EPR investigation on radical trap reactions of 2-methyl-2-nitrosopropane encapsulated by cyclodextrins with external organic radicals produced by photolysis of coenzyme B_{12} and its analogues

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^{1}H NMR studies reveal that 2-methyl-2-nitrosopropane (MNP) in the dimeric state can decompose into monomer effectively in the presence of cyclodextrins (Cds) in aqueous solution. The inclusion complexes formed are more soluble than MNP itself and stable to light. MNP–Cds are used as radical traps in the photolysis of coenzyme B_{12} and alkylcobalamins in the present study. The N and H hyperfine splitting parameters of the spin adducts are obtained by the EPR technique. Models for the interaction between Cds and aminoxyls are proposed, which mimic coenzyme B_{12}–enzyme interactions to a certain extent.

Introduction

α-, β- and γ-cyclodextrins (Cds) are cyclic oligosaccharides composed of six, seven or eight D-glucose units through α(1,4)-linkages. The oligosaccharide ring forms a torus, where the outer surface is hydrophilic and the inner cavity is hydrophobic, which can include various organic molecules.[1–3] The inclusion phenomena of Cds have been studied as models for enzyme–substrate interactions and further, Cds as well as their modified complexes have been invoked as 'artificial enzymes' to catalyze various reactions, even some systems in nature. Certainly, the specificities shown in the reactions are all related to the defined spatial relationship between the substrate and the Cds.[3–5]

Studies on the inclusion of organic radicals by Cds using the EPR technique have attracted a great deal of attention.[6–14] They are mainly focused on stable sterically-protected aminoxyls. Okazaki and Kuwata showed that di-*tert*-butyl aminoxyl, 2,2,6,6-tetramethylpiperidin-1-oxyl and 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl could be fully included into the β-Cd cavity in different dispositions, and this induced anisotropic rotational diffusion of the radical.[7] Kotake and Janzen have suggested that a Cd could recognize an aminoxyl functional group, resulting in the formation of bimodal inclusion complexes.[8] In addition, it has been reported that Cds could stabilize nitroarene radical anions[13] and the C_{60} radical anion.[14] Recently, Lucarini and Roberts described the EPR characterization of Cd inclusion complexes of short-lived radicals using the rapid-mixing continuous-flow technique.[6]

As reported by Lagercrantz, the spin-trapping technique is another powerful method for detecting the presence of short-lived radicals.[15] By using the technique with 2-methyl-2-nitropropane (MNP) as a spin trap, we have characterized the radicals produced in the photolysis of coenzyme B_{12} and its models.[16] However, the solubility of MNP in aqueous solution is poor and MNP undergoes decomposition on irradiation. In the present study, we explore the use of MNP–Cds inclusion complexes in the radical trapping reactions involved in the photolysis of coenzyme B_{12} and other alkylcobalamins.

The coenzyme B_{12} (5′-deoxyadenosylcobalamin, AdoCbl, see Fig. 1 for structure) is a natural coenzyme in over a dozen enzyme reactions. Homolysis of the Co–C bond is the key step in the catalytic cycle and the role of AdoCbl in enzyme reactions was suggested to be that of radical carrier. Methylcobalamin (MeCbl) is another kind of coenzyme form of B_{12}, which takes part in methyltransferase reactions.[17–20] Structure–function relationships such as why nature chooses 5′-deoxyadenosyl (Ado) as axial ligand are pivotal questions in B_{12} chemistry. Previous studies have suggested that the non-covalent interaction between Ado and proteins contributes to a weakening of the Co–C bond to form a geminate pair, then to moving the radical to the reaction site and finally to making Ado combine with cobalt again in the course of the enzymatic reaction.[17–21] Photolysis of AdoCbl and its alkylcobalamin analogs generates cob(II)alamin and corresponding radicals, in a process resembling many B_{12}-dependent enzymatic reactions.[17] In this study, radical trap reactions of MNP encapsulated by Cds with external organic radicals produced by photolysis of AdoCbl, MeCbl and their alkylcobalamin analogs are investigated by the EPR technique. The N and H hyperfine splittings (hfs), which reflect the interactions between aminoxyls and Cds, are given and inclusion models are proposed. The inference that Cds act as enzyme models helps in understanding the interaction between Ado and protein in enzymatic reactions.

