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Internal Spin Trapping of Thiyl Radical during the Complexation and Reduction of Cobalamin with Glutathione and Dithiothreitol

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Abstract

The activation of cobalamin requires the reduction of Cbl(III) to Cbl(II). The reduction by glutathione and dithiothreitol was followed using visible spectroscopy and electron paramagnetic resonance. In addition the oxidation of glutathione was monitored. Glutathione first reacts with oxidized Cbl(III). The binding of a second glutathione required for the reduction to Cbl(II) is presumably located in the dimethyl benzimidazole ribonucleotide ligand cavity. The reduction of Cbl(III) by dithiothreitol, which contains two thiols, is much faster even though no stable Cbl(III) complex is formed. The reduction, by both thiol reagents, results in the formation of thiyl radicals, some of which are released to form oxidized thiol products and some of which remain associated with the reduced cobalamin. In the reduced state the intrinsic lower affinity for the benzimidazole base, coupled with a trans effect from the initial GSH bound to the β -axial site and a possible lowering of the pH results in an equilibrium between *base-on* and *base-off* complexes. The dissociation of the base facilitates a closer approach of the thiyl radical to the Co(II) α -axial site resulting in a complex with ferromagnetic exchange coupling between the metal ion and the thiyl radical. This is a unique example of ‘internal spin trapping’ of a thiyl radical formed during reduction. The finding that the reduction involves a peripheral site and that thiyl radicals produced during the reduction remain associated with the reduced cobalamin provide important new insights into our understanding of the formation and function of cobalamin enzymes.

Keywords

Cobalamin; glutathione; dithiothreitol; electron paramagnetic resonance; visible spectroscopy; thiyl radicals

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¹The parameters obtained in X-band at a 3 fold excess of GSH (Fig. 4A) are somewhat different than the *base-on* parameters obtained in Q-band at a 10 fold excess of GSH. Although this may reflect 2 different *base-on* complexes (Scheme 1), we have not included these parameters in the scheme.

²A comparison of Fig. 5 and Fig. 6b indicates that the same spectra are observed in a large excess of GSH whether DTT or GSH are used to reduce the Co(III) to Co(II). We, therefore, investigated the saturation effects in both a 50 fold excess of GSH reacted Cbi(III) and DTT reduced Cbi(III) to which GSH was added.

INTRODUCTION

Cobalamin (Cbl) dependent enzymes catalyze three important types of reactions; viz., *intramolecular rearrangements*, *methylation* and the *reduction of ribonucleotides to deoxyribonucleotides*. Out of nearly 15 enzyme reactions that require vitamin B12, the conversion of L-methylmalonyl CoA into succinyl CoA and the formation of methionine by methylation of homocysteine are the only reactions occurring in mammals that are known to require coenzyme B12. The importance of regulating homocysteine is evident by the finding that mild to moderate elevations of serum homocysteine are observed in Alzheimer's disease [1] and that there is an increased risk of cardiovascular disease and stroke associated with elevated levels of serum homocysteine [2–5].

Cbl (Fig. 1) contains a cobalt ion which can be in the +1, +2 or +3 valence states and an intramolecularly coordinated 5,6-dimethylbenzimidazole in the α -axial position. The function of the Cbl cofactor depends on the oxidation state of the cobalt and the ligand bound in the β -axial position. While Cbl is generally isolated in the +3 valence state with cyanide, water or hydroxide coordinated in the β position, the activity of Cbl requires its reduction to the +1 or +2 valence state with the coordination of a methyl group or the 5' deoxyadenosyl group [6,7].

Glutathione (GSH) is the most abundant non-protein thiol and is a major intracellular reducing agent present in almost all biological tissues with a concentration in the mM range. Its concentration in cells is at least an order of magnitude greater than that of other thiols such as cysteine [8]. Vitamin B12 and its derivatives have been known to form complexes with GSH [9–11]. The complex formed between Cbl and GSH, glutathionylcobalamin Cbl(III)GSH, is believed to be one of the major forms of vitamin B12 found within mammalian cells. It is considered to be the substrate for Cbl(III)reductase [12] serving as an intermediate in the conversion of biologically inactive cyanocobalamin to the active coenzyme forms, adenosylcobalamin and methylcobalamin [6]. The pathway for this process is thought to involve the reaction of cyanocobalamin with β -ligand transferase, a cytosolic enzyme that utilizes FAD, NADPH and GSH to produce Cbl(III)GSH, which reacts with the NADH-linked microsomal cobalamin(III) reductase, that is then converted to adenosylcobalamin or methylcobalamin. It was, in fact, hypothesized by McCaddon [1] that Cbl(III)GSH might play a role in the treatment of various neuropsychiatric disorders associated with oxidative stress, including Alzheimer's disease.

In vitro, Cbl(III)GSH can be prepared by reacting aquocobalamin and GSH to form a stable complex of 1:1 stoichiometry [10,13–16]. The equilibrium constant for the formation of Cbl(III)GSH was estimated as $5 \times 10^9 \text{ M}^{-1}$. This Cbl(III)GSH complex was shown to be stable in the presence of air and a detailed study of the structure of this complex has been obtained by means of ^1H , ^{13}C 2D-NMR and X-ray absorption. These studies confirm that GSH is coordinated to the cobalt atom in the β -axial position via the cysteine sulfur atom [14,17].

The potential of low molecular weight thiols like GSH and dithiothreitol (DTT), as well as enzyme sulfhydryl groups, to form radicals when reacting with Cbl and related compounds has been well documented [18–22].

In this report we have investigated the reduction of Cbl(III)OH and cobinamide with GSH and DTT under anaerobic conditions by visible spectroscopy and electron paramagnetic resonance (EPR). For GSH we have also monitored the formation of oxidized glutathione (GSSG). The reduction by GSH is shown to involve a second GSH molecule in addition to the GSH that binds to the cobalt of Cbl(III). It is found that GSH and DTT radicals remain coordinated to the cobalt ion after reduction providing a model for 'internal spin trapping'.

The objective of this study was to identify by means of electron paramagnetic resonance (EPR) and optical spectroscopy the species formed during the reduction, providing an explanation for the overall reduction process. The species formed include the *base-on* species, the *base-off* species as well as radicalated Cbl(II), which is a 'spin trapped' exchange-coupled species.

