Synthesis, Characterization, Solution Stability, and X-ray Crystal Structure of the Thiolatocobalamin γ-Glutamylcysteinylcobalamin, a Dipeptide Analogue of Glutathionylcobalamin: Insights into the Enhanced Co−S Bond Stability of the Natural Product Glutathionylcobalamin

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Glutathionylcobalamin (γ-glutamylcysteinylglycinylcobalamin; γ-GluCysGly-Cbl) is a natural product which functions as an intermediate in the biosynthesis of the active B_{12} coenzymes adenosylcobalamin and methylcobalamin. Of interest to the present studies is glutathionylcobalamin's unique stability in comparison to other thiolatocobalamins, notably the $\geq 6 \times 10^4$ fold less stable cysteinylcobalamin, Cys-Cbl. In order to determine which parts of the glutathione tripeptide contribute to the overall stability of glutathionylcobalamin, two cysteine-containing dipeptides, which are truncated versions of glutathione, were used to synthesize their corresponding cobalamins, specifically γ-glutamylcysteinylCbl (γ-GluCys-Cbl) and cysteinylglycinylcobalamin (CysGly-Cbl). As with glutathionylCbl, *the dipeptide γ-GluCys-Cbl forms a stable thiolatocobalamin. However and most interestingly, CysGly-Cbl is observed to be unstable much like Cys-Cbl*. The results require that the extra stability of glutathionylcobalamin and its congeners, compared to cysteinylcobalamin and its analogues, must be derived from *destabilization* by the γ-NH_3^+ group in cysteinylcobalamin, or *stabilization* by the γ-NHC(═O)− amide linkage in glutathionylcobalamin, *or both*. To probe any ground-state structural basis for the possible stabilization in γ-GluCys-containing cobalamins, γ-GluCys-Cbl was crystallized and yielded the first X-ray structural determination of a true *thiolato*cobalamin, and only the second structure of a cobalamin containing a Co−S bond, the first example being Randaccio and co-workers' 1999 structure of the thioketone complex, thioureacobalamin, $(NH_2)_2$CSCbl. Key features of the structure of γ-glutamylcysteinylcobalamin include (i) a normal Co−S bond length of 2.267(2) Å, (ii) a Co−N(axial) bond length of 2.049(6) Å, (iii) two alternate conformations of the γ-glutamylcysteinyl moiety, and (iv) folding of the corrin ring upward by 24.2°, the highest degree of folding yet observed for a cobalamin. These results do not show any strong stabilization (e.g., no shortened Co−S bond), although it is not clear for certain what the effect is (stabilizing or destabilizing) of the elongated Co−N(axial) bond; instead, the crystallographic results suggest that the metastable Cys-Cbl probably has a Co−S cleavage transition state that is stabilized (along with, possibly, any ground-state destabilization of the Co−S bond). Overall, the results strongly suggest that placing a positive charge on the γ-NH_3^+ stabilizes the Co−S bond cleavage transition state, thereby setting the stage for the needed full thermolysis product and kinetic studies—as a function of the axial-base on−off equilibrium—that will be required to understand in even greater detail the unique stability of glutathionyl- (γ-glutamylcysteinylglycinyl-) and γ-glutamylcysteinylcobalamins.

Introduction

Glutathione (γ-glutamylcysteinylglycine, γ-GluCysGly) is a tripeptide of unique structure, in that the glutamate residue forms the peptide bond through the γ-carboxyl group rather than the normal α-carboxyl group, Figure 1. Consequently, the elongated main chain is more conformationally flexible, and not suitable for protease digestion. Glutathione is a major intracellular reducing agent present in almost all biological tissues (with typical intracellular concentrations of 1−2 mmol/dm^{-3})[1] and also plays important roles in biosynthesis, metabolism, transport, and the protection against adventitious free radicals.[2,3]

Glutathionyl*cobalamin* (γ-glutamylcysteinylglycinylcobalamin) is a naturally occurring intracellular form of cobalamin found in mammalian cells, one that is more readily absorbed and retained longer than cyanocobalamin (CNCbl, vitamin B_{12}).[4−9] In vivo, glutathionylcobalamin is an intermediate in