Experimental

AdoCbl was purchased from Sigma. 2′,5′-Dideoxyadenosylcobalamin (2′dAdoCbl), 1-methyl-5-deoxy-β-D-(−)-ribofuranos-5-ylcobalamin (RibCbl), 1-methyl-5-deoxy-2,3-isopropylidene-β-D-(−)-ribofuranos-5-ylcobalamin (Rib*Cbl), neopentylcobalamin (NpCbl) and methylcobalamin (MeCbl) were synthesized according to the literature.[22] MNP was prepared according to the method suggested by Stowell.[23] The purity was confirmed by ^{1}H NMR.

^{1}H NMR spectra were obtained using a Bruker AM 500 MHz spectrometer. Chemical shifts were referenced in D_2O with respect to external sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

The EPR measurements were carried out on a JEOL-FE1XG spectrometer at room temperature with 100 kHz field modulation, 0.5–0.8 G modulation width, 25 mW microwave power and 0.1 s response time. Irradiation was performed in the cavity of the EPR spectrometer using a BM-501S high pressure mercury lamp with a wavelength in the ultra-violet range. Sample preparations were carried out under dim red lights. The solutions containing 10 mg of the cobalamin and 1 ml of solution (a) or solution (b) were injected into a flat quartz sample tube ($0.5 \times 4.2 \times 43.5$ mm). Solution (a) is 6×10^{-2} mol l^{-1} MNP aqueous solution without cyclodextrin, which was prepared by adding 25 mg MNP to 5 ml double-distilled water with continuous stirring for 4 h at 45 °C under nitrogen. Solution (b) is 2×10^{-2} mol l^{-1} MNP aqueous solution in the presence of

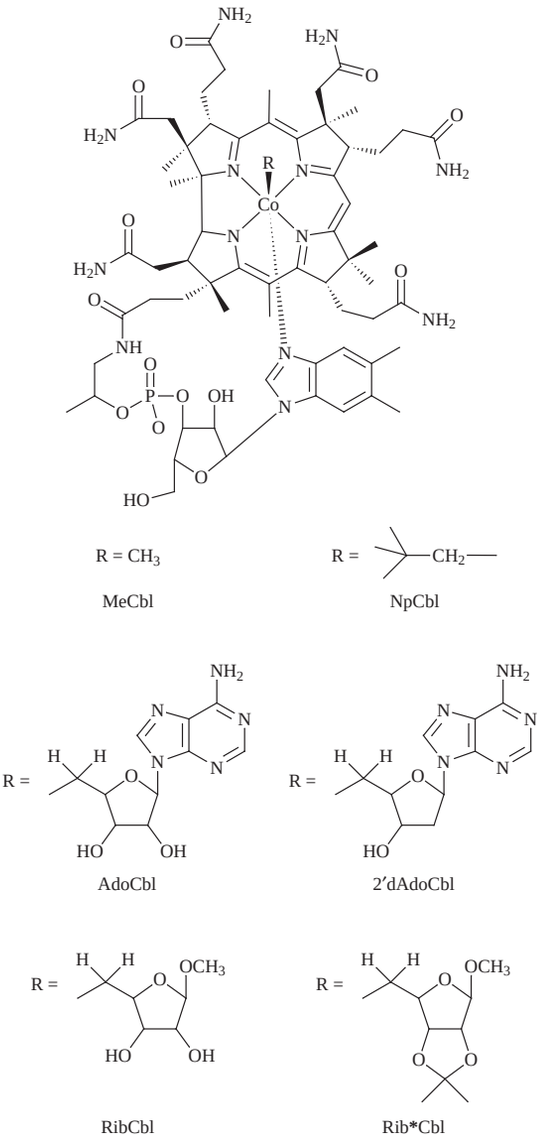


Fig. 1 Structures of coenzyme B_{12}, methylcobalamin and alkylcobalamins

cyclodextrin (1:1). In the resulting solutions, the concentrations of cobalamins, MNP and Cds were 3×10^{-3}, 2×10^{-2} M, respectively.

