
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Structure of an Intermediate of Coenzyme B₁₂ Catalysis by EXAFS: Cobalt(II) B₁₂

Irit Sagi, Michael D. Wirt, Eefei Chen, Suzanne Frisbie, and Mark R. Chance*

Contribution from the Department of Chemistry, Georgetown University, Washington, D.C. 20057.
Received October 23, 1989

Abstract: The prevalence and importance of B₁₂-dependent enzyme reactions is well-known; however, the structures of the catalytic intermediates are not well-understood. Homolytic cleavage of the cobalt-carbon bond occurs for a number of B₁₂ enzyme reactions, so that the reduced, Co(II), form of B₁₂ is well-established as an important intermediate. Using EXAFS spectroscopy, we have examined the structure of Co(II) B₁₂ in solution prepared by reduction of cyanocobalamin and by photolysis of adenosylcobalamin. The two EXAFS structures are very similar, with the average of the cobalt-nitrogen distances to the corrin ring at 1.88 ± 0.02 Å, and a cobalt-nitrogen (Co-N_d) distance to the dimethylbenzimidazole ligand of 1.99 ± 0.03 Å. In addition, X-ray edge measurements indicate that the Co(II) B₁₂ complex is five coordinate. Our results suggest that when the cobalt-carbon bond breaks, the Co-N_d bond becomes much stronger, while the Co-N equatorial distances show minimal changes.

Introduction

B₁₂-dependent enzyme systems have been actively studied and for a number of them, including diol dehydrase, ethanolamine ammonia-lyase, and ribonucleotide reductase, strong evidence has accumulated that homolytic cleavage of the cobalt-carbon bond occurs and that the Co(II) and radical species produced are kinetically competent.¹⁻⁷ Therefore, the Co(II) species (often referred to as B_{12r}) is a key intermediate in these reactions. Missing from the picture, however, is the direct structural characterization of Co(II) B₁₂, as its crystallization had proved difficult until recently. Krautler and co-workers⁸ have reported the crystal structure of Co(II) B₁₂ and find a number of similarities to the structure of adenosylcobalamin (**1**) and methylcobalamin (**2**). The Co(II) complex is a five-coordinate, low-spin species as expected from EPR measurements.⁹ Of interest, however, are the following: the equatorial nitrogen distances are within experimental error of the corresponding bond lengths for **1** and **2**, while the axial distance to the dimethylbenzimidazole (DMB) base is apparently shorter at 2.13 Å, and the cobalt ion is shifted to 0.12 Å out of the plane of the corrin nitrogens toward the DMB ligand. The ring structure of the Co(II) compound is also quite similar to that of **1** and **2**, especially the upward tilt of the corrin

ligand due to steric repulsion from the DMB base.

In order to obtain solution structural information on the Co(II) B₁₂ complex generated by reduction (from cyanocobalamin, **3**) and by photolysis (from **1**), we have carried out extended X-ray absorption fine structure (EXAFS) studies. Also reported here is the EXAFS structure of adenosylcobalamin that was studied as a control for our analysis. We find that the Co-N_d distance in Co(II) B₁₂ is much shorter than that seen in **1** and **2** and shorter than that reported by Krautler for the crystalline Co(II) B₁₂.

(1) Orme-Johnson, W. H.; Beinert, H.; Blakley, R. L. *J. Biol. Chem.* **1974**, *249*, 2388.

(2) Babior, B. M.; Moss, T. H.; Orme-Johnson, W. H.; Beinert, H. *J. Biol. Chem.* **1974**, *249*, 4537.

(3) Cockle, S. A.; Hill, H. A. O.; Williams, R. J. P.; Davies, S. P.; Foster, M. A. *J. Am. Chem. Soc.* **1972**, *94*, 275.

(4) Chepler, K. L.; Dunham, W. R.; Sands, R. H.; Fee, J. A.; Abeles, R. H. *Biochim. Biophys. Acta* **1975**, *397*, 510.

(5) Valinsky, J. E.; Abeles, R. H.; Mildvan, A. S. *J. Biol. Chem.* **1974**, *249*, 2751.

(6) Halpern, J. *Science* **1985**, *227*, 869.

(7) Finke, R.; Schiraldi, D.; Mayer, B. *Coord. Chem. Rev.* **1984**, *4*, 1.

(8) Krautler, B.; Keller, W.; Kratky, C. *J. Am. Chem. Soc.* **1989**, *111*, 8936.

(9) Bayston, J.; Looney, F.; Pilbrow, J.; Winfield, M. *Biochemistry* **1970**, *9*, 2164.

* Author to whom correspondence should be addressed.

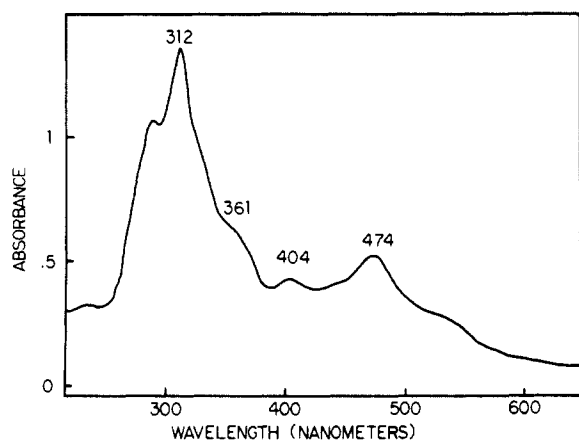


Figure 1. Optical spectrum of Co(II) generated by reduction from cyanocobalamin (methods described in the text). The remaining cyanocobalamin fraction seen in the small peak at 361 nm is <10% of the total.