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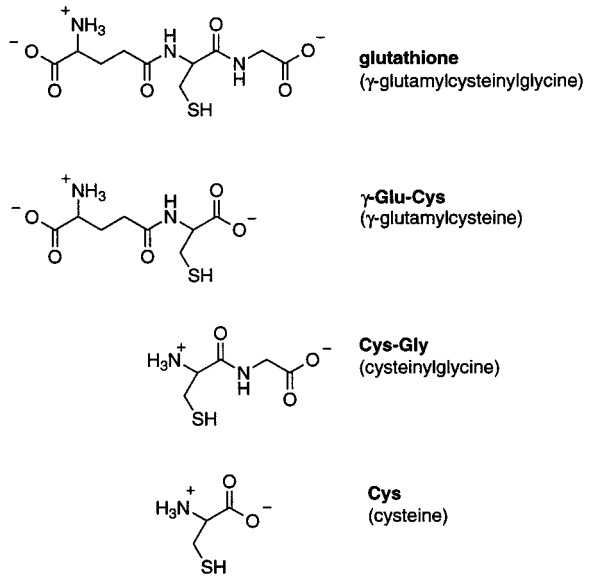

Figure 1. Structural formulas of the cysteine-containing peptides used to synthesize the thiolatocobalamins discussed.

the conversion of the biologically inactive cyanocobalamin to the active coenzyme forms, adenosylcobalamin (coenzyme B_{12}) and methylcobalamin.[6] Two enzymes are known to be involved in the intracellular metabolism of cyanocobalamin.[10] First, cyanocobalamin is decyanated by a cytosolic enzyme, using FAD, NADPH, and glutathione to generate glutathionylcobalamin (a process that may be related to other glutathione reductases that are involved in a variety of biological processes).[11] The glutathionylcobalamin can then be reduced by NADH-linked cob(III)alamin reductases, located on the microsomes and the inner mitochondrial membrane,[10] prior to the conversion to adenosylcobalamin or methylcobalamin.

In vitro, glutathionylcobalamin can be prepared by reacting glutathione and aquacobalamin (H_2OCbl^+) to form a stable thiolatocobalamin complex of 1:1 stoichiometry.[4,12,13] In contrast, other thiols such as cysteine, homocysteine, and mercaptoethanol form relatively unstable thiolatocobalamins, ones which decompose rapidly at 25 °C to yield, at least initially, a thiyl radical and cob(II)alamin.[13−18] The reason for the unique stability of glutathionylcobalamin in comparison to many other thiolatocobalamins, notably cysteinylcobalamin, has remained a mystery.

Previously, and in an effort to understand the high stability of glutathionylcobalamin, we obtained single crystals of glutathionylcobalamin, but X-ray crystallography exhibited unresolvable disorder in the glutathionyl moiety.[19] Herein, and as an alternative approach toward understanding any structural factors associated with the stability of glutathionylcobalamin and its glutathionyl tripeptide moiety, we used two cysteine-containing naturally occurring dipeptide fragments of glutathione,[2] γ-glutamylcysteine (γ-GluCys) and cysteinylglycine (CysGly), Figure 1 and Table 1, to synthesize their corresponding thiolatocobalamins, γ-GluCys-Cbl and CysGly-Cbl.[20] These two thiolatocobalamin analogues of glutathionylcobalamin were then examined for their relative stability in comparison to both the parent complex, glutathionylcobalamin, and cysteinylcobalamin. We were also able to obtain the X-ray crystal structure of γ-glutamylcysteinylcobalamin. When combined, the stability and X-ray crystallography results support a mechanism in which a positively charged $-NH_3^+$ in Cys-Cbl destabilizes the Co−S bond in comparison to the amide bond in γ-GluCys-Cbl and γ-GluCysGly-Cbl (=glutathionylcobalamin).

Table 1. Tabulation of the Physical Properties of the Thiols Used

compound	MW	pK_a[53]	% RS^- at pH 7.4[a]	apparent stability of thiolatocobalamin
γ-GluCys	250.3	9.9[b]	∼1	stable
γ-GluCysGly	307.3	9.2[b]	∼1	stable
Cys	121.2	8.53[c]	6	metastable
CysGly	178.2	7.87[c]	11	metastable

[a] Taken from Table 3 elsewhere.[53] [b] *Apparent* pK_as, i.e. not just of the RSH group. [c] The pK_a of $H_3N^+RSH \rightleftharpoons H_3N^+RS^- + H^+$.