MM calculations were performed using the Biosym program at the INDISO2 station.

Results and discussion

Formation of inclusion complexes of Cds and MNP

Many aliphatic nitroso compounds are present as dimers in solution. ^{1}H NMR investigations on MNP and MNP–Cds in D_2O show signals at ~1.5 and ~1.3 ppm, revealing that the dimer and the monomer are present[23] (Fig. 2). According to the integrated signal areas, the molar percentages of monomer are estimated to be 5% in the absence of Cds, 10% in the presence of α-Cd, 60% in the presence of β-Cd and 10% in the presence of γ-Cd, respectively. It suggests that Cds can catalyze the decomposition of MNP from its dimer into the monomer to different extents.

As discussed elsewhere, the *tert*-butyl group in an aminoxyl could be included into Cd cavities.[1,8] We suggest that Cds and MNP in the dimeric state might form 2:1 inclusion complexes through including *tert*-butyl into Cd cavities (see Scheme 1). MM calculations give an optimum structure for the dimer, in which the largest interatomic distance for axial D_3 symmetry is

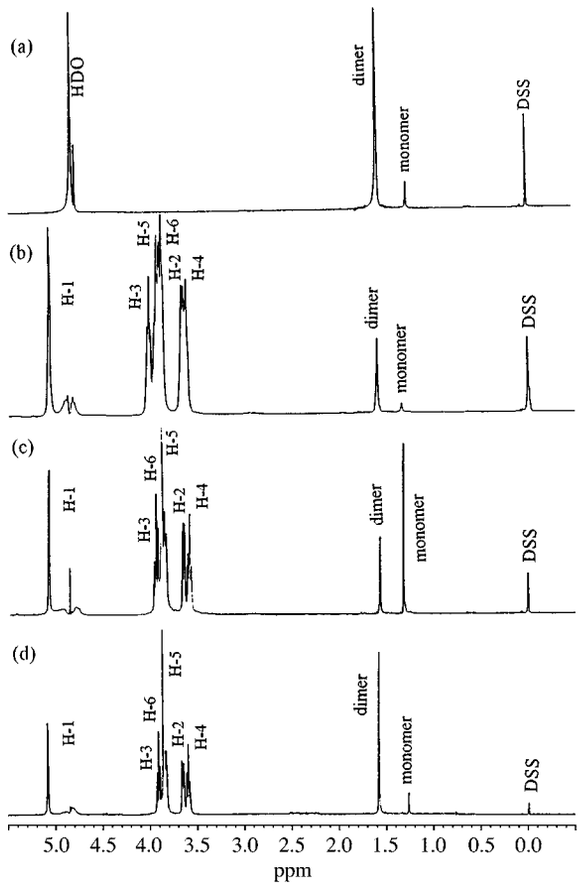
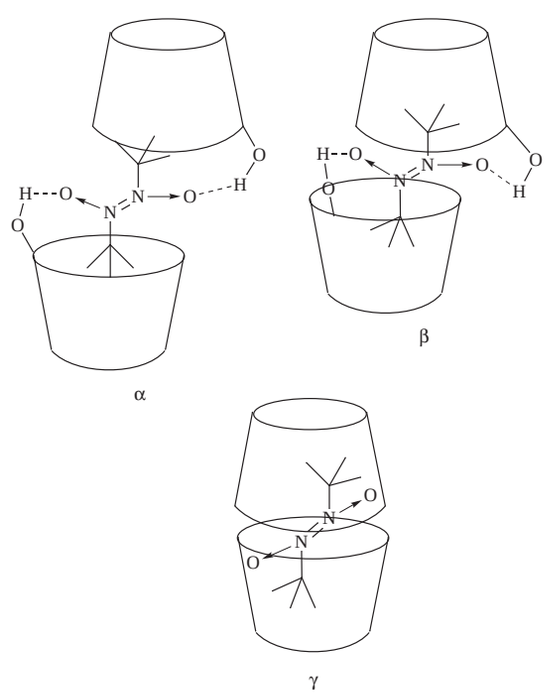


