Dioldehydratase Binds Coenzyme B_{12} in the "Base-On" Mode: ESR Investigations on Cob(II)alamin**

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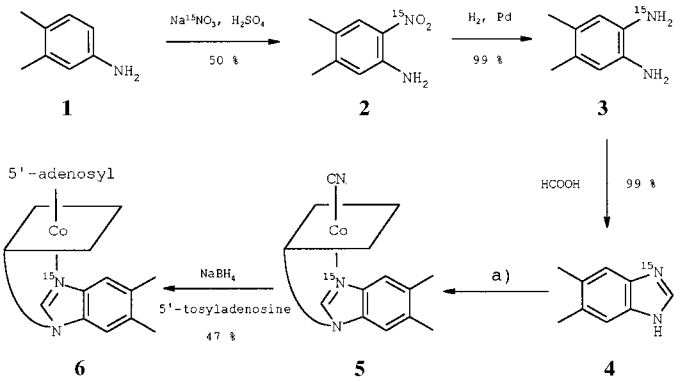
For several coenzyme B_{12}-dependent enzymes it has been shown that in the enzyme–coenzyme complex the axial dimethylbenzimidazole ligand of cobalt is replaced by the imidazole of a histidine residue in the polypeptide chain.[1] Two X-ray structure analyses[2, 3] as well as ESR spectra of the complexes with ^{15}N-labeled apoenzymes and unlabeled coenzyme led to this unexpected finding.[4, 5]

The ESR spectra of Cob(II)alamin is distinguished by an octet of triplets. The hyperfine splitting responsible for the octet arises from the interaction of the unpaired electron with the ^{59}Co nucleus [$I(^{59}Co) = 7/2$)].[6, 7] Interaction of the axial (but not the equatorial) nitrogen ligands with the unpaired electron, which resides mainly in the d_{z^2} orbital, leads to superhyperfine splitting. This results in a triplet (having lines with an intensity ratio of 1:1:1) in the case of ^{14}N, and in a doublet in the case of the heavy isotope ^{15}N [$I(^{15}N) = 1/2$).

More recent studies have indicated that the binding mode is not the same for all coenzyme B_{12}-dependent enzymes, but that there are "base-off" and "base-on" classes.[8, 9] In the latter, the dimethylbenzimidazole ligand is proposed to remain coordinated to the cobalt ion in the enzyme–coenzyme complex. To provide direct proof for the "base-on" binding mode for the dioldehydratase from *Salmonella typhimurium* we synthesized a sample of coenzyme B_{12} in which one of the nitrogen atoms of the dimethylbenzimidazole ligand is labeled with ^{15}N (Scheme 1).

3,4-Dimethylaniline (**1**) was nitrated with $Na^{15}NO_3$ in concentrated sulfuric acid, and the resulting 4,5-dimethyl-[2-^{15}N]nitroaniline (**2**) was catalytically reduced to [1-^{15}N]1,2-diamino-4,5-dimethylbenzene (**3**). Reaction of **3** with concentrated formic acid afforded [^{15}N]5,6-dimethylbenzimidazole (**4**), which was isolated in crystalline (m.p. 200°C) form. Compound **4** was biosynthetically incorporated into vitamin B_{12} (**5**) by using propionibacteria. The ^1H NMR spectrum of **5** unambiguously showed a geminal $^1H-^{15}N$ coupling. Reduction of **5** with $NaBH_4$ and subsequent reaction with 5′-tosyladenosine[11, 12]



Scheme 1. Synthesis of coenzyme B_{12} in which one the the nitrogen atoms of the dimethylbenzimidazole ligand is ^{15}N labeled. a) Propionibacteria.

afforded coenzyme B_{12} **6** labeled with one ^{15}N isotope in its dimethylbenzimidazole ligand.

The gene coding for dioldehydratase in *Salmonella typhimurium*[13] was subcloned in the expression vector pT7.7[14] and overexpressed in *E. coli*. When the cells were broken down by sonification, the enzyme remained in the insoluble cell debris, which was separated from the soluble proteins by centrifugation. Extraction of the membrane-bound enzyme with sodium cholate (46 mM) provided soluble dioldehydratase, which was subsequently purified by gel filtration (Pharmacia, HiLoad 26/60, Superdex 200 prep grade) in the presence of 125 mM 1,2-propanediol and 23 mM sodium cholate. The enzyme was electrophoretically homogeneous and had a specific activity of 95 U mg^{-1}.

For measuring the ESR spectra[16] the enzyme solution was concentrated to 60 mg mL^{-1} (250 μM), and 250 μL thereof was incubated with 60 nmol coenzyme B_{12} for 30 min at 37°C in the dark. Subsequent gel filtration on a Sephadex-G-25 column removed the possible excess free coenzyme. The high resolution of the axial spectrum at low field (2700–3000 G) provides additional evidence for the binding of the coenzyme to the enzyme. After addition of 60 nmol glycolaldehyde the samples were transfered into ESR vials, incubated for 24 h at room temperature without protection from light. The exposure to daylight led to homolysis of the cobalt–carbon bond and formation of cob(II)alamin and an organic radical. As a control, the free coenzyme was also submitted to homolysis in a potassium phosphate buffer (50 mM, pH 6.8) containing 10% glycerol and 5% 1,2-propanediol.