Experimental Section

Materials. All materials, unless otherwise noted, were obtained from Sigma and used without further purification. The γ-glutamylcysteine (γ-GluCys) dipeptide was obtained from Sigma as the trifluoroacetate salt and contained 73% peptide content, with 89% peptide purity (Sigma's analysis by HPLC). Distilled water was purified by a Barnstead NANOpure system.

Thiolatocobalamin in Situ Stability Studies. UV−visible analyses were conducted with a Hewlett-Packard 8452A diode array spectrometer. A 50 μM stock solution of H_2OCbl^+ was made with 50 mM KH_2PO_4/K_2HPO_4 (pH 7.2) as the buffer (extinction coefficients for H_2OCbl^+: $\epsilon_{411} = 3700$; $\epsilon_{499} = 8100$; $\epsilon_{527} = 8500$).[21] Each thiol compound was dissolved in H_2O to roughly 50 mM, and the exact thiol concentration was determined using Ellman's reagent (4 mg of 5,5′-dithiobis(2-nitrobenzoic acid) in 1 mL of 0.1 N Na_2HPO_4, pH 8).[22] Specifically, 10 μL of thiol solution and 100 μL of Ellman's reagent were mixed with 5 mL of 0.1 N Na_2HPO_4 (pH 8) and the thiol concentration was determined by measuring the absorbance at 412 nm ($\epsilon_{412} = 13600$).[22] The formation and relative stability of each thiolatocobalamin were ascertained by following the changes in the absorbance spectrum (300−800 nm). To 1 mL of 5×10^{-5} M H_2OCbl^+ in 0.05 M potassium phosphate buffer (pH 7.2) was added 1.5 μL of 50 mM thiol solution (1.5 equiv of thiol to cobalamin), followed by mixing. The UV−visible spectra were observed over 1 h, at 25 °C, under aerobic conditions, with loss of H_2OCbl^+ monitored at 374 nm and formation of thiolatocobalamin monitored at 370 nm (see the Supporting Information for further details).

Bulk Synthesis, Characterization, and Crystallization of γ-Glutamylcysteinylcobalamin. γ-Glutamylcysteinylcobalamin was prepared in milligram quantities by the following procedure. A solution (22 mM) of 22 mg of $H_2OCbl{\cdot}X$ (HCl or acetate form; $X = Cl^-$ or OAc^-) in 0.58 mL of 0.1 M MES (Na^+ form, pH 5.5) was syringe filtered (using a 0.2 μm membrane filter) and added to 7 mg of solid γ-GluCys (1.5 equiv). The reaction solution was mixed and then stored in a glass vial in the dark at 0 °C for several hours. During this time precipitate and

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Table 2. Crystal Data for γ-Glutamylcysteinylcobalamin

empirical formula	$C_{70}H_{102}N_{15}O_{20}PCoS$
fw	1578.6
temp	168(2) K
wavelength	0.71073 Å
cryst syst, space group	orthorhombic, $P2_12_12_1$
unit cell dimens	$a = 16.3183(8)$ Å
	$b = 21.1052(9)$ Å
	$c = 25.8890(12)$ Å
	$\alpha = 90$ deg
	$\beta = 90$ deg
	$\gamma = 90$ deg
vol	8916.2(7) Å^3
Z, calcd density	4, 1.333 Mg/m^3
abs coeff	0.32 mm^{-1}
$F(000)$	3764
cryst size, color	0.16 × 0.28 × 0.32 mm, red
θ range for data collection	1.24−28.31 deg
limiting indices	$-19 \le h \le 21$, $-28 \le k \le 24$, $-34 \le l \le 32$
reflns collected/unique	59247/21226 [R(int) = 0.1018]
abs correction	SADABS (T_{max} 0.98; T_{min} 0.81)
refinement meth	full-matrix least-squares on F^2
data/restraints/params	21210/58/1169
GOF on F^2	1.047
final R indices [$I > 2\sigma(I)$]	R1 = 0.1088, wR2 = 0.2513
R indices (all data)	R1 = 0.1868, wR2 = 0.3011
absolute structure param	0.02(3)
largest diff peak and hole	1.06 and −0.46 e A^{-3}

small crystals of γ-glutamylcysteinylcobalamin formed. The precipitate and crystals were removed from the bulk solution by filtration and washed with cold acetone, and this material was then used in the further characterization studies which follow. Crystals suitable for structure determination were obtained by storing the above reaction solution at 4 °C for several days before harvesting individual single crystals suitable for X-ray diffraction.