However, the EXAFS solution structure for adenosylcobalamin is in good agreement with the X-ray structure.¹⁰

Materials and Methods

Materials. Cyanocobalamin, adenosylcobalamin, and sodium borohydride were obtained from Sigma Chemical Co. Aluminum oxide and (5,10,15,20-tetraphenyl-21*H*,23*H*-porphine)cobalt(II) (CoTPP) were purchased from Aldrich Chemical Co. Cobalt(III) hexacyanide was purchased from Alfa Morton Thiokol Inc., and glycerol was obtained from Fisher Scientific. All reagents were used without further purification. Cobalt(III) hexamine was synthesized by standard methods.¹¹ We tested the glycerol, borohydride, and aluminum oxide in solution and solid form for the presence of cobalt by using the X-ray spectrometer. No detectable edge jump was observed for any of the test samples; therefore the cobalt concentration in these samples was less than 0.5% of that in the experimental samples and could be ignored.

Sample Preparation. Cobalt(II) B₁₂ was prepared by reduction of 1.5 mL of 12 mM cyanocobalamin with 0.5 mL of 0.8 M NaBH₄ under argon as described by Dolphin.¹² The cyanocobalamin and NaBH₄ solutions were degassed with argon for 30 min prior to reduction. Photolyzed Co(II) B₁₂ was prepared by degassing a 8 mM solution of adenosylcobalamin for 30 min, and then the sample was exposed to a 65-W Halogen lamp with constant stirring and bubbling of nitrogen for 5 h. All samples were prepared as solutions in 35% glycerol to reduce sample cracking upon freezing. Samples were placed in 25 × 2.5 × 2 mm deep lucite sample holders covered with mylar tape (approximate volume 200 μL) and were transferred into the sample holders under argon and immediately submerged in liquid nitrogen. Sample preparation was repeated several times and characterized by optical absorption spectroscopy. Optical characterization was accomplished by transferring concentrated samples under argon to stoppered cuvettes containing deionized water that were previously degassed for 15 min. Immediately following sample transfer, the cuvette was sealed and an optical spectrum taken. The cobalt(II) B₁₂ spectra were consistent with earlier absorption spectra of Beaven and Johnson¹³ as shown in Figure 1. Percent conversion to cobalt(II) B₁₂ for both preparations was calculated at 93 ± 3%. The presence of cyanocobalamin in the cobalt(II) B₁₂ samples was measured by observing the remaining height of the 361-nm peak. This was accomplished by fitting a cubic polynomial to the baseline of both the pure cyanocobalamin and Co(II) spectra to normalize the peak heights. The absolute peak height was determined, allowing the calculation of the percent cyanocobalamin remaining (5% by this method). A second method for analyzing Figure 1 involves fitting the region from 250 to 390 nm by a sum of Gaussian bands. There were four peaks in the region, two due to Co(II) (at 288 and 312 nm) and two due to cyanocobalamin (at 322 and 361 nm). Fitting of the four components gave a ratio of Co(II) to cyanocobalamin of 9:1, or 10% cyanocobalamin remaining. Analysis of Co(II) produced by photolysis gave similar results. Base-off B₁₂ was generated from a degassed sample of adenosylcobalamin by addition of 6 N HCl to a final pH of 2.0 and characterization by optical

spectroscopy. Solid samples of cobalt hexamine, cobalt hexacyanide, and CoTPP were prepared by diluting the pure compound in aluminum oxide powder and mixing in a mortar and pestle to a fine mixed powder. The mixed powder was then packed in a lucite sample holder. All solid samples were diluted to a 1:10 sample-aluminum oxide (by weight) mixture.

Data Collection. Data were collected at the National Synchrotron Light Source, Brookhaven National Laboratory, on beam line X-9A, using a double flat Si(111) crystal monochromator with fixed exit geometry. All experiments were carried out at 115–120 K, and sample temperature was maintained by flowing cooled nitrogen gas through a low-temperature cryostat as described previously.¹⁴ EXAFS data with 3-eV resolution was recorded by counting at a specific energy for 4 s and incrementing the energy by 10 eV from 120 eV below the cobalt edge to 20 eV below the edge, then in 1.0-eV steps to 15 eV above the edge, and finally in 3.0-eV steps from that point to 600 eV above the edge. Twelve scans were taken for both photolyzed and reduced B₁₂ compounds. Photon flux was 3.75 × 10¹¹ photons/s at 100 mA beam current; data were generally taken in the range of 90–180 mA. Cyanocobalamin was used as a standard to account for any shifts in the monochromator.

Cobalt Kα fluorescence was detected with a zinc sulfide coated photomultiplier tube, 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 PDP 11/23+ 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 reference detectors.

Data Analysis and Errors. Fourier filtered data are analyzed by a nonlinear least-squares fitting procedure in the standard manner,¹⁵ using the University of Washington EXAFS package on the Georgetown 8700 VAX computer. Data manipulation with use of a linear pre-edge fit, cubic polynomial spline background (isolated atom) subtraction, wave-vector cubed weighting, Fourier transformation, filter, and back-transform have all been described previously.^{16–18} The data were first smoothed by a three-point averaging routine. Then monochromator and other obvious noise glitches were removed before further processing by replacement of cubic polynomial fits across the glitches, with the polynomial determined by the boundary regions of the glitch.

CoTPP, cobalt(III) hexamine, and cobalt hexacyanide model compound data were treated in the same manner as the unknown samples. These compounds have known crystallographic structures.^{19–21} CoTPP was used as a standard in the fitting procedures for Co(II) B₁₂ and adenosylcobalamin; cobalt hexacyanide was also used in the fitting of adenosylcobalamin. CoTPP has four nitrogen ligands at 1.949 (3) Å average distance,¹⁹ cobalt hexacyanide has six ligands at 1.893 (11) Å average distance,²⁰ while cobalt hexamine has six ligands at 1.966 (9) Å average distance²¹ (using a 1/√*r*² average). To give one estimate of the errors in our data analysis procedures we fit the two Co–N model compounds to each other using CoTPP as the model compound and the cobalt hexamine as the unknown.

