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Temperature Dependent Coordination Effects in Base-Off Adenosyl and Methylcobalamin by X-Ray Edge Spectroscopy

Mark R. Chance
Case Western Reserve University, mark.chance@case.edu

Author(s) ORCID Identifier:

Mark R. Chance

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Michael D. Wirt and Mark R. Chance

Department of Chemistry, Georgetown University, Washington, DC

ABSTRACT

Examination of the role of base-off cobalamin species (where the 5,6-dimethylbenzimidazole ligand coordinated to cobalt is detached by protonation of the imidazole nitrogen) in differentiation between homolytic and heterolytic cobalt-carbon bond cleavage mechanisms is a primary step in better understanding B_{12}-dependent enzyme catalysis. X-ray absorption edge spectroscopy provides the first direct structural evidence of five-coordination in base-off adenosyl- and base-off methylcobalamin complexes at room temperature. Integration of 1s-3d pre-edge transitions of the base-off species reveals the dependence of coordination number on temperature. Gradual increases in 1s-3d transition intensities, as the temperature is increased from 180 K to 298 K, reflect a change in the coordination number from six (where a water molecule is presumed to occupy the coordination site vacated by the 5,6-dimethylbenzimidazole ligand) to primarily five-coordinate. Base-off configurations that strengthen the Co-C bond may be both decreasing the tendency for homolytic cleavage while increasing the tendency for hetrolytic Co(I) B_{12} formation.

INTRODUCTION

5′-Deoxyadenosylcobalamin and methylcobalamin are essential cofactors in B_{12} dependent enzyme catalysis. Extensive studies of base-off forms of cobalamin complexes (where the 5,6-dimethylbenzimidazole (DMB) ligand coordinated to cobalt in the sixth axial position (Fig. 1) is detached by protonation of the imidazole nitrogen) have been conducted for several years [1–9]. A series of detailed studies of five-coordinate organocobalt and cobalamin compounds by

Address reprint requests and correspondence to: Dr. Mark Chance, Department of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.

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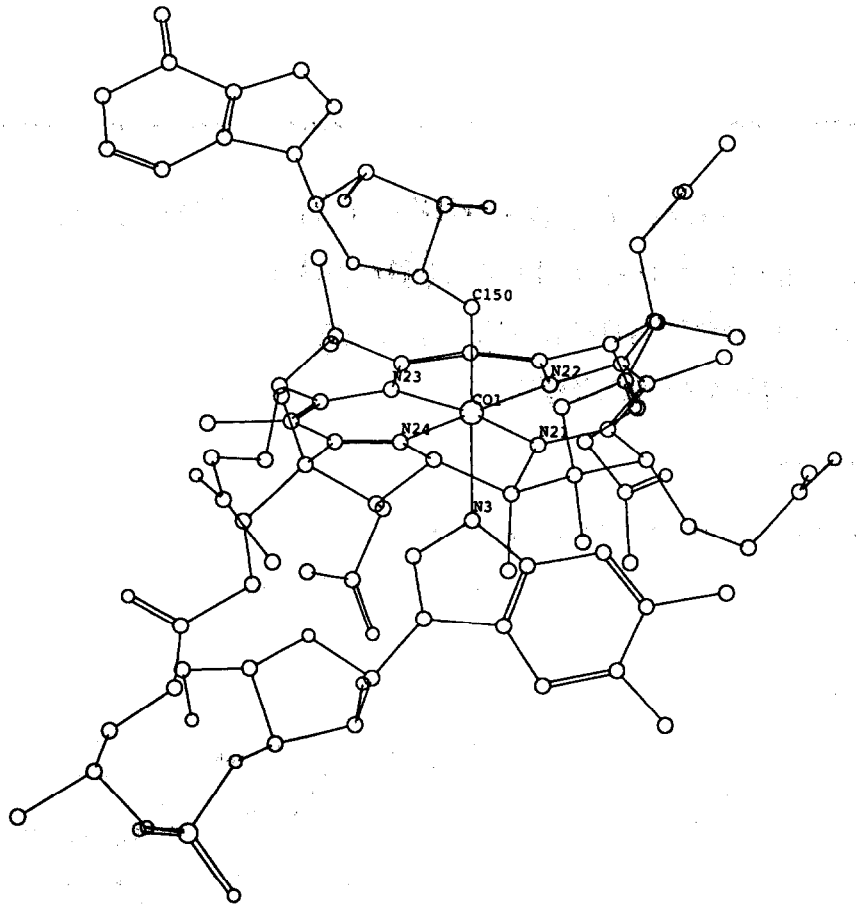

FIGURE 1. Structure of adenosylcobalamin (AdoCbl).