EXPERIMENTAL

Materials

Cbl(III)OH (vitamin B_{12a}) acetate salt, DTT, GSH, GSSG, glutathione reductase, Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and NADPH were obtained from Sigma and used without any further purification. 2-vinylpyridine was obtained from Aldrich. Cobinamide, in which the benzimidazole is chemically removed, was a gift from Dr. Vijay Sharma. All other reagents were of analytical grade and all solutions and buffers were prepared with deionized water. All solutions of Cbl's were prepared in 100 mM sodium phosphate buffer, pH 7.3.

UV-Visible Studies

For UV-Visible spectral measurements, 100 μ M Cbl(III)OH samples were prepared in 100 mM phosphate buffer, pH 7.3 in septum sealed 10 mm quartz cells and purged with argon to remove oxygen. The reaction of Cbl(III) with GSH and DTT was initiated by adding the thiol to the anaerobic Cbl(III) solution. Spectra were run anaerobically in the 250–650 nm regions at ambient temperature ($\sim 23^\circ\text{C}$) using a Perkin Elmer Lambda 6 spectrophotometer.

Electron Paramagnetic Resonance Studies

For EPR studies a 5 mM solution of Cbl(III)OH was prepared in 100 mM phosphate buffer, pH 7.3. The solution was deoxygenated by bubbling with pure argon. After the reaction was completed, 300 μ l samples were transferred to 4 mm quartz sample tubes under a positive argon pressure and immediately frozen by submerging the tube in liquid nitrogen. For GSH, excess thiol was added anaerobically and incubated for 3 hrs prior to freezing. For DTT, the samples were frozen immediately after the addition of the thiol. EPR spectroscopic measurements were carried out at X-band frequency on a Bruker EMX 6 spectrometer in the temperature range from 5K to 77K. The Temperature was maintained using an Oxford ESR 900 continuous flow cryostat with an ITC-502 temperature controller. X band measurements were carried out at a microwave power of 2mW and a modulation amplitude of 1mT. Some measurements were also made on a Varian E-112 instrument. For Q band measurements, a Varian E-9 system was used at the National Biomedical ESR Center, Medical College of Wisconsin. The Q-band bridge was modified with addition of a GaAs field-effect transistor signal amplifier and low-noise Gunn diode oscillator. The Q-band measurements were carried out at a microwave frequency, 35.0GHz; power, 10dB; modulation amplitude, 10G; gain, 400; temperature, -140°C ; seven scans averaged.

GSSG Assay

Griffith's method, which was a modification of the method developed by Tietze, was followed for the determination of GSSG [23–24]. The following stock solutions were made up in 125 mM Na-phosphate buffer containing 6.3 mM Na-EDTA adjusted to pH 7.5 and stored at 0°C . (1) 0.3 mM NADPH, (2) 6 mM DTNB, (3) ~ 50 units of glutathione reductase/ml. To 0.6 ml of the sample were added 2.1 ml of the NADPH solution and 0.3 ml of the DTNB solution giving a final volume of 3 ml in a cuvette with a 1 cm path length and equilibrated to 30°C . To the warmed solution, 30 μ l of the glutathione reductase solution was added and the absorbance change was monitored at 412nm until the absorbance

exceeded 2. Based on comparing the rate observed for known amounts of GSSG, a standard curve was generated. To prevent any contribution from the unreacted GSH present in the sample, the GSH (for pH's >5.5) was derivatized by adding 2 μ l of neat 2-vinylpyridine per 100 μ l solution and mixing the solution vigorously for 1 min. Depending on the final pH, GSH will be fully derivatized after 20–60 min at 25 °C.

RESULTS

UV-Visible Spectral Studies

The reaction of glutathione with Cbl(III)OH—Figure 2a shows the time dependent spectral changes produced by the reaction of a 10 fold excess of GSH with Cbl(III)OH under anaerobic conditions. A color change from red to violet is observed upon complex formation with absorption peaks changing from 273nm, 351nm, 412nm and 525nm to 287.5nm, 331.5nm, 370.5nm, 427.5nm and 534nm. Clear isosbestic points are observed at 338nm, 364nm, 447nm and 540nm. This spectral change is attributed to the displacement of the –OH group by GSH to form Cbl(III)GSH complex. Under these conditions no reduction of Co(III) to Co(II) is observed. With a 50 fold excess of GSH (Fig 2b) the formation of Cbl(III)GSH occurs within the mixing time of the reagents (< 5sec). This initial change is followed by a slow color change from violet to brown with the absorption peaks changing to 311nm, 404nm and 474nm. Clear isosbestic points are now observed at 499nm, 388nm, 330nm and 296nm. This slower spectral change is attributed to the reduction of Co(III) to Co(II). Other investigators interested in the reaction of GSH with cobalamin have either focused on the binding to Cbl(III) or the reduction of Cbl(III) to Cbl(II). However, by comparing both reactions it becomes evident that completely different concentrations of GSH are required for both reactions.

Job Plot of the Reduction of Cbl(III)OH by GSH—In order to determine whether the GSH that reduces Cbl(III) to Cbl(II) is distinct from the initial GSH bound to Cbl(III) a continuous variation study was performed changing the relative molar ratios of GSH and Cbl(III), maintaining the total molar concentration the same (Job plot). The samples were then permitted to reach equilibrium by incubating at room temperature for 3 hrs. The resultant UV-Visible Spectroscopy based Job plot (Fig. 3a) shows that at low molar ratios of GSH there is no reduction. At low GSH:Cbl ratios GSH binds to Cbl(III) resulting in the formation of Cbl(III)GSH. This reaction is completed by approximately a 1:1 ratio of Cbl(III) to GSH before any reduction takes place. Fig.3b shows the EPR based Job plot where we can directly measure the reduced Cbl(II) species, in contrast to the requirement for a fitting procedure to obtain Cbl(II) from visible spectroscopy (Fig.3a). The more precise EPR data indicates that only low levels of reduced species are formed at low GSH molar ratios (<1:1) when nearly all the added GSH binds to Cbl(III) without reduction. Both of these experiments clearly indicate the requirement for a second molecule of GSH to form the reduced species.

The reduction of Cbl(III)OH by DTT—Unlike the reduction of Cbl(III)OH by GSH, the reduction by DTT is very fast, even with a slight excess of DTT relative to Cbl(III)OH. The addition of DTT immediately resulted in a color change from red to brown with very clear spectral changes. It is to be noted that the Cbl(II) visible spectrum obtained immediately with a 3:1 molar ratio of DTT:Cbl(III)OH is similar to that obtained with a 50 fold excess of GSH after 3 hours of incubation (Figure 2c).

