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# DETECTION BY ELECTRON SPIN RESONANCE OF AN EXCHANGE-COUPLED COB(II)ALAMIN...FREE RADICAL PAIR SPECIES GENERATED BY ANAEROBIC PHOTOLYSIS OF POLYCRYSTALLINE ADENOSYLCOBALAMIN

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## Summary

Laser induced photolysis of highly purified polycrystalline adenosylcobalamin has been studied under anaerobic conditions. Electron spin resonance of photolyzed samples of adenosylcobalamin were studied versus a convenient new variable: the degree of hydration of the crystalline state. Photolysis of partially dehydrated samples of adenosylcobalamin leads to a reproducible, new type of electron spin resonance signal which is interpreted as an exchange coupled Co(II)...free radical species. We conclude that this new species may be the Cob(II)alamin...5'-deoxyadenosyl radical pair.

## Introduction

Homolytic cleavage of the Co-C bond is generally accepted as an initial step in enzymatic reactions which require adenosylcobalamin as a cofactor (1, 2,3). Several research workers have reported their ESR findings on homolysis of the Co-C bond of AdoCbl in enzyme-coenzyme systems (4,5,6,7). Due, however, to the lack of X-ray structural data on any such enzyme-coenzyme system, the structural and electronic factors responsible for the primary events of the homolytic process are still unknown. Since excellent crystal structure information is available for AdoCbl (8), the photochemical study of poly-

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Abbreviations used in text:
ESR=electron spin resonance
NMR=nuclear magnetic resonance
AdoCbl=adenosylcobalamin
DMBZ=dimethylbenzimidazole
BAE=dianion of N,N'-ethylenebis(acetylacetoneimine).

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crystalline AdoCbl appears to be a promising model system for developing an understanding of the primary chemical and physical steps of the homolysis of the AdoCbl cofactor. We report here our ESR results on the anaerobic photolysis of polycrystalline AdoCbl. The experimental methods for irradiation of polycrystalline samples under anaerobic conditions were developed in an earlier study of the photolysis of the model compound $C_6H_5CoBAE$ (9), in which it was determined that detection of a Co(II)...free radical complex depends critically on the complete removal of traces of $O_2$. The success of that study was the motivating factor in the design of the experiments reported here.

## Materials and Methods

Samples of AdoCbl purchased from Sigma Chemical Co. were found to contain paramagnetic impurities such as transition metal ions, some photolytic products and probably other cobalamins in trace amounts. We have purified the samples of AdoCbl used in these studies by employing two chromatographic techniques successively: Gel filtration using a Sephadex G-25 column, and ion-exchange using a Chelex-100 resin column. Details of the purification procedure will be published at a later date. Purified AdoCbl was recrystallized from aqueous acetone according to a published procedure (10). All chemicals used in these studies were analytical grade and the solvents were predistilled. The Chelex-100 resin (200-400 mesh, Na form) was purchased from Bio-Rad Laboratories. The Sephadex G-25 (20-80 μ) was a Sigma Chemical Co. product. Distilled, deionized water was used in all experiments. All operations were carried out in subdued light or (whenever possible) in total darkness.

The samples of pure AdoCbl were prepared in conventional ESR tubes, to which was attached a quartz/pyrex graded seal so that the tube could be pulled off the vacuum line (with a torch) while still under vacuum, and with the lower (sample) portion immersed in liquid nitrogen. In a typical experiment (which was repeated many times, to assure reproducibility) one sample was freeze(77K)-pumped-thawed (room temperature) on the vacuum line several times in order to remove $O_2$ but retain water of crystallization. The other sample was evacuated for 3 hours at room temperature. The laser source used for irradiating the samples was a Spectra-Physics Model 125 CW He-Ne gas laser ($\lambda$ = 632.8 nm). The samples were irradiated for 15 minutes (or more) at room temperature. ESR spectra were recorded on a Varian V-4500 ESR spectrometer. The advantages of using a laser for sample irradiation are: precisely known wavelength, high efficiency of power transfer from source to target, and ease of determination of radiation power.