The EXAFS solution for cobalt hexamine was consistent with the X-ray solution. The average Co–N ligand distance was 1.95 ± 0.02 Å for six ligands in a one atom type fit. The Co–N average distance in cobalt hexamine, as indicated by X-ray, may range from 1.948 to 1.984 Å within a limit of two standard deviations. The X-ray structure of cobalt hexamine showed that the hexagonal Co–N distances were of nearly identical lengths. With this in mind a two atom type fit was attempted in order to find out how the EXAFS fitting procedure deals with the similarity among the bond distances. Despite the additional free parameters, two-atom fits were no better than the one-atom fit above, with identical ligand distance results. This was encouraging, in that the fitting procedure does not derive artificial resolved contributions by pushing the ligand distances apart.

The errors above and in the analyses that follow were estimated by three methods. In the first, partial sums of the total number of scans for

(14) Powers, L.; Chance, B.; Ching, Y.; Angiolillo, P. *Biophys. J.* **1981**, *34*, 465.

(15) Lee, P.; Citrin, P.; Eisenberger, P.; Kincaid, B. *Rev. Mod. Phys.* **1981**, *53*, 769.

(16) Chance, M.; Powers, L.; Kumar, C.; Chance, B. *Biochemistry* **1986**, *25*, 1259.

(17) Chance, M.; Powers, L.; Poulos, T.; Chance, B. *Biochemistry* **1986**, *25*, 1266.

(18) Chance, M.; Parkhurst, L.; Powers, L.; Chance, B. *J. Biol. Chem.* **1986**, *261*, 5689.

(19) Madura, P.; Scheidt, W. R. *Inorg. Chem.* **1976**, *15*, 3182.

(20) Kruger, G. *Acta Crystallogr.* **1978**, *B34*, 915.

(21) Krueger, G.; Reynhardt, J. *Acta Crystallogr.* **1978**, *B34*, 915.

(10) Savage, H.; Lindley, P.; Finney, J.; Timmins, P. *Acta Crystallogr.* **1987**, *B43*, 296.

(11) Cotton, F. A.; Wilkinson, G. *Advanced Inorganic Chemistry*, 4th ed.; Wiley and Sons: New York, 1980; p 774.

(12) Dolphin, D. *Methods Enzymol.* **1970**, *18C*, 34.

(13) Beaven, G.; Johnson, E. *Nature (London)* **1955**, *176*, 1264.

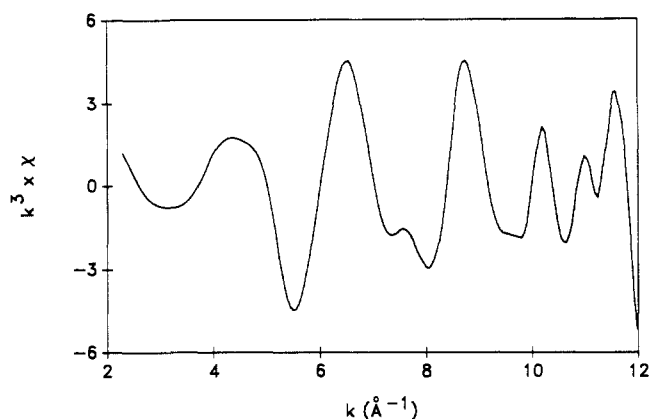


Figure 2. Wave vector cubed, background subtracted data of Co(II) B₁₂ generated by reduction. Data are smoothed and monochromator glitches have been removed from the data by replacement of cubic polynomial fits across the glitches as described in the text. Data were Fourier transformed from 1.5 to 11.0 Å⁻¹ in *k*-space with cosine squared tapered windows as described in the text.

a single sample were independently fit. The differences in fit distances were noted and provided an estimate of the errors due to random noise. Second, independently prepared samples were analyzed separately to note errors due to sample preparation, occupancy of states, and other related non-random fluctuations. Third, the method of mapping out the minimum solution by examination of χ^2 (sum of the residuals squared) was applied. This method was carried out in the following fashion: The error bar associated with a particular parameter in the fit was obtained by systematically varying and holding fixed that parameter, while least-squares refining the others, until χ^2 was double that of the minimum solution.²² These error estimates gave similar results, and the reported errors were the square root of the sum of the squares of these errors. Also to overcome systematic errors that arise from sample degradation we examined each scan for major changes in edge position. The Fourier transform filter window width and position were also varied along with the *k*-range in order to find the dependence of the solution on these parameters. A combination of all of the above techniques suggested errors of ± 0.01 – 0.02 Å for the average of the equatorial ligand distances and ± 0.02 – 0.03 Å for the axial ligand distances.