Electron Paramagnetic Resonance

EPR spectrum of Cobalamin(III) reduced by GSH—Because EPR requires a much higher concentration than visible spectroscopy, mass action results in significant reduction

even at a 3:1 molar ratio of GSH to Cbl(III)OH (results not shown). Figure 4 shows the Q-band spectrum at 12 K of the reduced Cbl(II) obtained when Cbl(III)OH reacts with a 10 fold excess of GSH. The improved resolution obtained in the Q-band provides direct evidence for two species as indicated by two broad $g_{\perp}$ lines with $g=2.23$ and $g=2.27$, respectively, and more than 8 cobalt-59 hyperfine lines in the parallel component. The observation that ^{14}N hyperfine splitting on the Cobalt-59 are partially resolved for some bands and completely absent for other bands indicates that one of the two species has the base dissociated from the cobalt. The EPR parameters for the two components obtained by Q-band simulation are: (1) For the base-off species: $g_{\parallel} = 1.992 \pm 0.003$, $g_{\perp} = 2.272 \pm 0.004$, $A_{\parallel} = 105 \times 10^{-4} \text{ cm}^{-1}$, $A_{\perp} = 3.5 \pm 0.5 \times 10^{-4} \text{ cm}^{-1}$ and (2) For the base on species: $g_{\parallel} = 2.010 \pm 0.003$, $g_{\perp} = 2.234 \pm 0.0005$, $A_{\parallel} = 102.8 \times 10^{-4} \pm 1.0 \text{ cm}^{-1}$, $A_{\perp} = 3.5 \pm 0.5 \times 10^{-4} \text{ cm}^{-1}$. Figure 5 shows the EPR spectrum at 77 K when a 50 fold excess of GSH is added to Cbl(III)OH and incubated for 3 hrs. With this large excess of GSH the *base-on* complex has a spectrum similar to that shown in Fig. 4 with the same A tensors, but better resolved nitrogen hyperfine lines. However, the *base-off* complex is completely different with changes that include: (a) sharpening of the $A_{\parallel}$ lines; (b) the shift and broadening of the $g_{\perp}$ component from its earlier value of 2.272 to almost 2.39. There is also the apparent formation of a buried low intensity line with a 'g' value of 2.19, (marked by a *).

EPR spectrum for cobalamin reduced by DTT and the effects of added GSH on the DTT reduced species—

The EPR spectrum at 77 K of Cbl(II) formed by the reduction of Cbl(III) with a 4 fold excess DTT (Fig. 6a) is produced rapidly and is a simple axial spectrum with poorly resolved ^{14}N hyperfine splitting. The addition of 50 fold excess GSH to the already DTT reduced sample results in the conversion of the spectrum of DTT reduced cobalamin (Fig 6a) into a spectrum (Fig.6b) that is very similar to that obtained when a 50 fold excess of GSH reacts directly with Cbl(III) (Fig. 5).

Cobinamide Experiments—In order to characterize the complexes formed without the base bound and particularly the nature of the *base-off* complex with the broad $g_{\perp}$ at 2.39 and sharp $A_{\parallel}$ (^{59}Co) lines as well as the small band observed at $g = 2.19$ marked by a * in Figures 5 and 6b we have used cobinamide (Cbi(III)), which has no benzimidazole nucleotide. The EPR measurement of Cbi (III) reduced by DTT yields a spectrum shown in Figure 7a similar to those of Figures 5 and 6b. It is, however, puzzling to see that the Cbi(II) spectrum (Fig. 7a) includes a *base-on* component with ^{14}N hyperfine splitting in addition to the *base-off* components.

Figure 7b shows the EPR spectrum obtained on the addition of excess GSH to already DTT reduced Cbi(III). The GSH causes (i) the obliteration of the apparent *base-on* species accompanied by a large increase in the intensity of the species with $g_{\perp} = 2.39$ (a broad and intense line) along with its sharp parallel hyperfine components at higher field; (ii) a substantial increase in the intensity of the $g = 2.19$ line.

To determine whether the 2.19 band is part of the $g_{\perp} = 2.39$ species, we carried out a computer simulation of the Co(II) *base-off* species with $g_{\perp} = 2.39$. The simulation (Fig. 7c-bottom) provides a very good fit with $g_{\perp} = 2.39$, $g_{\parallel} = 2.00$ and sharp lines with $A_{\parallel} = 130 \times 10^{-4} \text{ cm}^{-1}$. No $g=2.19$ was generated indicating that the $g = 2.19$ line is not part of the spectrum of this species (see Figure 7c, middle). Also shown in figure 7c (top) is the simulation of a spectrum with $g_{\perp} = 2.19$ involving a thiyl radical.

A molecule with no base bound to the cobalt, analogous to Cbi(II), can be generated directly from Cbl by lowering the pH to 2.0 where protonation of the axial benzimidazole results in the dissociation of the base forming a *base-off* Cbl(III). Under these conditions the addition of 50 fold excess GSH yields a spectrum (Fig. 7d) very similar to that that obtained when

GSH was added to the DTT reduced cobinamide (Fig. 7b). The pronounced $g = 2.19$ band in this spectrum and the Cbi(III) spectrum with GSH added (Fig 7b) indicates that it is formed when the base is dissociated from the corrin, but does not require the cleavage of the base from the corrin. We infer that this line is also present in figures 6b and 5 (see *), but at a much lower concentration.

Figure 7d is very similar to that of figure 7b except that many of the lines are broader. Since 7d was run at 8K instead of 77K, the difference can be attributed to saturation even though it was run at a lower microwave power of 0.02 mW instead of the 2mW microwave power used in Fig. 7b. To further investigate the saturation properties of the species formed, spectra were run varying the temperature and the power. The saturation produced by increasing the microwave power from 40 dB to 20 dB is shown in Figure 8a for a sample of GSH reduced Cbi(III), and by decreasing the temperature from 77 K to 10 K, is shown in Figure 8b for a sample of the DTT reduced Cbi(III), which was then reacted with excess GSH. A general increase in the intensity along with the broadening of the whole spectrum occurs with increased power or decreased temperature. The changes are, however, not the same for all bands. Initially the intensity of the $g = 2.19$ line increases and then decreases indicative of saturation only at the highest power and lowest temperature. However, the $g = 2.39$ line as well as the sharper $A_{||}$ lines are continuously broadened by a decrease in temperature or an increase in power indicating that these signals are saturated more readily than the $g = 2.19$ line. The different saturation of the $g = 2.19$ indicates that it is from a different species and not part of the main high intensity spectrum.