## Results and Discussion

Chromatographically purified and recrystallized AdoCbl gave a flat ESR baseline at room temperature, as expected (11). The anaerobic photolysis of hydrated samples of AdoCbl and a series of partially dehydrated samples was

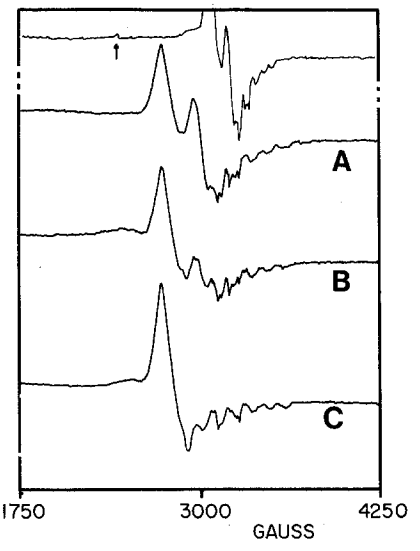


Figure 1. Effect of hydration on the ESR spectrum of photolyzed anaerobic polycrystalline AdoCbl. All ESR spectra were recorded at 50 mw microwave power and 77K. Laser irradiation (632.8 nm) power was 10 mw of intercepted power, and irradiation time was carefully measured with a digital watch.
A. 50 mg of hydrated AdoCbl was placed in a quartz sample tube, and evacuated to $10^{-6}$ torr on a vacuum line for 3 hours. Irradiation time 15 minutes. Field modulation=1000 (13 gauss peak-to-peak modulation amplitude), gain=2000 (arbitrary units), microwave frequency 9.1225 gHz. The sample temperature during photolysis was ambient (external air conditioning ca. 24°C) temperature, and the sample temperature rise, due to laser irradiation, was determined to be less than 1°C.
B. Hydrated sample which was freeze-thawed (77K, room temp.) three times on the vacuum line, and pulled off under vacuum with sample maintained at 77K. Irradiation time (room temperature) 15 minutes. Field modulation=1000, gain=2000. Microwave frequency same as in A.
C. Sample similar to B, except that the irradiation time was one hour. Field modulation=500, gain=2000. Same microwave frequency. The insert at the top of this figure is a tracing of sample A, on a 5000 gauss range, which displays the half-field line indicated by the arrow. Detection of this line is crucial for establishing the spin greater-than-1/2 character of the new ESR spectrum.

monitored by ESR. Some typical resulting ESR spectra, recorded at 77K, are shown in Fig. 1. Experimental conditions are given in the figure caption. A partially dehydrated sample of AdoCbl (Figure 1A) exhibits a new broad ESR signal which is barely detectable in the ESR spectrum of the hydrated sample of AdoCbl (Figure 1B). A half-field line was also detected, (see the uppermost tracing in Figure 1), the intensity of which appears to correlate with the presence of the new ESR signal. The g-value of the new band was determined to be $2.119 \pm .005$. The new band is also clearly independent of the ordinary

axial cob(II)alamin band since under irradiation at 632.8 nm it grows at a slower rate than the axial cob(II)alamin signal. (Compare Figs. 1B and 1C). It is reasonably stable at room temperature, but vanishes rapidly on heating the sample at 50-60°C for 15 minutes.

This newly observed ESR band can be attributed to an exchange-coupled cob(II)alamin...free radical pair. This interpretation rests on three observations (17): the line shape of the new band is symmetrical; the isotropic g-value of this band falls within the general range expected for an exchange coupled free radical and an axial cob(II)alamin (assuming a free radical g = 2.0023, and typical Co(II) g-values from accurate line-shape fits to enzyme bound cob(II)alamin ESR spectra (12)); and the observation of an associated half-field line. This interpretation is also in accord with our lineshape calculations concerning the effect on the ESR spectrum of decreasing the distance between an exchange and dipole-dipole coupled pair of openshell molecules. As we observe considerably more of this new exchange-coupled state mainly in partially dehydrated crystals, rather than in fully hydrated or dehydrated samples, the stabilization of the new intermediate state seems to be related to the removal of water of crystallization about the Co-C bond, and, in particular, from the vicinity of the 5'-methylene group of the nucleoside.