Fig. 2 ^{1}H NMR spectra for MNP in D_2O (a) in the absence of Cd, (b) in the presence of α-Cd, (c) in the presence of β-Cd and (d) in the presence of γ-Cd



Scheme 1 Proposed inclusion model involving Cds and MNP in the dimeric state

about 2.2 Å. The cavity diameters of α-Cd, β-Cd and γ-Cd are 4.9, 6.2 and 7.9 Å, respectively, and the shape of the Cds is a torus.[3] Therefore the *tert*-butyl group cannot fit into the α-Cd cavity properly, while it can fit snugly into the cavity of β-Cd.[24] Hydrogen bonding between the hydroxy in the Cd rim and oxy-

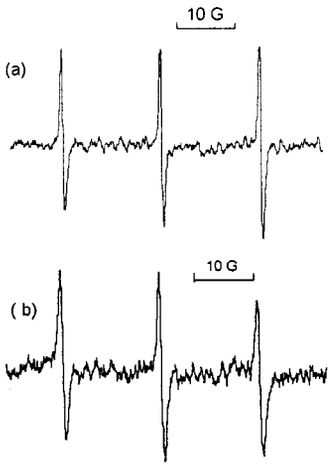
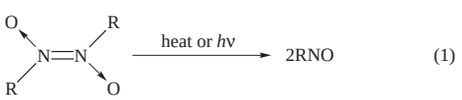


Fig. 3 EPR spectra of DTBA (a) in the absence of Cds, (b) in the presence of β-Cd

gen in nitroso may occur. In the case of the γ-Cd inclusion complex, the nitroso is fully included into the cavity, so no hydrogen bonding occurs. We suppose that more hinderance would exist in the β-Cd inclusion complex than in either the α-Cd or the γ-Cd inclusion complex. Therefore, β-Cd can catalyze the dissociation of the dimer into monomer more effectively.

In order to confirm our proposed inclusion model, we first investigated the photolysis of NpCbl, which can form inclusion complexes with Cds. The results are shown in Scheme 1.

As previously reported, the nitroso compound in the dimeric state cannot trap radicals. The dimers can be split into the monomeric state either thermally or by UV irradiation [eqn. (1)]. In the meantime, the nitroso compound in the monomeric



(1)

state can also dissociate into alkyl radicals under the reaction conditions [eqn. (2)]. The radicals add to undissociated molecules of the nitroso compound in a subsequent reaction to give the di-*tert*-butyl aminoxyl radical [eqn. (3)], which shows strong triple peaks in the EPR spectrum [Fig. 3(a)].

$$2\mathrm{RNO} \xrightarrow{\text{heat or } h\nu} \mathrm{R}^{\bullet} + \mathrm{NO}^{\bullet} \qquad (2)$$

$$\mathrm{R}^{\bullet} + \mathrm{RNO} \longrightarrow \mathrm{R_2NO}^{\bullet} \qquad (3)$$

On irradiation by UV light of a nitroso compound solution containing β-Cd, and γ-Cd for a long time, weak signals of the di-*tert*-butyl aminoxyl (DTBA) could be detected by EPR. It reveals that after formation of inclusion complexes, the nitroso compound is stable to light. Dissociation of free nitroso compound in solution contributes to the weak signals. The N hfs become smaller (see Table 1 and ref. 24), suggesting that DTBA is in a less polar environment than pure water, *i.e.* it is included into the Cd's cavities. Further, the relatively high peak in the spectrum [Fig. 3(b)] clearly indicates that the DTBA radical undergoes *y*-axis rotational diffusion in the inclusion complexes.[7] MM calculations present an optimum structure for DTBA. If we suppose that C_α–C_α (C_α is adjacent to the N atom) is along the C_n axial symmetry of Cds, the distances of atoms in DTBA from the axis are less than 2.2 Å and the height is 6.9 Å. The results provide evidence that the DTBA can fit into β- and γ-Cd cavities properly (see Scheme 2). The result is in agreement with the previous report.[7]