Further characterization of the γ-glutamylcysteinylcobalamin was accomplished as follows: ^{1}H NMR (5 mg in 0.8 mL of CD_3OD at room temperature) was obtained in the 7.4−5.8 ppm region according to our recently developed ^{1}H NMR method for assaying for the presence of any cobalamin impurities;[23] the ^{1}H NMR aromatic-region signals (referenced to tetramethylsilane) are δ 7.17, 7.01, 6.45, 6.20 (d), and 6.06 with no significant level of impurities (save a few small peaks known to be due to impurities in the commercial $H_2OCbl{\cdot}X$ starting material;[23] Supporting Information Figure E). LSIMS (in *m*-nitrobenzyl alcohol matrix) was also obtained and is consistent with the expected composition and molecular weight,$C_{70}H_{101}N_{15}O_{19}PSCo$ (calcd molecular mass = 1578.6; found m/z $[M]^+$ = 1578.7; Supporting Information, Figure F).

Crystallographic Data. A red crystal of γ-glutamylcysteinylcobalamin, with dimensions 0.16 × 0.28 × 0.32 mm, was placed under oil and rapidly cooled to −105 °C in the cold stream of the X-ray diffractometer. The diffraction data were obtained on a Bruker SMART CCD diffractometer using Mo Kα radiation. Crystal data is listed in Table 2. A total of 21220 unique reflections within the 2θ range from 2.48° to 56.62° were measured.

Structure Determination by X-ray Diffraction. The Bruker SHELXTL software package (version 5.03) was used to solve the crystal structure by direct methods and difference Fourier techniques. The atoms of the γ-glutamylcysteine moiety were found in two alternate conformations of roughly equal occupancy. Two water networks were also modeled around these two conformations. All non-hydrogen atoms were refined anisotropically, except for the solvent water molecules associated with each of the two γ-glutamylcysteine moiety conformations. Hydrogen atoms were placed at calculated positions and refined by using a riding model. All Fourier peaks less than 1.07 e Å^{-3}, that were associated with the solvent, were not refined as individual atoms. The Na^+ cation necessary for charge balance was definitively observed only for a single conformation of the γ-glutamylcysteine moiety. All peaks associated with the second conformation were modeled as a collection of water molecules, with the assumption that the remaining Na^+ cation was disordered within this region. The two γ-glutamylcysteine conformations were subjected to a rigid bond restraint (DELU) and a similarity restraint (SIMU). Several side chain amide atoms and R5 had elevated thermal displacement parameters, but we were unable to satisfactorily resolve the disorder at these positions.

γ-Glutamylcysteinylcobalamin Thermal Stability and Red Light Photosensitivity Studies. A solution of γ-GluCys-Cbl was made (∼0.5 mg of solid γ-GluCys-Cbl dissolved in 3.0 mL of 0.05 M potassium phosphate buffer, pH 7.2). Under red light illumination only, the solution was placed in a UV−visible spectrophotometer at 25 °C and left there between spectra which were taken at 4 h intervals over 5 days. The slight increase in the absorbance at 350 nm and slight decrease at 376 nm were plotted vs time and apparent first-order rate constants were obtained for the two sets of data using the Hewlett-Packard Chemstation Software (1996). From the resultant first-order rate constants, an average half-life of $\sim 1.1 \times 10^4$ min was estimated. A separate but otherwise identical sample which was left outside the UV−visible spectrophotometer between spectra, and thus which was exposed essentially continuously to red light, showed a noticeably higher amount of decomposition over the 5 day period; that is, γ-GluCys-Cbl is photosensitive (as presumably the other RSCbl's also are) to even red light over longer periods of time.

Results and Discussion

γ-Glutamylcysteinylcobalamin and Cysteinylglycinylcobalamin in Situ Formation and Apparent Stability Studies. To begin, we surveyed the ability to form the truncated glutathionylcobalamin analogues, γ-GluCys-Cbl and CysGly-Cbl, in situ and then compared their stability in solution to Cys-Cbl: would they be stable? Could we then isolate and fully characterize at least one of them and, perhaps, even obtain the first single-crystal X-ray structure of a thiolatocobalamin, the first structure related to the naturally occurring glutathionylcobalamin, γ-GluCysGly-Cbl? By way of reference, glutathionylcobalamin is relatively stable in light and air (but will decompose under strong light) and is isolatable out of an aqueous solution.[13,15] However, under these conditions, cysteinylcobalamin is metastable in solution only in the presence of excess cysteine.[12,13,15] For our stability studies, a physiologically relevant pH value of 7.2 and temperature of 25 °C were employed.