Chemaly and Pratt et al. have provided considerable insight into both pH-dependent and pH-independent equilibria of base-off species [1–3]. These studies have identified a reversible protonation and displacement of the DMB ligand (through optical spectroscopy) as well as a temperature dependent (pH-independent) equilibrium favoring a detached DMB ligand at higher temperatures. However, no direct structural data have been available for two biologically relevant base-off alkyl-cobalamins, base-off 5′-deoxyadenosylcobalamin (AdoCbl) and base-off methylcobalamin (MeCbl), to confirm their coordination number at room temperature. Our previous x-ray edge studies of base-off AdoCbl at 180 K are consistent with a six-coordinate complex where a water is presumed to occupy the sixth-coordination position vacated by the DMB ligand [10]. Recent temperature dependent studies of phosphitocobalamins [6, 7] suggest a shift in the coordination number favoring the five-coordinate base-off conformation as temperature is increased from 15°C to 55°C. In these studies, a water molecule is presumed to occupy the sixth-coordination position in the lower temperature regime as well. Examination of the structure of base-off

AdoCbl and MeCbl at room temperature provides a means for understanding the structural role of the five-coordinate base-off species in the mediation of Co-C bond cleavage mechanisms.

We present structural data, derived from x-ray absorption edge spectroscopy, demonstrating that both base-off AdoCbl and MeCbl species are primarily five-coordinate at room temperature. The relevance of base-off species to the heterolytic cleavage mechanisms of enzyme systems such as methionine synthase [11] and the corrinoid/iron-sulfur protein of *Clostridium thermoaceticum* [12] has recently been identified. Additionally, it is established that the base-off forms of cobalamins are resistant to homolytic thermolysis relative to the base-on species [8, 9]. Marzilli et al., have characterized five-coordinate alkylcobalt compounds of the type Co(saloph)R, where saloph denotes a dianon of disalicylidene-*o*-phenylenediamine [5]. In this study, crystallographic data for two monomeric cobalt saloph species ($R = i\text{-}C_3H_7$, and $R = CH_3$) yield a five-coordinate, distorted square-pyramidal complex where the cobalt atom is displaced by 0.16 Å and 0.11 Å out of the saloph plane toward the alkyl moiety, respectively, with Co-C distances of 2.031(8) Å, $R = i\text{-}C_3H_7$ and 1.957(13) Å, $R = CH_3$. Comparison of five-coordinate saloph species with relevant six-coordinate counterparts (where a pyridine is coordinated in the sixth axial position) shows a decrease in Co-C bond length for the five-coordinate complexes [5], indicating a strengthening of the Co-C bond. Since Co-C bond homolysis is seen to be less facile in both the five-coordinate Co(saloph)R and base-off alkylcobalamin species [5], the absence of a coordinated water molecule in the sixth position of base-off AdoCbl and MeCbl at room temperature favors a heterolytic cleavage mechanism and formation of a four-coordinate Co(I) B_{12} intermediate.

EXPERIMENTAL

Materials

AdoCbl, MeCbl, and cyanocobalamin were obtained from Sigma Chemical Co. and were verified by optical spectroscopy. Glycerol was obtained from Fisher Scientific. All were used without further purification. We tested the glycerol for the presence of cobalt by x-ray absorption prior to sample preparation. No detectable edge jump was observed for the test samples, therefore the cobalt concentration in the glycerol was less than 0.5% of that in the experimental samples and could be ignored.