Photolysis of NpCbl

The concentrations of NpCbl, Cds and MNP (in terms of

Table 1 EPR parameters for free and included radicals in aqueous media at 293 K

Radical	Additive	A_N/G[a]	A_{H1}/G[a]	A_{H2}/G	$A_{H1} + A_{H2}$/G
MNP	None[b]	17.1			
	α-Cd	17.1			
	β-Cd[b]	16.7			
	γ-Cd	16.8			
Me–MNP	None[c]	17.3	14.3		
	α-Cd	17.1	14.3		
	β-Cd	16.4	13.3		
	γ-Cd	16.5	14.2		
Np–MNP	None	17.1	10.4		
	β-Cd	16.3	9.5		
	γ-Cd	16.7	9.6		
Ado–MNP	None[c]	16.4	7.9	14.6	22.5
	β-Cd	16.0	8.0	14.4	22.4
		15.1	6.3	12.9	19.2
2′dAdo–MNP	None[c]	16.8	7.9	14.5	22.4
	β-Cd	15.8	7.2	14.7	21.9
Rib–MNP	None	16.5	10.6	14.8	25.4
	β-Cd	16.9	10.1	14.9	25.0
		19.0	7.9	14.3	22.2
Rib*–MNP	None	16.5	10.6	14.6	25.2
	β-Cd	17.0	10.3	14.9	25.2
		19.0	7.9	14.3	22.2

[a] Error is ±0.1 G for A_N and A_H. [b] A_N = 17.2 (H_2O) and A_N = 16.6 (β-Cd) in ref. 24. [c] Values from ref. 16.

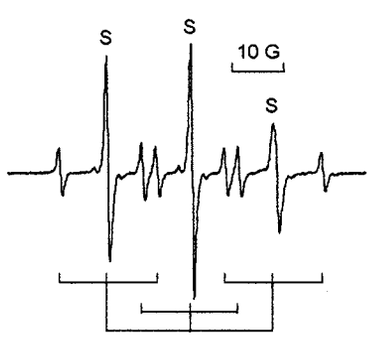
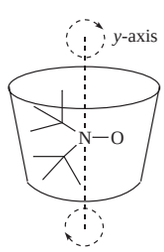


Fig. 4 EPR spectrum of Np–MNP–β-Cd at 293 K



Scheme 2 Proposed inclusion model for DTBA–β-Cd[7]

monomer) are 3×10^{-3}, 2×10^{-2} and 2×10^{-2} M, respectively. The NMR results suggest that the nitroso compound in the dimer state forms 2:1 inclusion complexes with Cds. As discussed elsewhere, NpCbl might form inclusion complexes with Cds, in which the Np group enters into the Cd's cavities.[1–3] Competition for inclusion into the Cd's cavities between NpCbl and the nitroso compound could result in the concentration of free MNP being somewhat high, which results in the concentration of the DTBA radical being also somewhat high on irradiation. Therefore, in the EPR spectra (see Fig. 4), the relatively strong peaks of DTBA are shown, even though they are overlapped by those of Np–MNP (1:2:1 splitting). In the EPR spectrum for α-Cd there are no detectable signals for

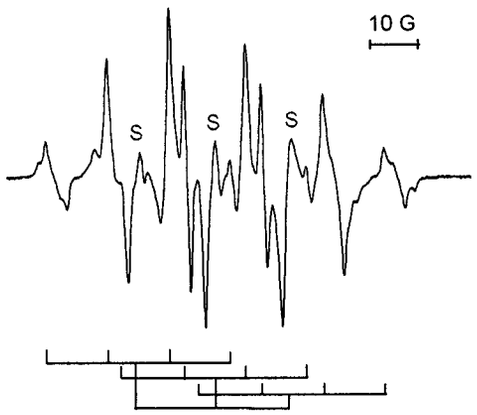
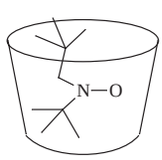


Fig. 5 EPR spectrum of Me–MNP–β-Cd at 293 K

Np–MNP, which is probably due to the low concentration of the active monomer of MNP.