EPR Saturation Experiments on Reduced Cbl(III)OH at pH 2—We have also performed saturation experiments on Cbl (III) at pH 2 after its reduction by (i) DTT, (ii) GSH and (iii) DTT followed by the addition of GSH. Their EPR spectral features are shown in figures 9a, 9b, and 9c, respectively. The saturation properties of DTT reduced Cbl(III) at pH 2 are substantially different from those of either GSH or DTT/GSH reduction, even though all of them give similar non-saturated signals at the lowest microwave power of 0.02 mw. For DTT without added GSH the broadening of the parallel component with ^{59}Co hyperfine lines is a continuous process, but the $g = 2.19$ and $g = 2.39$ lines do not saturate at all until we go to the highest microwave power of 5 dB. The two reduced species with GSH respond like the Cbi experiments (Fig.8) where the only line that is not saturated until the highest power is reached is the $g = 2.19$ line. This again supports the contention that the origin of this line is different from that of the others.

The Formation of the $g = 2.19$ and $g = 2.39$ bands by lowering the pH of DTT Reduced Cobalamin—Fig. 10a shows the DTT reduced cobalamin (III), which is the same as that shown in Fig. 6a. In Figure 10b the pH of this sample was reduced to pH 2. At this low pH, the Bzm base is no longer coordinated to cobalt (II). Although the DTT can now bind in the base binding site no additional reduction takes place and, therefore, no thiyl radicals were formed. The observation of the thiyl radical coordinated exchange coupled species with its $g_{\perp} = 2.19$ similar to that found in figures 7b and 7d indicates that, even though the signal is not detected, the thiyl radicals are present when the base is coordinated.

The Formation of GSSG during the Reaction of GSH with Cobalamin

The reduction of cobalamin by GSH produces $\text{GS}^{\bullet}$. This thiyl radical can either remain coordinated with Cbl or released into solution. If the e thiyl radical is released from Cbl it will rapidly undergo the reaction,

To determine whether thiyl radicals are associated with the reduced Cbl, the formation of GSSG was determined during the reaction. The results indicate that the level of GSSG formed is about half of that necessary to account for the Cbl being reduced. Thus, a significant fraction of the thiyl radical formed must be associated with the reduced Cbl.

DISCUSSION

The reduction of Cbl(III) to Cbl(II) by glutathione

The GSH complex with Cbl(III), Cbl(III)GSH, is found in mammalian cells and is believed to be one of the major forms of vitamin B12 serving as a substrate for Cbl(III) reductase [12]. These findings have been the impetus for extensive *in vitro* studies characterizing the complex. It is a very stable 1:1 complex formed by reacting aquocobalamin with GSH [10,13–16]. The detailed structure of this complex obtained by means of ^1H , ^{13}C 2D-NMR and X-ray absorption confirm that GSH is coordinated to the cobalt atom via the cysteine sulfur atom [14,17]. In figure 2a we show the rapid formation of the Cbl(III)GSH complex. This complex is stable and does not undergo reduction. However, with a larger excess of GSH there is a slow reduction of the Cbl(III) to Cbl(II), as shown in Fig.2b. The Job plots obtained both from the visible spectra (Fig. 3a) and the EPR data (Fig. 3b) indicate that a second GSH molecule is required for Cbl reduction.

The requirement for a second GSH indicates that the tightly bound GSH is not involved in the reduction. As the X-ray absorption data [19] indicates, the first GSH is docked in the –OH position of Cbl(III)OH attached to the Co(III) atom at the β axial site where it is protected by the phytyl chains stretching out providing a “basket” kind of arrangement (see the structure of Cbl(III), Fig.1). This GSH replaces the ligand (OH or water) at the β -axial site and may actually stabilize the Cbl(III) oxidation state inhibiting reduction [25]. Reduction takes place only when a second GSH binds.

Where does the second GSH involved in the reduction (Fig. 2b) bind? It is unlikely that a second GSH binds directly to cobalt, with the first GSH tightly bound to the β axial site and the benzimidazole bound trans to the GSH on the other side of the corrin at the α axial site. With no available cobalt coordination site the most reasonable area that seems to permit the GSH to approach the metal center and facilitate the reduction would be the void space available in the 5,6-dimethyl benzimidazole ribonucleotide ligand cavity (Fig. 1). The kinetics of the slow reduction (Fig. 2b) can be explained by the requirement for the relatively large GSH to enter this restricted cavity and achieve the proper location and orientation necessary for the transfer of an electron from the thiol to Co(III). The first GSH already coordinated to Cbl(III) in the β -axial site may contribute to the reduction process by lowering the reduction potential.

The general spectral features of GSH reduced cobalamin shown in the EPR spectra of figures 4 and 5 are similar to earlier observations [26,27]. In figure 5 at 77K, with the excess of GSH required for reduction, the nitrogen superhyperfine lines originating from the bound benzimidazole are partially resolved in one of the species. The improved resolution of these lines relative to DTT reduced cobalamin (Fig. 6a) can be explained by GSH still bound in the β axial position when cobalamin is reduced. GSH at this site will decrease the intermolecular interactions reducing dipolar interactions, resulting in less broadening of the superhyperfine lines.

With GSH there is clear evidence from both X-band and Q-band EPR for an additional species even with a 10 fold excess of GSH (Fig. 4). A single species should have eight equally spaced hyperfine lines that originate from the quadrupolar effect of the cobalt-59 ($I=7/2$) nucleus. The presence of two species is evident by the number of hyperfine lines and

the unequal splitting between these hyperfine lines. In addition nitrogen superhyperfine splitting is observed for only some of these lines, while other lines are sharp indicating the absence of any nitrogen interactions. These two species are the *base-on* species with triplet superhyperfine due to ^{14}N and the *base-off* species with sharp lines due to ^{59}Co hyperfine lines with no ^{14}N splitting. Using the Q-band EPR spectra, it was possible to simulate both spectral components (Fig. 4) obtaining the EPR parameters (see above). The *base-off* species is stabilized when Co(III) is reduced to Co(II), by a trans effect on the base due to GSH still bound in the β axial site and by a reduction of the pH when excess GSH is added.