This hypothesis is supported by the three dimensional molecular structure of crystalline coenzyme $B_{12}$ (8,13). The unit cell contains 4 molecules of AdoCbl and about 68 molecules of water. Of the 17 water molecules per AdoCbl, about 13 unique sites (but 40 in total) were determined in Lenhert's work. Thus, a fair number of water molecules per unit cell (ca. 28 molecules) are unlocatable by X-ray structure analysis and are presumably mobile at room temperature. The water molecules mainly fill in the voids between neighboring AdoCbl molecules. The major water containing region of each unit cell has the shape of a twisted cavity extending along the c-axis into which project two 5,6-dimethylbenzimidazole and two 5'-deoxyadenosyl moieties of 4 neighboring

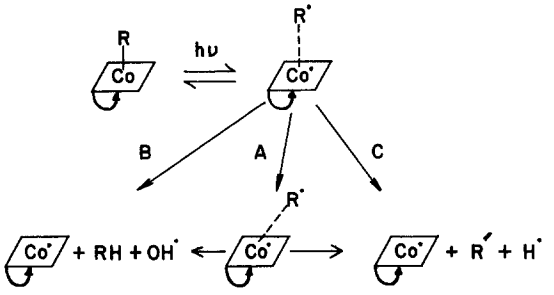


Figure 2. Scheme showing major reaction pathways for anaerobic photolysis of adenosylcobalamin for the three states of hydration, corresponding to the three different types of experimental ESR results. RH = 5'-deoxyadenosine, R'=5'-deoxy-8,5'-cycloadenosine, B = hydrated state, A = partially dehydrated state, C = "dehydrated state".

molecules. An occupied portion of this region lies near the 5'-deoxyadenosyl moiety, and the structure indicates that some of these water molecules have access to the region about the 5'-methylene group of the nucleoside. In addition, hydrogen bonding gives structural rigidity to the bonded 5'-deoxyadenosyl moiety. Using this information and knowledge of the chemical reactivity of the 5'-deoxyadenosyl radical, we propose the photochemical reaction pathways of Figure 2.

According to our proposal: in the hydrated sample of AdoCbl, the 5'-deoxyadenosyl radical (formed as a result of the photolytically induced "incipient homolysis") readily abstracts a hydrogen atom from a neighboring $H_2O$ molecule forming 5'-deoxyadenosine and a OH· radical. The latter radical rapidly diffuses away and is annihilated at another photochemical reaction site. Hence, the ESR spectrum for this case shows mainly the spectrum of cob(II)alamin. Stabilization of the cob(II)alamin...free radical pair is brought about by the partial removal of $H_2O$ molecules from the void structure of the crystal, including the vicinity of the 5'-methylene group of the nucleoside. A reasonable surmise is that the free radical is stabilized at a different (adjacent) site in the unit cell (e.g. 5-10A away), with the stabilizing forces being both the hydrophobic "stacking" interaction between the adenine of deoxyadenosyl and one of the neighboring DMBZ molecules in the unit

cell, the hydrogen bonding to nearby AdoCbl molecules and the remaining water molecules. These stabilization forces are proposed to be sufficient to prevent cyclization of the 5'-deoxyadenosyl radical. In the dehydrated state, a sufficient amount of water has been removed so that the deoxyadenosyl radical may now cyclize to 5'-deoxy-8,5'-cycloadenosine with the elimination of a hydrogen atom. Here again, only the cob(II)alamin signal is observed.