It has been found that EPR spectral parameters are sensitive to changes of environment.[7,8] The N hfs can detect the polarity around the aminoxyl group. In apolar media the N hfs becomes smaller and in polar media the N hfs becomes larger. The magnitude of the β-H splitting constant will depend on the time-average values of $\cos^2 \theta$, where θ is the dihedral angle between a β-C–H bond and the unpaired electron orbital, according to the well-known Heller–McConnell relationship.[25]

Comparison of the values of N and H hfs of Np–MNP radicals obtained from irradiation of solutions containing either β-Cd or γ-Cd with those of free Np–MNP radicals reveals that the aminoxyl radical is included into β-Cd and γ-Cd cavities, respectively. If it is assumed that C_α–C_α in the optimum structure given by MM calculations is along the C_n symmetry axis of Cds, except that the distances of some atoms of methyl at neopentyl from the axis are between 2.5 and 3.2 Å, the others are less than 2.2 Å and the height is 8.0 Å. The results also suggest inclusion of Np–MNP into the Cd's cavities (see Scheme 3).



Scheme 3 Proposed inclusion model for Np–MNP–Cds (β- and γ-)

Photolysis of MeCbl

Photolysis of MeCbl generates the methyl radical, a small and very short-lived radical with a highly localized unpaired electron, which was suggested to be more easily trapped than larger and more stable species with the spin density distributed over the molecule.[15] EPR signals can be detected in solution for nitroso–α-Cd, –β-Cd and –γ-Cd. The signal intensity in β-Cd solution is much stronger than those in the others, which is due to the concentration of active monomer of the nitroso compound as mentioned above. Weak signals of DTBA and free methyl–MNP appear in the spectra. Some peaks overlapped (see Fig. 5).

We have compared the EPR parameters of free radical and included species. It was revealed that: (a) N and β-H hfs values in α-Cd solution are similar to those in the absence of Cd, suggesting that the methyl–MNP is not suitable to fit into the α-Cd cavity; (b) N hfs, and β-H hfs in β-Cd and γ-Cd solution, are smaller than those of free species, suggesting that the aminoxyl radicals are included into the cavities. The ratios of inclusion complex to free species for β-Cd are larger than those for γ-Cd. This was probably due to the cavity diameter of γ-Cd being larger, resulting in the hydrophobic interaction between

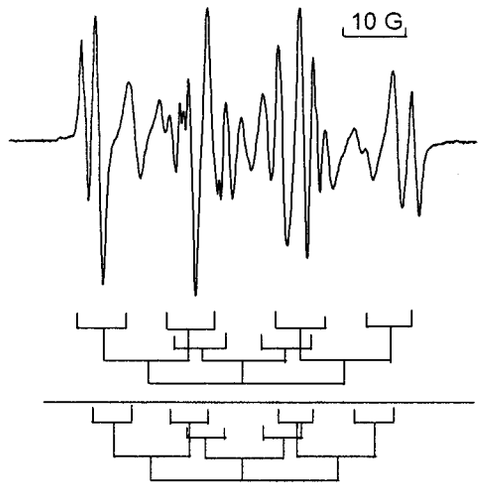
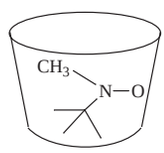


Fig. 6 EPR spectrum of Ado–MNP–β-Cd at 293 K

aminoxyl and γ-Cd being weaker. As described above, the distances of atoms in methyl–MNP from the axis along C_α–C_α are less than 2.2 Å and the height of the molecule is about 5.6 Å in the structure given by MM calculations. The results further suggest that methyl–MNP can be included into β- and γ-Cd cavities fully (see Scheme 4).