With a large excess of GSH (Fig.5) the relative intensity of the *base-on* species is reduced and there is an increase in the *base off* species, with sharp $A_{\parallel}$ (^{59}Co) lines not broadened by nitrogen superhyperfine splitting. However, at these high concentrations of added GSH the base-off complex with $g_{\perp} = 2.272$ obtained at a 10 fold excess of GSH (Fig. 4) is no longer detected. Instead there is an increase in $g_{\perp}$ to 2.39 caused by the coordination of a second GSH in the α -axial site originally occupied by benzimidazole.

The Thiyl Radical Associated with GSH Reduced Cobalamin

The basis for radical involvement in Cbl chemistry goes back to the original Schrauzer [18] proposal that the reduction of Cbl by GSH involves the formation of thiyl radicals.

The involvement of these radicals in Cbl dependent enzymes has been extensively studied [28,29].

In order to determine if any of the thiyl radical formed when GSH reduces Cbl stays associated with Cbl(II), we measured GSSG formed during the reaction. Interestingly, the oxidized GSSG accounted for only ~ 50% of the thiyl radicals formed during the reduction. The other 50% of the thiyl radicals must, therefore, be associated with reduced Cbl.

Two distinct species involving additional interactions with GSH are detected by EPR. These species are only detected in the *base-off* configuration, which is stabilized during the reduction by GSH (see above). The $g_{\perp} = 2.39$ line together with the sharp Co hyperfine lines are associated with the coordination of a sulfur in the α -axial position. These lines are formed when excess GSH binds to reduced Cbl (complex F, see Scheme 1, vide infra). If the GSH on the α -axial side is a thiyl radical, similar EPR parameters can be observed if the sulfur remains $>2.2 \text{ \AA}$ from the Co atom. A closer approach results in the exchange coupled species ($g=2.19$), which reflects the capture of a spin from the thiyl radical by Cbl(II), referred to as 'internal spin trapping'.

In order to prove that the $g=2.19$ band is not part of the species with $g_{\perp} = 2.39$, we simulated the spectrum of the $g=2.39$ species. As shown in Figure 7c (bottom), this simulation fits all the lines in the reduced cobinamide spectrum except for the line with $g=2.19$. Confirmation that the $g=2.19$ line is not part of the $g_{\perp} = 2.39$ species is also provided by differences between these two lines in saturation studies performed by increasing the power (Fig. 8a) or decreasing the temperature (Fig. 8b). These studies clearly indicate that the $g=2.39$ line and the sharper $A_{\parallel}$ lines are saturated before the $g=2.19$ line.

The relatively broad (15 gauss) line with a g -value of 2.19 is clearly not an isolated free radical, which should have a g value much closer to the g -value of 2.0023 for a free electron [30]. Even for sulphur based radicals, which have higher g -values, the value should be between 2.03 and 2.09 for an isolated radical [31,32]. Very large exchange coupling or dipolar coupling between the metal center and the thiyl radical can give rise to the broad $g=$

2.19 isotropic line. This suggestion is, however, ruled out by the predicted very small exchange coupling constant (*vide infra*). Hence this large-intensity line is interpreted to represent the $g_{\perp}$ of an exchange coupled species involving “internal spin trapping” between base-free Cbl(II) and the coordinated thiyl radical. The $g_{\perp}$ for this species should be the average between that of Cbl(II)(GSH)₂ with GSH bound in the α and β axial position and that of the free thiyl radical. The ligand field for two GSH molecules bound would result in $g=2.39$ (complex F, see Scheme 1 *vide infra*) and the estimated $g_{\perp} \sim 2.00$ for a free thiyl radical can be based on the theoretical calculations and experimental data reported by van Gastel and coworkers [31]. The average results in a value of 2.195 in good agreement with the observed intense line at 2.19. It should, however, be noted that the $g_{\perp}$ -value of 2.00 being used for the thiyl radical is only a rough estimate. Van Gastel and coworkers [31] have cautioned that the largest of the anisotropic g -parameters *viz.* g_{xx} , which decides the magnitude of g_{av} or g_{iso} is very sensitive to the conformation of the cysteine, the environment of sulfur and polarity. This is because the EPR g -values are very sensitive to the energy difference between the singly occupied almost degenerate levels and one of the lone-pair orbitals, the ΔE being of the order of 1000 to 4000 cm⁻¹ [31].

The parallel component of the exchange coupled species would be in the 2.0–2.05 range taking an average of 2.0 for the g_{zz} for the cobalt species and 2.0 to 2.10 for the thiyl radical [31,32]. Similarly, the parallel component will have its ⁵⁹Co hyperfine lines at half the $A_{\parallel}$ value observed for the $g=2.39$ species i.e. $\sim 50\text{--}60 \times 10^{-4} \text{ cm}^{-1}$ at much reduced intensities in comparison to the amplitude of $g_{\perp} = 2.19$. The simulated spectrum of this species with $g_{\parallel} = 2.00$, $g_{\perp} = 2.19$, $A_{\perp} = 1 \times 10^{-4} \text{ cm}^{-1}$ and $A_{\parallel} = \sim 50 \times 10^{-4} \text{ cm}^{-1}$ is shown on the top of Figure 7c. At least one or two of the predicted lines are poorly resolved as weak lines with + signs in the experimental spectrum (Fig. 7d).

The temperature dependence in the 77–20 K reveals that the Co(II)-thiyl species is ferromagnetically coupled. The intensity variation of this data was used to calculate a ‘J’ value, i.e. Heisenberg exchange coupling, [34–36] of $2.45 \pm 0.2 \text{ cm}^{-1}$. In our analysis, we could not detect any effects due to dipolar coupling. Confirmation of a triplet state due to the exchange-coupled species is frequently obtained by the observation of a half-field transition. However, the intensity of the band due to this forbidden transition is proportional to the zero-field splitting (2D) and cannot be detected for the small D-value expected for our complex

Support for the assignment of the $g=2.19$ complex as a Co(II) thiyl exchange coupled species can be drawn from two important works involving ribonucleotide triphosphate reductase: (i) A protein based thiyl radical has been proposed as an intermediate in this enzyme from *Lactobacillus leichmannii*, which catalyzes adenosyl cobalamin-dependent reduction as well as the exchange of the 5 hydrogens of adenosylcobalamin with solvent [37]. (ii) Modeling of this intermediate as a thiyl radical coupled to cobalamin(II) by electron-electron exchange and dipolar interaction yields a reasonable fit to both X- and Q-band spectra. The J-value calculated by Gerfen et al [33,37] was $|J_{ex}| > 0.45 \text{ cm}^{-1}$ for the cysteine thiyl radical) detected in cobalamin(II) reacted ribonucleotide triphosphate reductase. This determination of the J value by simulation was in the same range as the 2.45 cm^{-1} (see above) that we calculated from the temperature dependence of the intensity, which indicates that both complexes are similar. They also observed a minimal amount of dipolar broadening.