Background subtracted data were Fourier transformed with use of square windows with tapered ends—the taper ends had centers of 1.5 and 11.0 Å⁻¹ in *k*-space for the Co(II) reduced data and centers of 1.5 and 10.5 Å⁻¹ for the Co(II) photolyzed data. The Fourier filter back-transform window taper centers were set to 0.8–2.2 Å; all data were fitted from 4 to 11.5 Å⁻¹ in *k*-space. The Washington package provides cosine squared tapers for use at the square window edges. When the *D* value of the tapers (see below) was set to zero, the window function was a square cutoff at *k*₁ and *k*₂ (or *r*₁ and *r*₂). Application of the taper function to the window edges introduced some tailing and smoothing to the window function. At *k*₁ – *Dk*₁/2 and *k*₂ + *Dk*₂/2 the window function tailed to zero, and at *k*₁ + *Dk*₁/2 and *k*₂ – *Dk*₂/2 the window function was entirely square and left the data unchanged. The back-transform or the *r* window function was of the same type as the *k* window function. The taper functions were set at *Dk*₁ = *Dk*₂ = 2 and *Dr*₁ = *Dr*₂ = 0.2. For example, this means that for the Co(II) reduced data above, the window left the data unchanged at *k* = 10 Å⁻¹ and tapered to zero at *k* = 12 Å⁻¹. To test the effects of these tapered windows we also fit data generated using square windows and no tapers in the Fourier transform. The data range for this fitting had the *k*_{max} cutoff of the transformed data (*k* = 11.5). The results were within 0.01 Å of the reported solutions, although the χ^2 was higher. All unknown and model compound data that were used for a reported series of one- or two-atom fits were treated with identical windows and tapers. These windows and fitting ranges yielded data with 6–7 independent degrees of freedom.^{15,16} The *E*₀ shift was varied from –8 to 8 while choosing the solution with the best χ^2 .

Results

Figure 2 shows the background subtracted data for the reduced (Co(II) B₁₂). The data in Figure 2 are smoothed and deglitched as described above, in order to remove noise and beam glitches. The Fourier transform of Figure 2 before any deglitching is shown

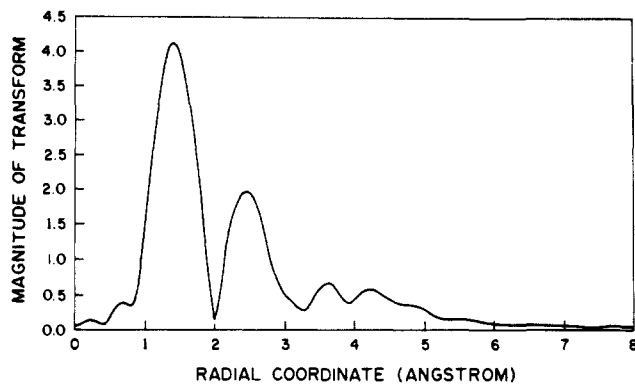


Figure 3. Fourier transform of undeglitched and unsmoothed background subtracted data (Figure 8). The noise level represented by the magnitude above 5 Å is very low.

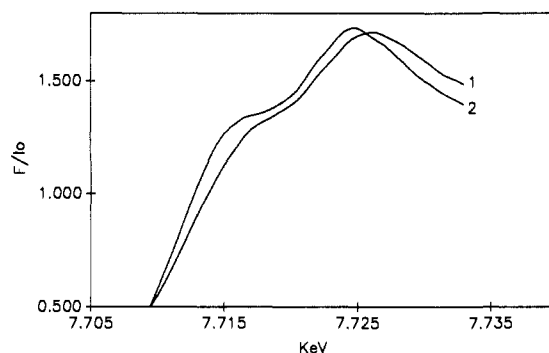


Figure 4. X-ray fluorescence edge data of 1s–4p transitions observed in cobalamin compounds. Spectra are calibrated by comparison to a cyanocobalamin standard, and absolute energies are not calibrated: (1) “base-off” adenosylcobalamin; (2) Co(II) B₁₂.

in Figure 3. The noise level, represented by the transform magnitude above 5 Å, is very low. The back-transformed data are first fit to CoTPP with use of a one atom type fitting procedure, with free parameters of distance (*r*), energy shift (*E*₀), and the Debye–Waller factor (σ^2) shift (both with respect to CoTPP), while the coordination number is fixed at five ligands. This was considered reasonable because EPR measurements of Co(II) B₁₂⁹ and the recent crystal structure of Krautler⁸ provide strong evidence that it is five coordinate, with four ligands to the corrin equatorial nitrogens and one axial ligand to the DMB base. Also, although EXAFS is not exceedingly accurate in determining coordination number, when four parameters, including the coordination number (*N*), are allowed to float in the one atom type fitting procedure, 5.4 ± 0.2 nitrogen ligands gave the best fit.

Edge studies were performed in order to verify the oxidation state and the coordination number of the Co(II) B₁₂ samples. The main edge peak position corresponds to a 1s–4p bound state transition that is sensitive to the oxidation state of the metal ion in the complex. We found the 1s–4p transition of Co(II) B₁₂ to be shifted by 1.0 eV to lower energy compared to that of the presumed five-coordinate “base-off” B₁₂²³ (see Figure 4). This is consistent with a one-electron reduction to Co(II) compared to the five-coordinate Co(III) form and provides an internal check of the compound's identity.^{16,24,25}

Variations in intensity of the symmetry-forbidden 1s–3d pre-edge transition can be correlated with coordination number and distortions in the symmetry. The size of the peak increases with a reduction in coordination number and its intensity also increases with deviations from centrosymmetric geometry. The Co(II) B₁₂ pre-edge spectrum, shown in Figure 5, shows a strong 1s–3d transition, larger in size than both cobalt hexamine, an undistorted

(23) Glusker, J. P. *B₁₂*; Dolphin, D., Ed.; John Wiley & Sons: New York, 1982; Vol. 1, p 23.

(24) Bair, R.; Goddard, W. *Phys. Rev. B* **1984**, *22*, 2767.

(25) Wong, J.; Lytle, F.; Messmer, R.; Maylotte, D. *Phys. Rev. B* **1984**, *30*, 5596.