Scheme 4 Proposed inclusion model for methyl–MNP–Cds (β- and γ-)

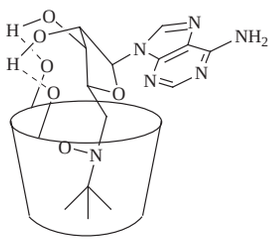
Photolysis of AdoCbl and 2′dAdoCbl

EPR studies show that (a) only in the β-Cd solution are there detectable signals of aminoxyl radicals for both AdoCbl and 2′dAdoCbl, due to the low concentration of active monomer in α-Cd and γ-Cd solution. (b) There are two different sets of 12 lines in the spectrum of Ado–MNP–β-Cd solution (see Fig. 6). After careful analysis, two sets of A_N and A_H values corresponding to both free and included Ado–MNP are obtained and shown in Table 1. (c) The EPR peak intensity in photolysis of 2′dAdoCbl in β-Cd is much weaker than that in the photolysis of AdoCbl. Complete assignment of the spectrum for 2′dAdo aminoxyl is difficult, Table 1 only presents the A_N and A_H values for the included radical. (d) The N hfs, the sum of β-H1 hfs and β-H2 hfs in β-Cd solution both for coenzyme B_{12} and its analog, are smaller than those for the corresponding free radicals, in the absence of β-Cd. Obviously, Ado is too large to fit into the Cd cavity, while in accordance with the bimodal inclusion of aminoxyls, we propose that the *tert*-butyl-in inclusion model is appropriate for Ado and 2′dAdo aminoxyl.[8] Upon addition of β-Cd the N hfs values for Ado aminoxyl are smaller than those for 2′dAdo aminoxyl. It is probably attributed to the Ado–MNP being included deeper into the β-Cd cavity than the 2′dAdo–MNP.

It is interesting to note that the difference between 2′dAdoCbl and AdoCbl is that the oxygen atom in the hydroxy group at the 2′-position of the deoxyadenosyl moiety is absent. Investigations revealed that the analog is very similar both electronically and structurally to the natural cofactor.[26] Therefore, the concentrations of adenosyl radical on irradiation of AdoCbl and 2′dAdoCbl may be similar. The obvious difference in the EPR intensity might be due to the rate of the trapping. We suggest that the 2′-hydroxy group of the adenosyl is involved in a hydrogen-bonding interaction with the hydroxy group at the wider opening of β-Cd, which contributes to the

higher rate of trapping involving Ado, and further, deeper inclusion into the cavity for Ado aminoxyl.

Previous studies of the crystal structure of AdoCbl[27] revealed that the deoxyadenosine glycosidic bond is in the usual β configuration, which points both hydroxy groups away from the base and allows a torsion angle of −68.9°. The adenine and ribose planes make angles of 20.5 and 72.5°, respectively, with the plane of the four corrin nitrogen atoms. Therefore, based on the crystal structure and EPR parameters, we propose the inclusion model shown in Scheme 5.



Scheme 5 Proposed inclusion model for Ado–MNP–β-Cd

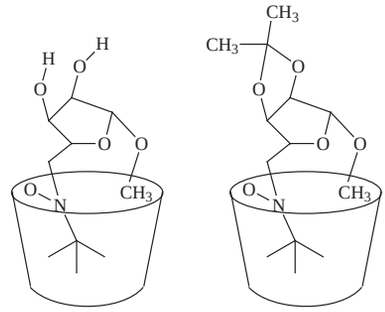
Recently, it has been reported that the activity of enzymes in the presence of the analog is much lower than that in the presence of the natural cofactor (only ~1–2%). Calafat *et al.* supposed that the 2′-hydroxy group is involved in a hydrogen-bonding interaction at the active site that contributes to the steric enzyme-induced distortion of AdoCbl proposed to be important for labilizing the Co–C bond, and recombination of radical pairs after homolysis.[26] Therefore, the present radical trap system can be taken as a model for the hydrogen-bonding interaction of the natural cofactor in the enzyme-binding form.