Sample Preparation

Base-off AdoCbl and MeCbl were prepared by acidification of 12 mM solutions in 500 mM phosphate buffer, with 6 M HCl to a final pH of 2.0 and 1.0, respectively. All samples were characterized by optical absorption spectroscopy before and after data collection to verify sample integrity and were prepared as solutions in 35% glycerol to reduce sample cracking upon freezing. All samples were placed in $25 \times 2.5 \times 2$ mm deep lucite sample holders covered with mylar tape (approximate volume 200 μL). Preparation of model compounds used for 1s-3d intensity comparisons were described previously [10].

Data Collection

Data were collected at the National Synchrotron Light Source, Brookhaven National Laboratory, on beam lines X-9A, X-11A, and X-10C using double flat Si(111) (X-9A, X-11A) and Si(220) (X-10C) crystal monochromators. To insure consistency between data collected on different beamlines, x-ray edge data from X-9A, X-10C, and X-11A were compared and gave 1s-3d intensities with $< 5\%$ variation. Temperature dependent base-off cobalamin data were collected at 298 ± 2, 250 ± 2, and 180 ± 2K. Sample temperature was maintained by flowing cooled nitrogen gas through a low temperature cryostat as described previously [13]. Temperature was monitored using a Lake Shore Cryogenics DRC 82C temperature controller with a silicon diode probe. X-ray edge data having 3 eV resolution were recorded by counting at a specific energy for 3 sec and incrementing the energy by 0.5 eV from 20 eV below the cobalt edge to 100 eV above the edge. A cobalt foil was used as a standard to account for any shifts in the monochromator. K-α cobalt fluorescence was detected with a zinc sulfide coated photomultiplier tube [14] and incident photon scattering was rejected by an iron oxide filter. Output signals were amplified with a Keithley amplifier, converted to frequency, and counted in a scaler interfaced to a computer via CAMAC. For reference signals, mylar tape was mounted at a 45° angle to the x-ray beam to scatter photons counted by a similar photomultiplier tube positioned perpendicular to the x-ray beam. This method provided excellent linearity between the sample and the reference detectors.

Data Analysis and Errors

Data were converted to two column ASCII format and the files were processed using Galactic Industries *Spectra Calc* (1988) software package. First, linear fits were separately applied to the pre-edge and EXAFS regions of the spectra to determine the absorption step-jump for each spectrum. This method has been described in detail by a number of previous investigators [15–17]; and allows samples of different concentrations to be normalized to each other, so that the integrated intensities of pre-edge features can be compared. The step-jump for a particular sample is the numerical difference between the pre-edge and EXAFS region's linear fits. In order to compare spectra under different conditions, each spectrum is normalized to the step-jump of the other spectrum by multiplying one spectrum by the ratio of the two. This process allows for direct comparison of pre-edge intensities of any pair of spectra. Integration of the 1s-3d peak areas was accomplished by fitting the pre-edge region containing the 1s-3d transition with a cubic polynomial to establish a baseline. The resulting baseline corrected peak was then integrated to determine the peak area. Data are presented in ratio form referenced to the area of the cobalt hexamine 1s-3d peak (data collected previously [10] which was arbitrarily given the value of one. The actual area of the Co(III) hexamine 1s-3d transition is 2.0×10^{-2} eV with the step-jump normalized to 1.

Errors in the analysis were estimated from a combination of two factors: errors due to statistical noise and errors introduced due to the method of analysis. Statistical noise was measured in the region prior to the 1s-3d transition. Contributions of the statistical noise to the integrated 1s-3d peak area were determined by calculating the percent contribution of the statistical noise to the

total peak area. This provides a statistical error measure for each and every scan. Statistical error varied from 0.5–10% depending on the transition intensity, sample concentration, beam conditions, and signal averaging. Errors introduced due to the method of data analysis were calculated by comparison of the integrated peak areas of different scans and from duplicate or triplicate samples. These systematic errors were generally $\leq 5\%$. Errors seen in Tables 1 and 2 represent the expected error for this measurement taking into consideration the statistical and systematic errors discussed above.