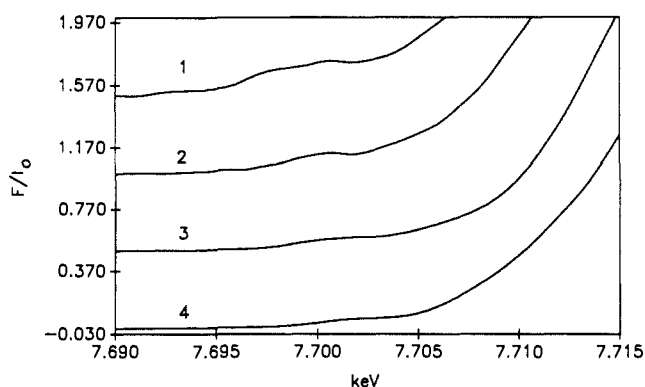


Figure 5. Comparison of X-ray fluorescence edge data of 1s-3d transitions observed in cobalamins: (1) base-off adenosylcobalamin; (2) Co(II) B₁₂; (3) cyanocobalamin; (4) cobalt(III) hexamine.

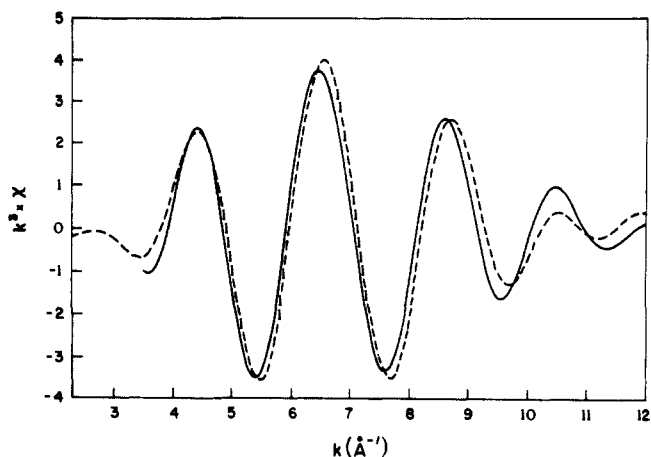


Figure 6. Back-transformed data of Co(II) (---) compared to the one-atom fit simulated solution A (—), $\chi^2 = 8.3$. Data are fitted from 4 to 11.5 Å⁻¹ in k -space.

Table I. Nonlinear Least-Squares Fitting Solutions to Cobalt(II) B₁₂^a

fit type	soln	model	r	N	ΔE_0	$\Delta\sigma^2$	χ^2
one-atom fit	A	Co-N	1.90	5	-7	4×10^{-3}	8.3
	B	Co-N	1.91	5	-6	3×10^{-3}	3.3
two-atom fit	C	Co-N	1.87	4	-5	1×10^{-3}	1.3
		Co-N	1.97	1	0	5×10^{-3}	
	D	Co-N	1.89	4	-3	3×10^{-3}	>30
		Co-N	2.13	4	-5	1×10^{-2}	
	E	Co-N	1.89	4	-7	3×10^{-3}	0.7
		Co-N	2.00	1	4	2×10^{-3}	
	F	Co-N	1.89	4	-7	2×10^{-3}	>30
		Co-N	2.13	1	0	1×10^{-2}	

^aNonlinear least-squares fitting solutions for Co(II) B₁₂ EXAFS spectra. Parameters: r , distance in Å; N , coordination number; ΔE_0 , energy shift relative to model compound; $\Delta\sigma^2$, Debye-Waller factor shift relative to model; χ^2 , sum-of-residuals squared. CoTPP was the model compound used for these solutions: Co(II) by reduction, solution A, C, D; Co(II) by photolysis, solution B, E, F. Solutions D and F show the results of inserting the crystal structure bond distances and holding them fixed in the fitting routine; these results did not show a minimum.

octahedral complex and cyanocobalamin, a distorted octahedral complex, and the parent compound. A slightly larger transition is observed for the five-coordinate base-off B₁₂. This indicates that the samples examined by EXAFS are authentic Co(II) B₁₂ five-coordinate species, possibly with a distorted square-pyramidal configuration.²⁶

(26) Bart, C. *Adv. Catal.* **1986**, *34*, 203.

(27) Rossi, M.; Glusker, J. P.; Randaccio, L.; Summers, M. F.; Toscano, P. J.; Marzilli, L. G. *J. Am. Chem. Soc.* **1985**, *107*, 1729.

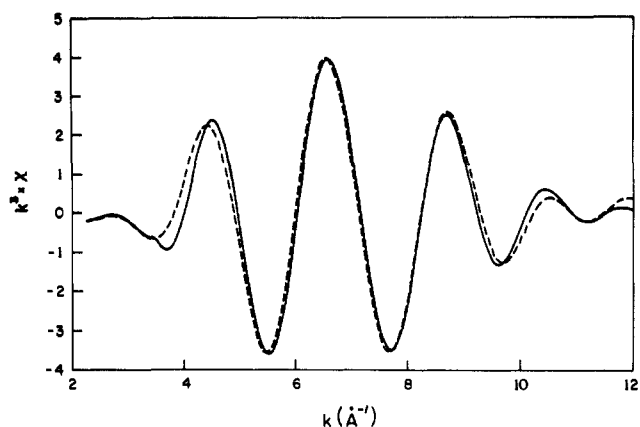


Figure 7. Back-transformed data of Co(II) (---) compared to the two-atom fit simulated solution B (—), $\chi^2 = 1.3$. Data are fitted from 4 to 11.5 Å⁻¹ in k -space.