Photolysis of RibCbl and Rib*Cbl

As in the photolysis of AdoCbl, the EPR signals (more than 20 peaks or shoulders, see Supplementary Material†) appear only in β-Cd solution and the complete assignment indicates that two species, *i.e.* free and included adducts, occur in solution; their EPR parameters are also listed in Table 1. However, N hfs, β-H1 hfs, β-H2 hfs and even peak intensity for Rib–MNP are close to those for Rib*–MNP. Furthermore, the N hfs are somewhat higher than those for the corresponding free species; β-H1 hfs are smaller, while β-H2 hfs are larger.

The structural difference between Rib and Rib* lies in the absence or presence of the 2′,3′-isopropylidene group at ribofuranose. There is no possibility of hydrogen bonding between β-Cd and Rib*–MNP. The results perhaps reveal that no hydrogen bonding exists between β-Cd and Rib–MNP either. Although Rib only differs from Ado in that the bulky adenine of Ado is replaced by a methoxy group, the inclusion model for Rib–MNP (see Scheme 6) is different from that for Ado–MNP. Inspection of a space-filling molecular model suggests that if it were not for the *tert*-butyl group part being included into the β-Cd cavity, the methoxy group might also be included. It induced an altered conformation of the ribofuranose ring, in which the hydroxy groups at the 2′- and 3′-positions of ribofuranose stand vertically relative to the wide opening of β-Cd, and no hydrogen-bonding interaction can be formed between the β-Cd and ribofuranose aminoxyls. Based on the EPR parameters, the inclusion model for Rib*–MNP is similar to that for Rib–MNP. Since the β-H hfs function as a detector of the distortion of the aminoxyl upon inclusion while the hfs of nitrogen acts as a detector of the polarity of the environment about the aminoxyl, we propose that (i) in the case of Rib–MNP and Rib*–MNP, the NO group may be located on

† Available as supplementary data (SUPPL. NO. 57384, 2 pp.) from the British Library. For details of the Supplementary Publications Scheme, see 'Instructions for Authors', *J. Chem. Soc.*, *Perkin Trans. 2*, available *via* the RSC Web page (http://www.rsc.org/authors).



Scheme 6 Proposed inclusion model for Rib–MNP–β-Cd and Rib*–MNP–β-Cd

the rim of the β-Cd cavity, a more polar environment than in water; (ii) the distortion of ribofuranose contributes to a decrease in one β-H hfs and an increase in another. In short, the inclusion model, different from that for adenosyl aminoxyl–β-Cd, may facilitate elucidation of why adenine is necessary in B_{12}-dependent enzyme reactions,[17] *i.e.* the absence of adenine might result in an altered conformation of the ribofuranose ring of the enzyme-bond cofactor, with a consequent impact on the Co–C bond. The impossibility of the homolysis of Co–C, which is the first step in the enzymatic reactions under ambient conditions means that the catalytic cycle cannot occur.

Conclusions

(a) Cds, especially β-Cd, can catalyze the decomposition of MNP from its dimeric state into monomer in aqueous solution. The formed MNP–Cds inclusion complexes are more water soluble and light stable. (b) MNP–Cds complexes are useful radical traps. It suggests an interesting way to investigate the interaction between Cds and short-lived radicals by the EPR technique. (c) In this study, we focused our attention on radicals produced by the photolysis of coenzyme B_{12} and alkylcobalamins. The EPR parameters (N and H hfs) suggest that the corresponding aminoxyls are included into Cd cavities. (d) As discussed, the interactions between Cds and the corresponding aminoxyl radicals might mimic the interaction between coenzyme B_{12} and proteins in natural enzymatic reactions.

Acknowledgements

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