Figure 1 shows the ESR spectrum of free, unlabeled cob(II)alamin and the expanded region around 3400 G. In contrast, the spectrum of cob(II)alamin with (1-^{15}N)dimethylbenzimidazole as ligand has a doublet instead of the triplet in the same region (Figure 2). Although based on the labeling pattern half of the coordinating ligands should bear a ^{14}N atom in the first ligand sphere of the cobalt, the resulting triplet is apparently buried. Simulation of the ESR spectrum in the same region (3400 G, Figure 3) shows that at a linewidth of 8 G no superhyperfine splitting due to ^{14}N is discernible. The experimental spectrum shows the sum of the superhyperfine splittings arising from the coordination of both nitrogen atoms to the cobalt.

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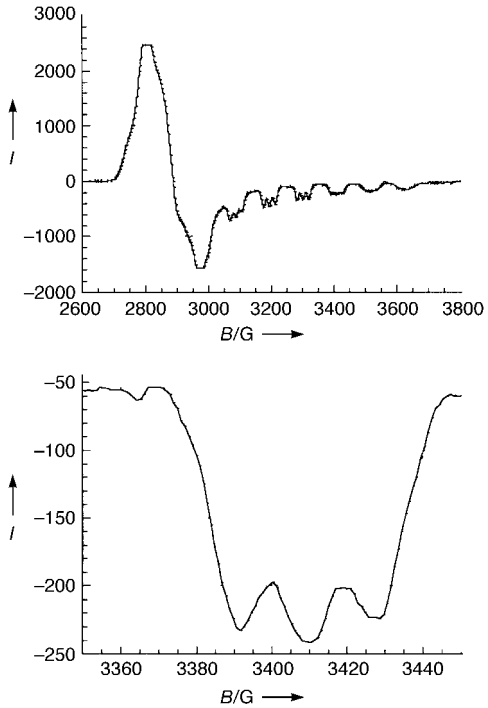

Figure 1. ESR spectrum of the free, unlabeled cob(II)alamin (top) and expansion of the triplet at 3400 G (bottom). Intensity I in arbitrary units.

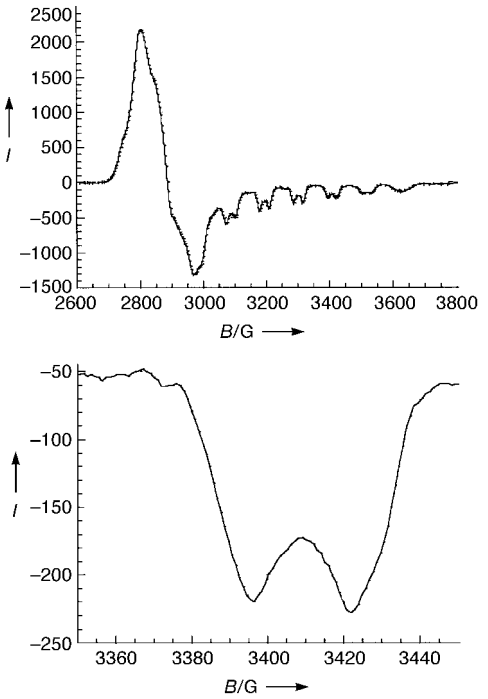

Figure 2. ESR spectrum of the free cob(II)alamin labeled with ^{15}N in the dimethylbenzimidazole ligand (top) and expansion of the doublet at 3400 G (bottom). Intensity I in arbitrary units.

The ESR spectra of the dioldehydratase B_{12} inhibitor complexes are similar but not identical (Figure 4). The dominant signal of the inhibitor radical at 3300 G ($g = 2$) obscures the superhyperfine splitting at lower fields. However, the multiplet structure of the superhyperfine splittings can be readily seen at higher fields. The region at 3400 G

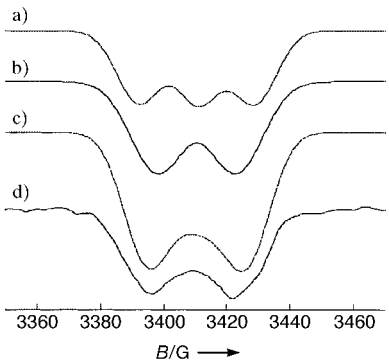

Figure 3. Section of the simulated ESR spectra (3350 – 3470 G) for the free (not enzyme-bound) cob(II)alamin in solution. a) The spectrum expected for coordination of the cobalt with ^{14}N and b) that with ^{15}N coordination. c) Sum of spectra a) and b). d) The experimental spectrum. Intensity I in arbitrary units.