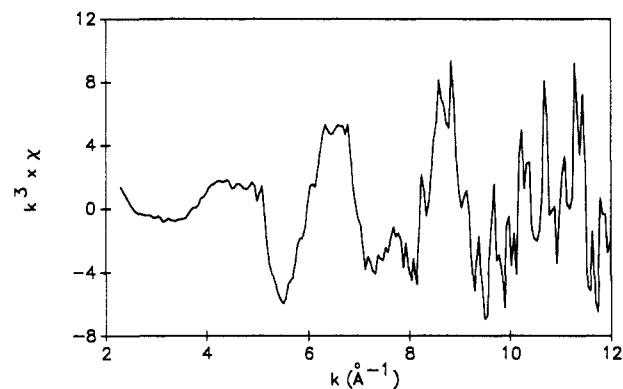


Figure 8. Undeglitched, unsmoothed, wave vector cubed, background-subtracted data of Co(II) generated by reduction.

The results of nonlinear least-squares fitting for Co(II) B₁₂ are summarized in Table I. Both E_0 and Debye-Waller factor shifts observed in the table for all solutions are chemically reasonable.¹⁵⁻¹⁸ Solution A represents the results of a one atom type fit for the Co(II) generated by reduction. The minimum for solution A had an average Co-N distance of 1.90 Å; however, χ^2 was poor at 8.3. This can be seen by examining Figure 6, where the B₁₂ data are compared to the simulated fit.

The poor quality of the one atom type fit is not unexpected, since the equatorial distances to the corrin ring nitrogens are not expected to be of the same type as the axial ligand. However, it provides an excellent starting place for understanding the structure. The best fits clearly require a negative E_0 value, and the average of the equatorial and axial distances should be close to 1.90 Å. With this in mind, we attempted a two atom type fit with six free parameters by fixing the coordination numbers for the two atom types to four and one nitrogen contributions. Since the data have nearly seven degrees of freedom, six free parameters are supported. In addition, the knowledge that four ligands are corrin nitrogens and one is to the axial ligand allows us to distinguish them in the fitting procedure and to search for different solutions for the different atom types.¹⁶⁻¹⁸

Solution C shows a result of four ligands at 1.87 Å and one ligand at 1.97 Å. χ^2 at 1.3 is excellent as is the fit seen in Figure 7. The $1/r^2$ average of the two distances in solution C does not exactly agree with that of the one atom type solution, but it is within experimental error at 1.89 Å. This difference is not unreasonable, since the one-atom fit is relatively poor. The results for Co(II) by reduction are the same as those for solution C within experimental error when undeglitched (raw) data, shown in Figure 8, are used for fitting, although χ^2 is higher. In addition, the solution is relatively insensitive to small variations in the back-transform filter width and small variations in the k range of data fitting. These kinds of tests are necessary to assure that the results are not dependent on data processing or fitting artifacts. The

Table II. Nonlinear Least-Squares Fitting Solution to Adenosylcobalamin^a

fit type	soln	model	<i>r</i>	<i>N</i>	ΔE_0	$\Delta\sigma^2$	χ^2
one-atom fit	A	Co-N	1.92	6	-5	3×10^{-3}	3.8
two-atom fit	B	Co-N	1.91	5	-4	2×10^{-4}	0.5
		Co-N(C)	2.03	1	-5	3×10^{-4}	
	C	Co-N	1.90	5	-4	8×10^{-4}	0.6
		Co-N(C)	2.19	1	-5	1×10^{-3}	
three-atom fit	D	Co-N	1.90	4	-4	2×10^{-3}	0.55
		Co-N	2.02	2	-5	3×10^{-3}	
	E	Co-N	1.90	4	-5	2×10^{-4}	1.1
		Co-N	2.19	1	-4	1×10^{-3}	
		Co-N	2.03	1	-4	8×10^{-2}	
	F	Co-N	1.90	4	-5	2×10^{-4}	0.6
		Co-N	2.19	1	-4	1×10^{-3}	
		Co-C	2.03	1	-4	2×10^{-3}	
errors:		Co-N ± 0.01					
		Co-C ± 0.03					

^a Nonlinear least-squares fitting solutions to adenosylcobalamin EXAFS spectra. Parameters are the same as in Table I. CoTPP and cobalt hexacyanide were the standards used for these solutions.

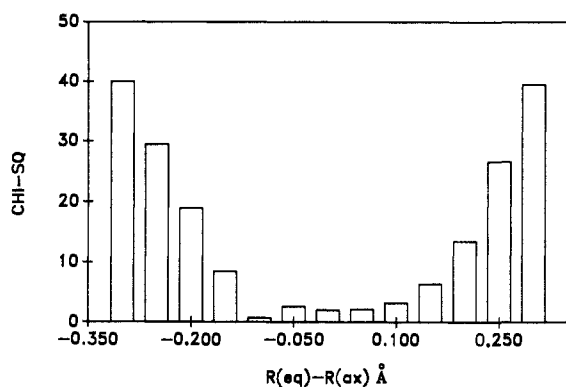


Figure 9. Plot of two-atom-fit results (χ^2) vs R (axial) distance (Å), showing a minimum at the EXAFS solution presented in Table I ($R_{eq} - R_{ax} = -0.1$ Å). The distance difference is varied while maintaining the overall average distance.

solutions for Co(II) generated by photolysis are within experimental error for those of Co(II) generated by reduction. The one-atom fit, solution B, shows five nitrogen ligands at 1.91 Å, while the two-atom fit, solution E, shows four ligands at 1.89 Å and one ligand at 2.00 Å.

Solutions C and E show differences in the axial and equatorial ligands that are near the resolution limit of our data.²² In order to evaluate the variations in χ^2 as a function of the differences in equatorial and axial ligands, we performed a series of fits that vary the distance difference between the equatorial and the axial ligands while maintaining the overall average distance. The results are summarized in Figure 9. χ^2 is very sensitive to the difference between the equatorial and axial ligands; if they are allowed to wander from the minimum of -0.1 Å, χ^2 rises considerably. This shows that solutions C and E best represent the data and that other combinations of equatorial and axial distances that maintain the same overall average distance do not fit the data adequately. Solutions D and F show the fitting results when the crystal structure bond distances of 1.89 Å for the average of the equatorial ligands and 2.13 Å for the axial ligand bond distances are inserted and held fixed in the fitting procedure for both sets of Co(II) EXAFS data. No minimum solution was found that had chemically reasonable energy shifts or Debye-Waller factors; the results shown in both solutions D and F were obtained by not allowing the Debye-Waller shift to wander beyond a chemically reasonable value of 1×10^{-2} Å².