The reduction pathway of Cbl(III)OH by the addition of GSH is summarized in Scheme I. The GSH adduct of Cbl(III), already established earlier by UV-Vis experiments, is represented by A, in this Scheme. The formation of this complex, Cbl(III)GSH, requires one GSH molecule and involves the simple displacement of OH by GSH. The binding of a

second GSH in the α -axial ligand cavity is represented by B. The second GSH interacts with the cobalt as represented by C resulting in the reduction of Cbl(III) to Cbl(II) forming a thiyl radical (D and D'). The thiyl radical can be released and undergoes dimerization leading to the formation of oxidized glutathione GS:SG and the *base-on* reduced complex D. As indicated by our analysis of the formation of GSSG, only ~ 50% of the radical goes on to form GSSG and the remainder of the GS• radicals formed are still associated with Cbl. The EPR parameters with the base still coordinated (the *base-on* complex D with $g_{\perp}=2.234$) is not significantly affected by having the thiyl radical (complex D') in the pocket or if some of the excess GSH enters this pocket (complex D'').

Under conditions where the base is still coordinated, the evidence for the formation of thiyl radicals is, therefore, indirect. The released thiyl could not be spin-trapped using an external spin trap in this experiment, since the formation of GS:SG through the process of radical recombination as well as the internal spin trapping by Cbl(II) are faster than the reaction of GS• with an external spin trap. At the low temperatures used, the EPR spectrum of the thiyl in the ligand pocket is broadened and not detected (*vide infra*).

In the pathway with the thiyl radical released, the initial low levels of a *base-off* complex with $g_{\perp}=2.272$ (E) is observed (Fig. 4). This is a 5-coordinated complex without any ligand substituting for the displaced base in the α -axial position. The dissociation of the base in this complex is facilitated by a trans effect associated with GSH bound in the β -axial site and the release of protons during the reduction process that lowers the pH. The affinity of GSH for the α -axial site is much less than that of the initial GSH bound to the β -axial site. However, with a large excess of GSH a second GSH binds in the α -axial site resulting in a complex with $g_{\perp}=2.39$ (complex F).

In the pathway with the thiyl in the ligand pocket, as soon as the base is released the thiyl radical will interact with Co(II). A transient species formed as the thiyl radical approaches Co(II) would have parameters similar to complex F with two thiols bound with $g_{\perp}\sim 2.39$. However, this species is only formed transiently, because the closer approach of the thiyl radical produces an exchange coupled complex E' with $g_{\perp}=2.19$. This complex is present whenever *base-off* complexes are observed (Figs. 5, 6b, 7a), but is most pronounced when the base is completely removed as in cobinamide (Fig. 7b) or by lowering the pH and protonating the base (Fig. 7d). At high GSH levels it is also possible that the thiyl radical bound in the α -axial position (species E') can be displaced by GSH producing complex F.

Comparison of Cbl reduction by GSH and DTT

DTT a smaller molecule with two terminal thiols reduces Cbl much more efficiently and rapidly than GSH (Fig 2). From the kinetics and spectral differences between GSH and DTT reduced Cbl, important insights can be provided into the reduction of Cbl by thiols.

Although the EPR spectrum at 77K obtained with DTT (Fig. 6a) is similar to that obtained for GSH (Figs. 4& 5), the nitrogen superhyperfine lines with DTT are more poorly resolved than for GSH. We have attributed the better resolution for GSH (see above) to the binding of the larger GSH in the β -axial position which reduces intramolecular dipolar broadening. The broadening of the hyperfine lines for DTT can, however, also be attributed to increased retention of the formed thiyl radicals in the ligand pocket.

Proof that thiyl radicals are associated with DTT reduced Cbl even when the base is bound is provided by figure 10b, where we show that lowering the pH of the DTT reduced *base-on* complex results on the formation of the exchange coupled species with $g_{\perp}=2.19$ (complex E'). The simultaneous formation of a *base-off* complex with $g_{\perp}=2.39$ (complex F) that involves the binding of two thiols instead of a $g_{\perp}=2.272$ (complex E) also indicates that

DTT is bound to the β -axial side of the corrin. Although, DTT binding to the β axial side without the interactions involving the glu and gly residues of GSH [22] is expected to be weaker than the binding of GSH, this linkage to the corrin can explain a greater retention of DTT thiyl radicals than the 50% retention observed for GSH. This retention can result in broadening of the nitrogen hyperfine lines observed with DTT. The initial β -axial binding also facilitates the access of the second DTT thiol to the α -axial ligand pocket, explaining the rapid reduction with DTT.

The binding of one of the DTT thiols in the β -axial position that increases the retention of the thiyl radical in the pocket is also expected to restrain the approach of the thiyl radical to cobalt when the base is dissociated. This can result in complex F where one of the sulfur atoms is part of a thiyl radical that does not approach Co(II) close enough to form the exchange coupled complex (E'). This is supported by the power saturation studies (Figs. 9a & 9b). Thus, for the GSH reaction (Fig. 9b) the 2.39 line (complex F), which does not involve thiyl radicals, is saturated before the 2.19 line (complex E'), which involves a thiyl radical and, therefore, has a shorter relaxation time. However, for DTT (Fig. 9a) the saturation of the 2.39 line is similar to the 2.19 line implying that the 2.39 line (complex F) also involves a thiyl radical. The resultant complex, nevertheless, has the properties of two sulfur axial ligands and has the same EPR parameters as the GSH complex F.