RESULTS AND DISCUSSION

X-ray absorption spectra of transition metal complexes containing empty 3d orbitals provide a sensitive indicator of coordination geometry about the central metal [16–18]. Although 1s-3d intensities are derived from a somewhat complicated set of structural dependencies (described previously [10, 16–18], coordination number has the most influential effect on intensity due to substantial changes in 3d,4p orbital mixing [17, 18]. 1s-3d transition intensities generally increase with decreasing coordination number [10, 16, 17] (Fig. 2).

We have compared the integrated areas of 1s-3d transitions for the base-off forms of AdoCbl, MeCbl, and selected model compounds of various geometries in Tables 1 and 2. Cobalt hexamine is used as a standard for 1s-3d intensity comparisons because it has an almost perfect octahedral structure with maximum deviations of only 2.1° from ideal octahedral bond angles [19]. 1s-3d transitions in perfect octahedral complexes are dipole-forbidden; consequently, cobalt hexamine has the smallest integrated 1s-3d transition area for the octahedral compounds presented.

Since the corrin ring system is not an ideal octahedron, AdoCbl and MeCbl are used as additional six-coordinate models for a more direct comparison to the two base-off species. Steric contacts between the DMB ligand and the corrin ring in combination with the fusion of tetrapyrrole rings A and D results in a tilt in the corrin ring plane (14.6° tilt angle for AdoCbl and 15.8° for MeCbl) [4, 20]. When cobalt hexamine is compared to the more distorted AdoCbl and MeCbl species, the integrated 1s-3d intensity increases from a value of 1.0 for cobalt hexamine to 14.9 and 12.5 respectively. Two factors primarily contribute to the observed increase in 1s-3d intensity: (1) the presence of a Co-C σ-bond in the AdoCbl and MeCbl species results in a loss of the electronically symmetric (all

TABLE 1. Temperature Dependent Integrated 1s-3d Transition Intensities for Base-Off Adenosyl- and Methylcobalamins and Five-Coordinate Model Compounds

Compound	Temperature (K)	1s-3d Intensity
Base-off adenosylcobalamin	298	20.9 ± 0.6
	250	16.3 ± 0.5
	180	13.4 ± 0.6
Base-off methylcobalamin	298	22.9 ± 0.2
	250	18.8 ± 0.9
	180	15.8 ± 0.8
Co(II) B_{12}	180	8.3 ± 0.6
Co(II) $(DMG)_2$pyridine	180	20.5 ± 0.1

TABLE 2. Integrated 1s-3d Transition Intensities for Cobalamin and Model Compounds

Compound	Coordination Number	1s-3d Intensity	Comments
Co(III) hexamine	6	1.0 ± 0.3	Octahedral, no distortions [19]
Co(III) $(DMG)_2$ methyl-pyridine	6	9.2 ± 0.2	Distorted octahedron, Co-C bond present [25]
Cyanocobalamin	6	5.1 ± 0.1	Distorted octahedron, ca 19° tilt in corrin ring [26, 27]
Adenosylcobalamin	6	14.9 ± 0.6	Distorted octahedron, 14.6° tilt in corrin ring, Co-C bond present [28]
Methylcobalamin	6	12.5 ± 0.8	Distorted octahedron, 15.8° tilt in corrin ring, Co-C bond present [29]
Co(II) B_{12}	5	8.3 ± 0.6	Distorted square-pyramidal, 16.3° tilt in corrin ring [23, 24]
Co(II) $(DMG)_2$ pyridine	5	20.5 ± 0.1	Square-pyramidal [10]

nitrogen) ligand field and increases the degree of 3d-4p orbital mixing [21]; (2) removal of the rigorous octahedral symmetry seen in cobalt hexamine due to the tilt angle in the corrin ring reduces the *g* character of the 1s-3d transition. For example, dicyanocobinamide, which contains cyano groups at both axial positions, has a more electronically similar ligand field to that of cobalt hexamine and a corrin ring tilt angle of only 8.0°. These two factors contribute to the relatively weak 1s-3d intensity of 3.6.