In order to determine the accuracy of the EXAFS procedure beyond the error analysis described above, and to determine our ability to assign axial and equatorial distance contributions, we analyzed EXAFS data of adenosylcobalamin as a control. Data are processed and analyzed by the same procedures as Co(II) B₁₂. The model compounds that are used for the fitting procedure are CoTPP for the Co-N ligands and cobalt hexacyanide for the Co-C ligand of adenosylcobalamin. The EXAFS results are shown in

Table III. X-ray and EXAFS Solutions for Adenosylcobalamin^a

ligand	X-ray	EXAFS
Co-N(eq)	1.90 Å	1.90 ± 0.01 Å
Co-N(DMB)	2.24 Å	2.19 ± 0.01 Å
Co-C	2.01 Å	2.03 ± 0.03 Å

ESD for X-ray = 0.02

^a Comparison of X-ray and EXAFS solution for adenosylcobalamin. The Co-N(eq) distance for the X-ray solution is the $1/r^2$ average of the individual equatorial distances.

Table II. Three different types of fits are performed: one atom type fits, a series of two atom type fits, and a three atom consistency test. All fits use a total fixed coordination number of six. Solution A represents the one atom type fit for six Co-N ligands; the average distance of six Co-N ligands is not consistent with the expected solution and in addition χ^2 is relatively high. This is not surprising since the EXAFS solution was forced to have six unresolved Co-N ligands; this is obviously not the case in adenosylcobalamin.

Solutions B, C, and D represent a series of two atom type fits used in searching for the Co-N and Co-C axial ligands; these are performed in order to distinguish the axial ligand distances from the equatorial ligand distances. Solutions B and C were fit with fixed coordination numbers of 5 and 1, five Co-N ligands modeled by CoTPP and alternately one Co-C ligand with cobalt hexacyanide and one Co-N ligand with CoTPP. This approach provides a search for any resolved axial ligand distances, which will be associated with the fixed coordination number of 1. Solution D is a fit with fixed coordination numbers of 4 and 2. This will identify the average of the equatorial ligand distances, which will be associated with the fixed coordination number of 4.

The results for the two atom type fits support an average distance of 1.90 ± 0.01 Å for the four equatorial Co-N ligands, 2.19 ± 0.01 Å for one axial ligand, and 2.03 ± 0.03 Å for the second axial ligand. Fits E and F represent a three atom type consistency test.¹⁶⁻¹⁸ Various distance and coordination number pairs are fixed at the solutions provided by the two atom type fit. The χ^2 is minimized by varying E_0 and Debye-Waller factors. Therefore, only six free parameters are allowed in the procedure. Solution E is fitted only with CoTPP for all six ligands, the χ^2 is two times higher than the best two-atom-fit, and the Debye-Waller shift for the second Co-N axial ligand is not chemically reasonable at 8×10^{-2} Å². When a Co-C contribution is added, as in solution F, the fit is in excellent agreement with the two atom type fit and the E_0 and Debye-Waller factors are chemically reasonable. The EXAFS solution was found to be in a good agreement with the X-ray data, shown in Table III. However, EXAFS apparently provides a more precise solution since the Co-N EXAFS errors are on the order of ± 0.01 Å and the Co-C distance error is ca. ± 0.03 Å. For the crystal-structure data, the expected uncertainty of the metal-ligand distances is ca. ± 0.05 Å (99% confidence limit assuming normally distributed errors).

Discussion

The structures of cobalt corrin compounds have been thoroughly studied for some time. However, few accurate cobalamin structures presently exist. The best structures are of adenosylcobalamin¹⁰ and methylcobalamin.²⁷ Cyanocobalamin *b* carboxylic acid,^{23,28} cyanocobalamin²⁹ (wet crystal form), and cyanocobalamin³⁰ (dry crystal form) have also been studied; however, the cobalt–ligand distances for these compounds are unreliable since the estimated standard deviations (ESD) for the metal ligand distances range up to 0.1 Å. Our data show that EXAFS can analyze these kinds of compounds and provide accurate cobalt–ligand distance information.

Our EXAFS solution for Co(II) B₁₂ does not fully agree with the recent X-ray solution of Krautler et al.⁸ Although the equatorial Co–N distances closely agree, there is a discrepancy in the axial ligand distance. Insertion of the distances provided by Krautler et al. into the EXAFS equation and fitting to both data sets gives an unreasonable χ^2 . Figure 9 also shows the minimum EXAFS solution is consistent with an ca. 0.1-Å spread between the equatorial and axial distances. Yet, this short axial distance is not expected from an examination of some other Co(II) model compounds. For example, Co^{II}TPP with a 1-methylimidazole ligand has Co 0.14 Å out of the heme plane, and a Co–N axial distance of 2.14 Å,³¹ while Co^{II}salen(pyridine) has a Co–N axial distance of 2.10 Å.³² Since very little structural information is available on cobalamin compounds, it is unclear how relevant these other Co(II) model compounds are to understanding the complex interactions present in the corrin system. The strain of the corrin plane and hence the steric repulsion of the axial ligands on the corrin ring are key parameters that will influence the resultant cobalamin structure.