Upon their addition to H_2OCbl^+ at pH 7.2 and 25 °C, both γ-GluCys and CysGly dipeptides were found to form thiolatocobalamins with clean isosbestic points, at least *initially*, as evidenced by the formation of the double-peaked UV spectrum in the 270−290 nm region (see the Supporting Information for spectra, Figures A−D). This spectral feature is diagnostic of cobalt−sulfhydryl bonds in cobalamins.[12] The γ-GluCys-Cbl retained its clean isosbestic points (Figure C of the Supporting Information) at 338, 364, 448, and 534 nm for >16 h with no detectable decomposition, so that its half-life for decomposition can be estimated as $t_{1/2} > 10 \times 16$ h × 60 min/h = $t_{1/2} \ge 1 \times 10^4$ min at room temperature. A separate study of the rate of decomposition of γ-GluCys-Cbl under red-light-only conditions yielded a $t_{1/2} \approx 1.1 \times 10^4$ min at 25 °C (see the Experimental Section). On the other hand, CysGly-Cbl proved to be metastable, beginning to lose its isosbestic points after only 30−40 min; its decomposition half-life can be estimated to be $t_{1/2}$ = ca. 30 min (Figure B of the Supporting Information). Of interest here is that at the same pH of 7.2, but at the even lower, stabilizing temperature of 15 °C, Cys-Cbl decomposes completely in <1 min, $t_{1/2} \le 10$ s.[13] Hence the *apparent* stability order is glutathionylcobalamin (γ-GluCysGly-Cbl) ∼ γ-GluCys-Cbl ≫ CysGly-Cbl ≫ Cys-Cbl and by factors in their $t_{1/2}$ of decomposition of $>6 \times 10^4$,1.8×10^2, and 1.0, respectively. There is, therefore, a difference of ca. 6 kcal/mol at 25 °C in

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the $\Delta G^{\ddagger}$(apparent) of decomposition of γ-GluCysGly-Cbl and γ-GluCys-Cbl vs Cys-Cbl.

Since the γ-GluCys-Cbl complex is stable like glutathionylcobalamin (γ-GluCysGly-Cbl), yet the CysGly-Cbl complex is less stable, it follows that these experiments have revealed that the γ-GluCys-Cbl complex is stabilized toward Co−S bond cleavage by either (i) the *absen*ce of the $-NH_3^+$ moiety that is otherwise present in in CysGly-Cbl and Cys-Cbl (Figure 1) or (ii) the *presen*ce of the amide bond to the γ-glutamate group in γ-GluCys-Cbl and γ-GluCysGly-Cbl (Figure 1), or (iii) the *presence* of the glutamate α-carboxylate (i.e., by this carboxylate somehow interacting with another part of the cobalamin). Alternatively, (iv) some combination of i−iii could be responsible for the enhanced stability of γ-GluCys-Cbl and γ-GluCys-Gly-Cbl. Note that these experiments do not reveal if this stability is primarily kinetic or thermodynamic in origin, *but they do pinpoint for the first time the source of the stability difference between the stable glutathionyl- and related cobalamins vs the at best metastable cysteinyl- and related cobalamins.*

Larger Scale Synthesis, Isolation, and Crystallization of γ-Glutamylcysteinylcobalamin. In order to begin to gain any possible structural insights into the stability of glutathionylcobalamin and its congeners, γ-GluCys-Cbl was synthesized in larger quantities, isolated, and then crystallized in a form suitable for single-crystal X-ray crystallography. Since γ-GluCys-Cbl is less soluble in aqueous solution than its precursor, aquocobalamin, the addition of the dipeptide γ-glutamylcysteine to a relatively high concentration of H_2OCbl^+ results in precipitation and crystallization of γ-GluCys-Cbl from aqueous solution, thereby driving the reaction to completion. The resultant γ-GluCys-Cbl was isolated simply by filtering it away from the reaction solution. Crystals suitable for X-ray crystallography were produced during one synthesis by prolonged storage of the reaction solution as detailed further in the Experimental Section. The structure of γ-GluCys-Cbl which follows is the first true *thiolato*cobalamin whose structure has been determined, and only the second structure of a B_{12} complex with a Co−S bond, the first being thiourea complex, $(NH_2)_2CSCbl$, by Randaccio's group in 1999.[24] Worth noting here, however, is the expected component of thiolatocobalamin electronic character in Randaccio's thiourea complex due to the thiourea resonance structures, $H_2N^+{=}C(S^-){-}NH_2 \leftrightarrow H_2N{-}C(S^-){=}N^+H_2$, especially when S-bound to the cobalt atom in cobalamin.