The edge spectra of base-off AdoCbl and MeCbl at 180 K are similar in intensity to that of base-on AdoCbl and MeCbl. It has been speculated that a water molecule may occupy the sixth-coordination position upon removal of the

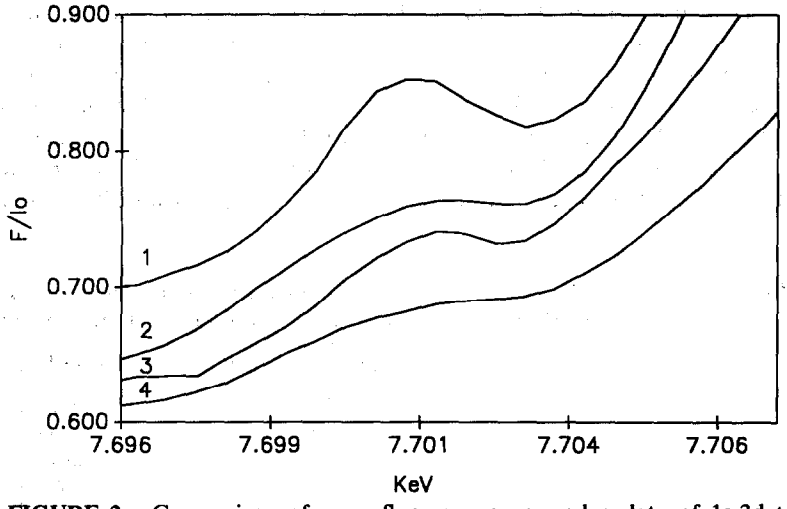

FIGURE 2. Comparison of x-ray fluorescence pre-edge data of 1s-3d transitions observed in model and cobalamin compounds. (1) Co(II) $(DMG)_2(py)$ (five-coordinate); (2) Co(III) $(DMG)_2Me(py)$ (six-coordinate); (3) Co(II) B_{12} (five-coordinate); (4) cyanocobalamin (six-coordinate).

DMB ligand at lower temperatures in several base-off cobalamin species [3, 4, 6, 7]. The minimal difference in 1s-3d intensity between the two base-off species at 180 K and base-on AdoCbl and MeCbl supports a predominately six-coordinate structure (Tables 1 and 2). Substitution of water for the DMB ligand is not expected to greatly influence the degree of 3d,4p mixing.

As the temperature is raised from 180 K to 298 K, an increase in 1s-3d intensity for both base-off AdoCbl and MeCbl is observed (Figs. 3 and 4). At 298 K, the 1s-3d intensity for both base-off AdoCbl and MeCbl species resembles that of a five-coordinate square-pyramidal complex such as bis(dimethylgloximato)pyridinecobalt(II) (cobalt(II) $(DMG)_2(py)$). Cobalt(II) $(DMG)_2(py)$ is known to be five-coordinate based on ESR data [22]. Co(II) B_{12}, formed by the loss of the alkyl ligand located in the fifth axial position upon homolytic Co-C bond cleavage, is also five-coordinate [23, 24] and provides a second comparison to the base-off cobalamins. The presence of a 16.3° tilt angle in the corrin ring [23], distorting the square-pyramidal geometry, reduces the 1s-3d intensity related to the Co(II) $(DMG)_2(py)$ species. Comparison of these intensities to those of the two base-off cobalamin species indicates that in both cases the geometric conformation at room temperature is consistent with a predominately five-coordinate square-pyramidal arrangement. Furthermore, the relatively strong 1s-3d transitions at room temperature for base-off AdoCbl and MeCbl may indicate a relaxation in the tilt angle forming a less distorted square-pyramidal structure when compared to the cobalt(II) B_{12} species.

To test the effect of temperature variation on 1s-3d transitions, we compared the integrated intensity of 1s-3d transitions of cyanocobalamin at 180 and 280 K. These results gave identical 1s-3d intensities (within 5%) for both temperatures. Since cycanocobalamin is six-coordinate at 180 and 280 K, these results indicate that differences in 1s-3d transition intensifies for the base-off AdoCbl and