The EPR results of Bayston et al.⁹ qualitatively confirm our measurement of a relatively strong Co–N_d bond. They examined a number of Co(II) cobinamides and cobalamin compounds with a range of axial ligands in the fifth position. For ligands in the fifth position like benzimidazole and dimethylbenzimidazole, the hyperfine coupling constant is lowered, indicating that less electron density is localized on the cobalt ion, and the superhyperfine (SHF) coupling constant indicated that unpaired spin density is present on the axial ligand. Increases in the SHF coupling constant are reasonably interpreted in terms of increasing Co–N_d bond strength.

Our EXAFS structural analysis for Co(II) B₁₂ generated by both reduction and photolysis shows a distance of 1.99 ± 0.03 Å for the Co–N axial distance while the average equatorial Co–N distance of 1.88 ± 0.02 Å is similar to that found in other cobalamin structures. Therefore, it is implied that upon cleavage of the Co–C bond there are insignificant changes of the corrin ring equatorial distances while the Co ion moves toward the DMB ligand. The minimal reorganization of the corrin ligand is consistent with a very low barrier for recombination of the Co(II) ion with radicals. This is supported by recombination studies subsequent to photolysis of methylcobalamin by Endicott and

Ferraudi³³ and adenosylcobalamin by Chen and Chance,³⁴ which have shown that recombination for the alkyl radicals is close to the diffusion limit. Under photolytic conditions where enzyme is not present, geminate recombination plays a significant role in the mechanism of homolysis.³⁵ For B₁₂-dependent enzyme systems, it is logical that Co(II) and radical must be separated in order for the enzyme-induced homolysis to be effective. This strongly suggests that one of the roles of the enzyme is to prevent radical recombination.

It has been shown through EPR and theoretical model studies that for B₁₂-dependent enzymes such as ethanolamine ammonia-lyase and ribonucleotide reductase, the distance between Co(II) and radical may be as much as 10–12 Å.^{36,37} Such a large separation may result if the enzyme pulls the geminate partners apart, perhaps, as suggested by Krouwer et al.,³⁸ through a conformational change of the enzyme. The steric strain exerted on adenine^{39–41} and the corrin ring^{38,42} by enzyme binding is well-established. The recombination results suggest that in the presence of enzyme the equilibrium of homolysis cannot simply be displaced toward homolysis but must approach complete separation of the fragments in order to avoid rebinding.³⁴ However, for both photolysis and enzyme-induced homolysis, the resulting Co(II) ion will be partially trapped by the reorientation of electron density toward the DMB ligand. However, the minimal differences in equatorial ligand distances between 1, 2, and Co(II) B₁₂ keep the activation barrier for radical recombination relatively low.

Conclusions

Analysis of the structure of Co(II) B₁₂ with use of EXAFS spectroscopy is carried out with CoTPP as a model compound for the Co–N bond distances. Our solution indicates that while the average equatorial distance in the corrin plane agrees with X-ray crystallographic data, the axial Co–N ligand distance is shorter. Attempts to model the X-ray solution with EXAFS are unsuccessful. The Co(II) B₁₂ structure indicates that when the Co–C bond breaks, the Co–N_d becomes stronger, while the equatorial distances show minimal changes.

Acknowledgment. We thank Syed Khalid and Mike Sullivan for technical assistance at beamline X-9. We also thank Linda Powers and Britton Chance for advice and encouragement. The construction and operation of beamline X-9 is supported by National Institute of Health Grant RR-01633 and National Science Foundation Grant DMR-85190959. The National Synchrotron Light Source is supported by the Department of Chemical Sciences. This research is supported by a grant from the USDA Program in Human Nutrition (No. 90-37200-5359).

- (28) Edmond, E. D. Ph.D. Thesis, Oxford University, 1970.
 (29) Brink-Shoemaker, C.; Cruickshank, D. W.; Hodgkin, D. C.; Kamper, M. J.; Pilling, D. *Proc. R. Soc. London, Ser. A* **1964**, *278*, 1.
 (30) Hodgkin, D. C.; Lindsey, J.; Sparks, R. A.; Trueblood, K. N.; White, J. G. *Proc. R. Soc. London, Ser. A* **1962**, *266*, 494.
 (31) Scheidt, W. *Acc. Chem. Res.* **1977**, *10*, 339.
 (32) Calligaris, M.; Nardin, G.; Randaccio, L. *J. Chem. Soc. A* **1970**, 2411.

- (33) Endicott, J. F.; Ferraudi, G. *J. Am. Chem. Soc.* **1977**, *99*, 243.
 (34) Chen, E.; Chance, M. *J. Biol. Chem.* **1990**, *265*, 1297.
 (35) Endicott, J.; Netzel, T. *J. Am. Chem. Soc.* **1979**, *101*, 4000.
 (36) Golding, B. *B₁₂*; Dolphin, D., Ed.; John Wiley & Sons: New York, 1982; Vol. 1, p 543.
 (37) Boas, J. F.; Hicks, P. R.; Pilbrow, J. R.; Smith, T. D. *J. Chem. Soc., Faraday Trans. II* **1978**, *74*, 417.
 (38) Krouwer, J. S.; Holmquist, B.; Kipnes, R. S.; Babior, B. *Biochim. Biophys. Acta* **1980**, *612*, 153.
 (39) Toraya, T.; Ushio, K.; Fukui, S.; Hogenkamp, H. P. C. *J. Biol. Chem.* **1977**, *252*, 963.
 (40) Toraya, T.; Matsumoto, T.; Ichikawa, M.; Itoh, T.; Sugawara, T.; Mizuno, Y. *J. Biol. Chem.* **1986**, *61*, 9289.
 (41) Toraya, T. *Arch. Biochem. Biophys.* **1985**, *242*, 470.
 (42) Babior, B.; Li, T. *Biochemistry* **1969**, *8*, 154.