Crystal Structure of γ-Glutamylcysteinylcobalamin. "Complete corrinoids" contain the corrin ring and the axial base, but the upper axial ligand can vary.[25] Generally, these crystallize in the $P2_12_12_1$ orthorhombic space group with four molecules per unit cell, and the crystals tend toward a box-like appearance. Despite variations in the upper (β) axial ligand, the similar strong packing interactions of the corrin and the axial base (α) regions of other cobalamins often result in similar crystal lattices. γ-GluCys-Cbl provides no exception, packing in the $P2_12_12_1$ space group with one of the four typical packing types (c/a = 1.59, b/a = 1.29; cluster I).[26,27]

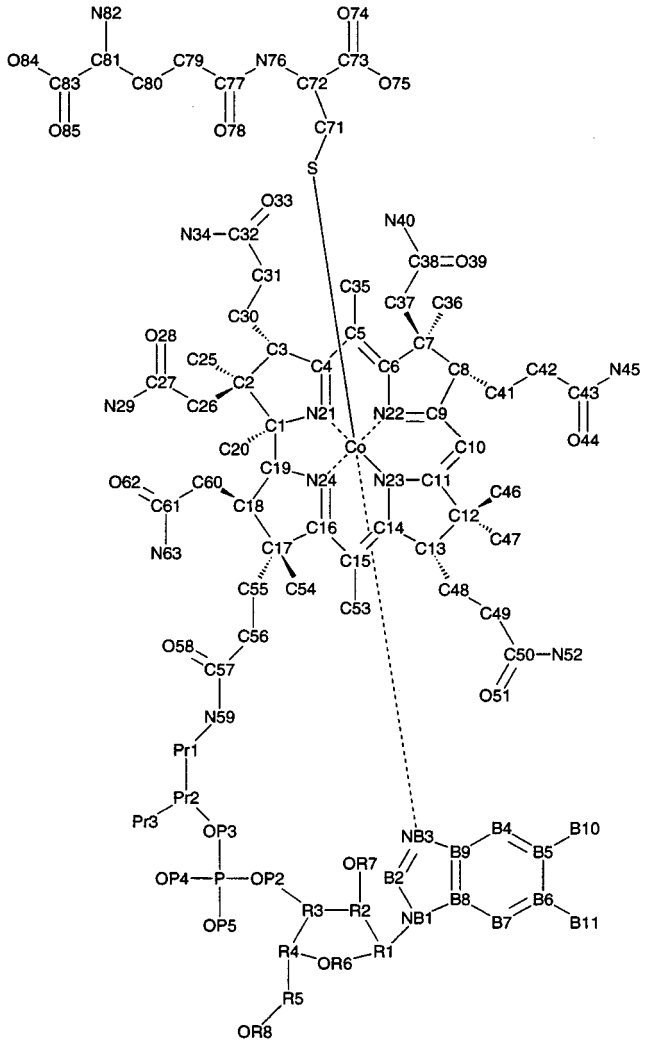

Figure 2. Atom-numbering system used for the X-ray structural analysis of γ-glutamylcysteinylcobalamin.

The *R*-factor that we observe of 10.9% for γ-GluCys-Cbl is in the middle of the range (4−16%)[26] of other published corrins, an *R*-factor range which includes the best structures obtained using synchrotron radiation. The large residual indices typically associated with corrin structures are often due to solvent disorder, side chain disorder within the cobalamin, and the strength of the radiation source.[27,28] The packing of the corrin in γ-GluCys-Cbl results in a large solvent-filled channel, extending parallel to the 2-fold screw along the *c* axis, into which the glutamylcysteine ligand extends. Two alternate conformations of the γ-glutamylcysteinyl moiety are observable, Figure 3. In solution the glutamic acid residue has multiple possible rotamers (e.g., see the nine rotamers considered in an NMR study elsewhere[29]), so that we are fortunate in seeing only two rotamers in the solid state. This type of disorder was also observed in our attempts to solve the structure of glutathionylcobalamin, but was unresolvable in that case and is why only a footnote describing that only partially refined structure was ever published.[19]