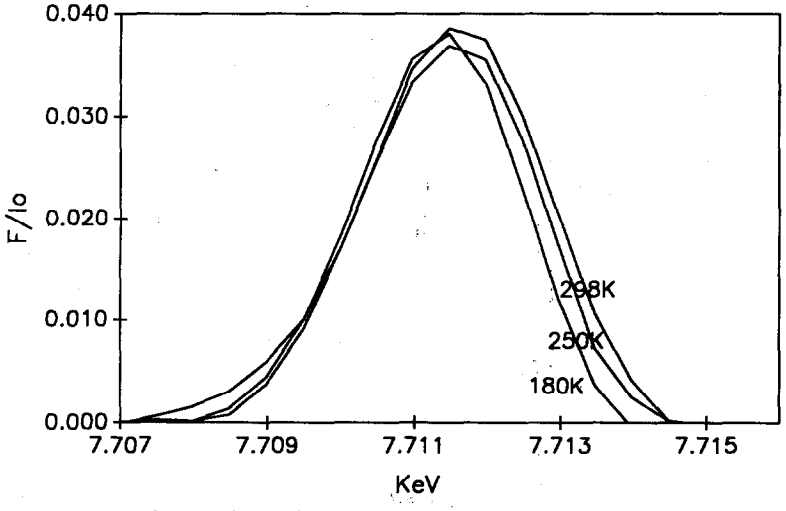

FIGURE 3. Comparison of temperature dependent x-ray fluorescence pre-edge data of 1s-3d transitions for base-off adenosylcobalamin. Data were collected at 180 K, 250 K, and 298 K. Note the decrease in 1s-3d transition areas with decreasing temperature, characteristic of an increase in coordination number.

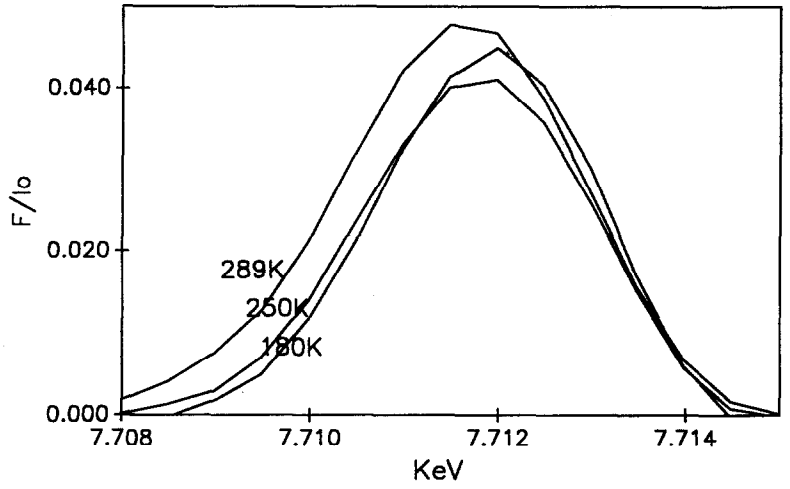

FIGURE 4. Comparison of temperature dependent x-ray fluorescence pre-edge data of 1s-3d transitions for base-off methylcobalamin. Data were collected at 180 K, 250 K, and 298 K. Note the decrease in 1s-3d transition areas with decreasing temperature, characteristic of an increase in coordination number.

MeCbl compounds are due to changes in coordination environment and are not due to changes in vibronic coupling.

CONCLUSIONS

Confirmation of a primarily five-coordinate structure for base-off cobalamin species at room temperature provides insight for differentiating the two established pathways of B_{12} dependent enzyme catalysis. The absence of a coordinated water molecule replacing the DMB ligand predisposes the five-coordinate species toward heterolytic fission to the Co(I) B_{12} intermediate [5]. Though further studies are needed to determine the enzyme's role in facilitation of the base-off species, substantial evidence exists for base-off MeCbl mediated heterolytic pathways for cobalamin-dependent methionine synthase [11] and for the corrinoid/iron-sulfur protein of *Clostridium thermoaceticum* [12]. In the latter study, Ragsdale et al. determined the DMB ligand to be uncoordinated to the cobalt ion in the 2+ and 3+ oxidation states. Temperature dependent Extended x-ray Absorption Fine Structure (EXAFS) studies of the base-off AdoCbl and MeCbl species as well as EXAFS investigations of the *Clostridium thermoaceticum* system will provide direct insight into the effects of the base-off configuration on modulation of the Co-C bond distance.

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