Thus, it has been possible to trap an exchange coupled cob(II)alamin... free radical pair in a solid state photochemical reaction, in contrast to previous studies on liquid or frozen solutions of AdoCbl which were only partially successful in detecting such a state (14,15).

There are some interesting similarities in parameter values between this new spectrum and the ESR spectra of "active coenzyme $B_{12}$" (recorded at both 9 GHz and 35 GHz) of Orme-Johnson et al. (7). ESR spectra of the latter system exhibited poorly resolved cobalt hyperfine structure, and Coffman et al. (16) found that the overall lineshapes (at both frequencies) could only be fitted by a model which assumed that the spectrum is a composite due to two independent cob(II)alamin species. First, the average $g_{yy}$ of the two "species" is $\langle g_{yy} \rangle = 2.117$ (compare to $g_{iso} = 2.119$ found here). Secondly, in an (as yet) unpublished experiment (Orme-Johnson, Blakley) the rapid quench ESR spectrum was discovered to have a half-field line, proving it to be exchange coupled. Finally, an explanation of the apparent doubling of the hyperfine lines (hence two "species) was discovered by a numerically accurate lineshape calculation of the interaction between a Co(II) and a free radical including all exchange and spin-spin interactions. In the strong exchange domain (relaxation times permitting) the spectrum exhibits two sets of Co(II) lines! We conclude that the exchange coupled cob(II)alamin...5'-deoxyadenosyl pair species is an interesting and relevant subject for study in the chemistry of coenzyme $B_{12}$.

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## References

1. Lee, H.A. Jr., and Abeles, R.H., (1963), J. Biol. Chem., 238,2367-2373.
2. Wagener, O.W., Lee, H.A. Jr., Frey, P.A. and Abeles, R.H., (1966), J. Biol. Chem., 241, 1751-1762.
3. Babior, B.M., (1975), Acc. Chem. Res., 8, 376-384.
4. Hamilton, J.A., Blakley, R.L., Looney, F.D., and Winfield,M.E., (1969), Biochim. Biophys. Acta, 177, 374-376.
5. Babior, B.M. and Gould, D.C., (1969), Biochem. Biophys. Res. Commun., 34, 441-447.
6. Hamilton, J.A., Yamada, R., Blakley, R.L., Hogenkamp, H.P.C., Looney, F.D., and Winfield, M.E., (1971), Biochemistry, 10, 347-355.
7. Orme-Johnson, W.H., Beinert, H., and Blakley, R.L., (1974), J. Biol. Chem., 249, 2338-2343.
8. Lenhert, P.G., (1968), Proc. Roy. Soc. (London), A303, 45-84.
9. Ghanekar, V.D. and Coffman, R.E. (1980), J. Organometal. Chem., 198, C15-C19.
10. Hogenkamp, H.P.C. and Pailes, W.E., (1967), Biochemical Preparations, 12, 124-128.
11. Hogenkamp, H.P.C., Barker, H.A., and Mason, H.S., (1963), Arch. Biochem. Biophys., 100, 353-359.
12. Pilbrow, J.R. and Winfield, M.E., (1973), Mol. Phys., 25, 1073-1092.
13. Lenhert, P.G. and Hodgkin, D.C., (1961), Nature (london), 192, 937-938.
14. Joblin, K.N., Johnson, A.W., Lappert, M.F., and Nicholson, B.K., (1975), J. Chem. Soc., Chem. Comm., 441-442.
15. Lowe, D.J., Joblin, K.N., and Cardin, D.J., (1978), Biochim. Biophys. Acta., 539, 398-401.
16. Coffman, R.E., Ishikawa, Y., Blakley, R.L., Beinert, H., and Orme-Johnson, W.H., (1976), Biochim. Biophys. Acta., 444, 307-318.
17. Abragam, A., and Bleaney, B., Electron Paramagnetic Resonance of Transition Ions. Clarendon Press, Oxford (1970).