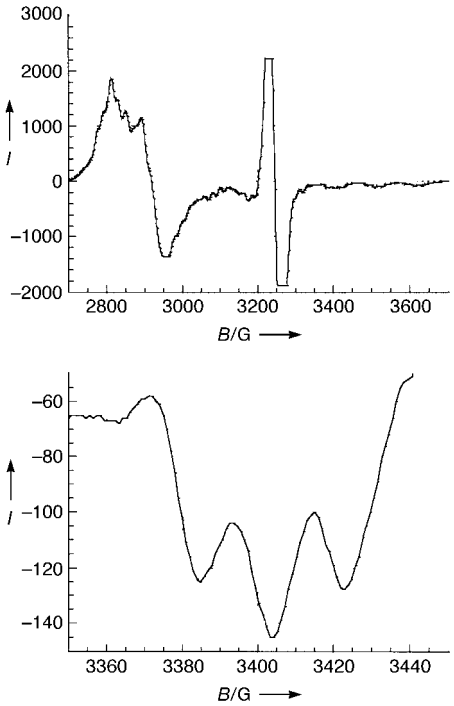

Figure 4. ESR spectrum of the unlabeled cob(II)alamin bound to dioldehydratase (top) and expansion of the triplet at 3400 G (bottom). Intensity I in arbitrary units.

contains a triplet for the enzyme complex with unlabeled cob(II)alamin (Figure 4), whereas a doublet is seen in the spectrum of the complex with [^{15}N]cob(II)alamin (Figure 5). It is noteworthy that the coupling constant A_N in free cob(II)-alamin is smaller than that in the enzyme-bound coenzyme. In the spectra of free cob(II)alamin we found $A_N = 18$ G for the unlabeled (^{14}N) and 26 G for the ^{15}N-labeled coenzyme. The ratio of the coupling constants $A_{^{15}N}/A_{^{14}N}$ compares favorably with the quotient of the theoretically expected gyromagnetic nuclear ratio of 1.44. The coupling constant for the cobalt is 105 G.

In the spectrum of the enzyme-bound cob(II)alamin A_N is 20 G for the unlabeled and 31 G for the ^{15}N-labeled coenzyme. Hence the ratio of the coupling constants is calculated to 1.5. Since the experimentally determined coupling constant

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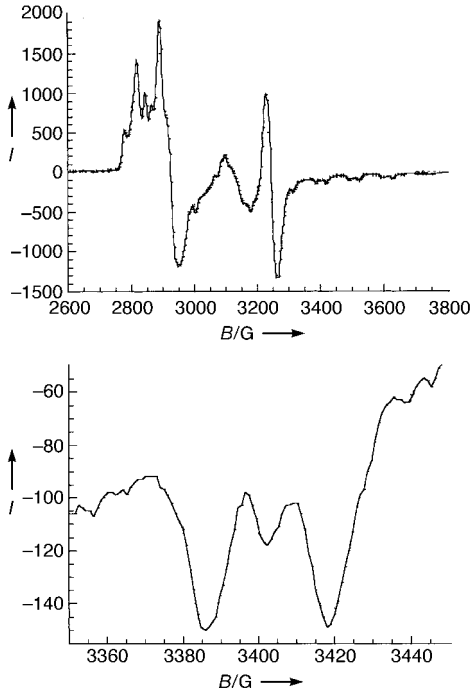

Figure 5. ESR spectrum of the ^{15}N-labeled cob(II)alamin bound to dioldehydratase (top) and expansion of the doublets with the partially obscured triplet at 3400 G (bottom). Intensity *I* in arbitrary units.

arises from the sum of the overlapping spectra, it is larger than that expected for the unlabeled (^{14}N) coenzyme. This conclusion is confirmed by the simulated spectra (Figure 6a, b).

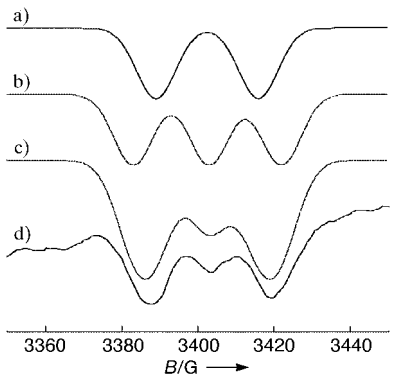

Figure 6. Section of the simulated ESR spectra (3350 – 3450 G) for the enzyme-bound cob(II)alamin in solution. a) The spectrum expected for coordination of cobalt with ^{15}N and b) that with ^{14}N coordination. c) Sum of spectra a) and b). d) The experimental spectrum. Intensity *I* in arbitrary units.

For these we assumed coupling constants of 20 G for ^{14}N and 28 G for ^{15}N (the gyromagnetic nuclear ratio is thus 1.4, the assumed linewidth 6 G). The simulated spectrum (Figure 6c) in the region around 3400 G is consistent with the experimental spectrum. The sum of the simulated individual spectra results in a spectrum similar to the experimental one.

We chose glycolaldehyde as a suicide inhibitor because it had been reported to cause immediate homolysis of the cobalt – carbon bond in the dioldehydratase-bound coenzyme B_{12}.[17] The initially formed 5′-deoxyadenosyl radical most likely abstracts a hydrogen atom from glycolaldehyde, leading to a stable enzyme-bound inhibitor radical. Characterization of this radical is in progress. The results presented here provide unambiguous evidence that dioldehydratase binds coenzyme B_{12} in the "base-on" mode.

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