The dominant species when DTT reduces Cbi (Fig. 7a) is a complex with nitrogen hyperfine interactions. These nitrogen hyperfine interactions in the EPR spectrum demonstrate that in the absence of the benzimidazole group one of the phytyl groups containing a nitrogen such as the longer $-\text{CH}_2-\text{CH}_2-\text{CO}-\text{NH}-\text{CH}_2-\text{CHOH}-\text{CH}_3$ or one of the two $-\text{CH}_2-\text{CH}_2-\text{CO}-\text{NH}_2$ (see Fig.1) swing back to provide axial coordination through nitrogen, presumably in the α -axial site.

Support for this bonding is provided by a careful analysis of the EPR spectra obtained, which indicates a difference in the hyperfine splitting from ^{14}N for the benzimidazole and phytyl nitrogens. The ^{14}N hyperfine splitting is less resolved in Figure 7a for Cbi than in Figures 5 and 6b, for the benzimidazole, attributed to a reduction in the hyperfine splitting from 22.3 G to 20.3 G that is due to weaker binding. Such differences in ^{14}N hyperfine splittings have been observed in a study of the coordination of hyperlong nitrogen ligands in a series of LCo(II)Cbi^+ adducts where L represents a series of substituted pyridines with ^{14}N hyperfine splittings varying from 18.3 to 19.8G [27].

These nitrogen hyperfine lines are eliminated with an excess of GSH (Fig.7b) implying that these relatively weak interactions with the phytyl nitrogens inhibit the coordination of the DTT thiyl radicals, but not GSH or the GSH thiyl radicals (Fig. 5) with Co(II). These results are consistent with constraints on the interaction of the DTT thiyl radical imposed by binding the same molecule to the β -axial site (see above). It is only at low pH where both the benzimidazole group and any phytyl nitrogens are protonated that in the DTT spectrum the $g_{\perp} = 2.19$ is clearly observed and the $g_{\perp} = 2.39$ becomes the dominant band (Fig. 10b).

Since the $g_{\perp} = 2.19$ line (complex E') requires that the thiyl radical interact strongly with the Co(II), complex E' formed with DTT would have the interaction on the β -axial side further weakened. When an excess of GSH is added to DTT reduced Cbl (Figs. 6b & 9c) no additional thiyl radicals are formed. However, GSH, which has a much higher affinity for the β axial Co(II) will displace DTT from the β axial side of the corrin. For the $g_{\perp} = 2.19$ complex E', this reaction will not affect the properties of the complex. However, complex F with a constrained thiyl radical (see above) will be converted into the $g_{\perp} = 2.19$ complex E'. The conversion of complex F to complex E' is seen by reduction in the F/E' ratio from ~12 for DTT (Fig. 10b) to ~8 in Fig 7b after an excess of GSH is added. Furthermore, the

saturation experiment (Fig. 9c) indicates that after an excess of GSH is added saturation of the 2.39 line occurs at a lower power than the 2.19 line indicating that thiyl radicals are no longer associated with complex F.

Physiological Relevance

The Complex of GSH with cobalamin—It was first proposed by Wagner and Bernhauer that the Co(III) Glutathionylcobalamin (γ -glutamylcysteinylglycylcobalamin) could be the precursor of cobalamin coenzymes [10]. The Cbl(III)GSH formed *in vivo* can be reduced by a number of cellular reducing agents including the large excess of reduced GSH found in cells.

While the significance of the Cbl(III)GSH complex has been discussed in the earlier literature, our studies show that GSH is one of the few ligands that also bind to reduced Cbl(II). With Cbl(III)GSH the putative substrate for Cob(III)alamin reductase and the presence of high concentrations of intracellular GSH, it would be expected that the initial reduced form of cobalamin is Cbl(II)GSH. It should, therefore, be this complex which serves as an intermediate in the biosynthesis of methylcobalamin and adenosylcobalamin.

Our results clearly show that the Cbl(II)GSH complex exists as an equilibrium between a *base-on* and *base-off* complex. The binding of a relatively strong ligand like GSH in the β -axial site is expected to weaken the benzimidazole bound in the α -axial site through a trans effect contributing to this equilibrium. A number of studies indicate that the incorporation of the Cbl cofactor into enzymes involve the *base-off* configuration, which our studies show to be stabilized by GSH.

An example of this is the recent X-ray crystallographic structure determination of methylmalonyl-CoA mutase at 2.0 Å, which reveals that, as discovered for methylcobalamin dependent methionine synthase, the adenosylcobalamin cofactor is also bound in a dimethylbenzimidazole *base-off* form. The nucleotide-loop appended 5,6-dimethylbenzimidazole serves to anchor the B12 cofactor to the enzyme, and a protein side chain, histidine imidazole, serves as cobalt's axial base [38–41]. It has also been reported that methylcobinamide which completely lacks the lower axial nitrogen donor ligand to cobalt, is the substrate for acetyl-coA synthase [42].

These studies indicate that although the dimethylbenzimidazole base binds to Cbl(III), the functional role for the benzimidazole base does not seem to involve coordination to the Co(II)metal center. The weakening of the bond between the base and the metal when GSH is bound to reduced Cbl may, thus, play an important role in producing the proper configuration of the corrin cofactor in biological systems.

These results support the hypothesis that lack of a lower axial nitrogen donor ligand to cobalt in the native corrinoid iron-sulfur protein from methanogenic and acetogenic microbes enhances its propensity for reductive activation and demethylation reactions [43].

The Reduction by Thiols- the second thiol and complexes involving the thiyl radical—The major new insight in our study involve the requirement for a second thiol when GSH or other thiols reduce Cbl(III) (Fig. 3) and the finding that ~50% of the thiyl radicals formed during the reduction process remain associated with Cbl (Scheme 1). Both of these conclusions provide new insights into understanding the redox reactions which play an important role in Cbl bioactivity.

The demonstration that a peripheral site presumably located in the dimethyl benzimidazole ribonucleotide ligand cavity is responsible for the reduction of Cbl(III) to Cbl(II) instead of

coordination to one of the axial positions may play a role in understanding the role of GSH and other thiols in reducing Cbl. Furthermore, such interactions may play a role in Cbl related enzymes.

