A.; Kozminski, W.; Dolgonos, G. *Supramol. Chem.* **2004**, *16*, 287−292.