The enzymatic activation of coenzyme B₁₂ (5'-deoxyadenosylcobalamin) [28,29] initiates enzymatic radical reactions thought to require the homolytic cleavage of the carbon cobalt bond producing the 5'-deoxyadenosyl radical. This radical has, however, not been directly detected, and a peripheral interaction with a thiol or other reducing agent may help explain the enzymatic activation process. Of particular relevance to our results are the studies with ribonucleotide triphosphate reductase [33,37], which contains the deoxyadenosyl cobalamin cofactor. In this case the formation of an intermediate involving a thiyl radical coordinated to the cobalamin has been detected [33,37] with similar properties to the thiyl complexes found in our study (see above). While it has usually been assumed that the deoxyadenosyl radical reacts with Cys-408 to produce the thiyl radical [44], our results suggest the need to consider a direct interaction of the protein cysteine with the cobalamin that is analogous to the observed interaction of GSH, in our study producing a thiyl radical that coordinates with the Cbl.

CONCLUSION

The reduction process of Cbl with GSH is analyzed. It is found that GSH rapidly forms a stable complex with Cbl(III). The slow reduction requires a second GSH molecule that does not initially bind to the cobalt, but is presumably located in the dimethyl benzimidazole ligand cavity. The reduction results in the formation of thiyl radicals. About 50% of these radicals are released from Cbl to form GSSG, but the other 50% remain associated with the reduced Cbl. In the reduced state the intrinsic lower affinity for the benzimidazole base coupled with a trans effect from the initial GSH still bound in the β -axial site and a possible lowering of the pH results in an equilibrium between a *base-on* and *base-off* complex. The dissociation of the base facilitates a closer approach of the thiyl radical to the metal center resulting in a complex with ferromagnetic exchange coupling between the metal ion in the corrin ring and the thiyl radical.

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Abbreviations

Cbl	Cobalamin
Cbl(III)OH	hydroxycobalamin
Cbl(III)GSH	glutathionylcobalamin
Cbi(III)	cobinamide
GSH	reduced glutathione
GSSG	oxidized glutathione
DTT	dithiothreitol
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
DTNB	Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)

Figure 1.
Molecular Structure of Cobalamin (III) with the OH in the β -axial site and the benzimidazole nucleotide in the α axial site.

2a

2b

2c

Figure 2.

a. Time-dependent UV-Vis spectral change monitored during the reaction of 3 fold excess of GSH with 100 μ M Cbl(III)OH under anaerobic condition. The time taken for the complete formation of the Cbl(III)GSH complex is 8–9 min. The first spectrum corresponds to that of Cbl(III)OH. After a dead time of 10 sec., the accumulation of the spectra was initiated. The running time for each spectrum is 38 sec.

b. Time-dependent UV-Vis spectral change during the reaction of 50 fold excess of GSH with 100 μ M Cbl(III)OH under anaerobic condition. The initial spectrum obtained after the 10 sec dead time is the same as that of Fig.2a at the end of the reaction with a 3:1 molar ratio of GSH and Cbl(III)OH. The subsequent reduction was followed for 5 Hrs.

c. The UV-Vis spectra of the final product formed from 100 μ M Cbl(III)OH with a 50 fold excess GSH (-----) is compared with that obtained with a 3 fold excess of dithiothreitol (_____).

3b

Figure 3.

- a.** Job's plot from the UV-Vis spectral results for the reduction of Cbl(III)-OH with GSH. The fraction of reduced Cbl(II) for the Job's plot was obtained by fitting the 3 hour spectra with the spectra of Cbl(III), Cbl(III)-GSH and Cbl(II) taken from Figures.2a and 2b.
- b.** Job's plot from the EPR spectral data for the reduction of Cbl(III)-OH when incubated with GSH for 3 Hrs. The intensity of Cbl(II) is directly measured.

Figure 4.
Q band EPR spectrum of 5mM Cbl(III)-OH with 10 fold excess of GSH at 12 K (solid line) and its simulation requiring two different species (dotted line).

Figure 5.
X-band EPR spectrum of 5mM Cbl(III)-OH to which a 50 fold excess of GSH is added and measured at 77 K.

Figure 6.

a. X-band EPR spectrum at 77 K of Cbl(III)OH (5mM) reduced by DTT (20mM) at 77 K.

b. X-band EPR spectrum at 77 K of Cbl(III)OH (5mM) reduced first by DTT (20mM) to which 250mM GSH was subsequently added.

7c

7d

Figure 7.

- a.** X-band EPR spectrum at 77 K of 965 μ M Cobinamide, Cbi(III), reduced by 10mM DTT.
- b.** X-band EPR spectrum at 77 K of 965 μ M Cobinamide, Cbi(III), reduced by 10mM DTT to which 50mM GSH was subsequently added (See text).
- c.** (Middle) The X-band EPR spectrum of Cbi(III)OH reduced first by DTT and followed by GSH addition (the same as in Figure 7b reproduced for comparison with simulated spectra). (bottom) Computer Simulation of the species with $g_{\perp} = 2.39$ which is attributed to the spectrum where the base is replaced by a second GSH (Cbl(II)(GSH)₂ (**F**)). (Top) Simulation of the exchange coupled species (**E'**).

d. X-band EPR spectrum of Cbl(III) (5m) at pH 2 reduced directly by GSH. Measurement done at 8K at a microwave power of 0.02 mw. * and + respectively identify the $g_{\perp} = 2.19$ and the low intensity $A_{g_{\parallel}}$ (^{59}Co) of the exchange coupled species.

8a

8b

Figure 8.

a. EPR power saturation measurement on sample of 965 μ M Cbi(III) reduced by GSH at 77 K.

b. Temperature dependent EPR spectra of 965 μ M Cbi (III), first reduced by DTT and later reacted with GSH at a constant power of 40db (0.02mw).

9a

9b

9c **Figure 9.**

- a.** Power variation EPR measurement at 77 K of 5mM Cbl(III) at pH 2 reduced by 20mM DTT.
- b.** Power variation EPR measurement at 77 K of 5mM Cbl(III) at pH 2 reduced by 250mM GSH.
- c.** Power variation EPR measurement at 77 K of 5mM Cbl(III) at pH 2 first reduced by 20 mM DTT with 250mM GSH added subsequently.

10a&b **Figure 10.**

The EPR spectra describing the effect of lowering the pH on DTT reduced cobalamin: (a) cobalamin reduced by DTT at pH 7.4; (b) the pH of the DTT-reduced cobalamin was then lowered to pH 2.

Scheme I.
Sequence of reduction of Cbl(III)OH with GSH.