In adenosylcobalamin two major solvent distribution networks have been described.[30−32] The solvent is distributed between

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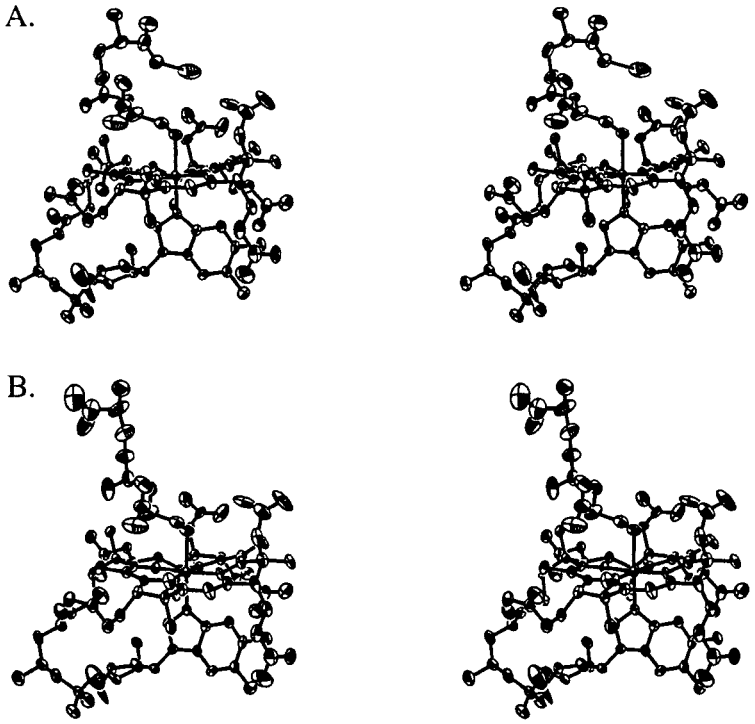

Figure 3. Stereoview of γ-glutamylcysteinylcobalamin crystal structures (50% probability) with the two observed, disordered conformation A (top) and conformation B (bottom) of the γ-glutamyl group.

Table 3. Comparison of Co−S Containing Cobalamin Features

feature	γ-GluCys-Cbl	$(NH_2)_2$CSCbl[24]	Δ γ-GluCys-Cbl − $(NH_2)_2$CSCbl
Co−S (Å)	2.267(2)	2.300(1)[34] [2.216(7)[24]]	−0.033
Co−NB3 (Å)	2.049(6)	2.032(5)[34] [2.01(1)[24]]	+0.017
Co−N21 (Å)	1.885(5)	1.853(9)	+0.032
Co−N22 (Å)	1.902(6)	1.896(9)	+0.006
Co−N23 (Å)	1.914(6)	1.91(1)	+0.003
Co−N24 (Å)	1.891(5)	1.881(9)	+0.010
corrin fold (deg)	24.2	14.9	+9.3

two regions: the pocket region with ordered waters, and the channel region with disordered water molecules. In the structure of γ-GluCys-Cbl, two water networks were also found, one for each of the two disordered conformations of the glutamyl residue. The water networks were evaluated according to the criteria developed previously for corrin water networks by Bouquiere et al. (see section 5, "Criteria for formulation of water networks" [32]).

The glutamylcysteinyl moiety is bound to the cobalamin through the sulfur atom with a typical, 2.267(2) Å Co−S bond length,[33] one a bit shorter, as expected, than the dative Co−S bond in the thioketone $(NH_2)_2$CSCbl characterized in the most recent structural work[24,34] by Professor Randaccio and coworkers, Table 3. The X-ray crystal structure of γ-GluCys-Cbl agrees well with the extended X-ray absorption fine structure (EXAFS) analysis of glutathionylcobalamin which gave a Co−S bond distance of 2.28 Å ± 0.05 Å.[33] However, and although the Co−S and Co−N_{eq} bond lengths are in agreement (within experimental error) of that predicted for glutathionylcobalamin by EXAFS,[33] the Co−N(DMB) bond length in γ-GluCys-Cbl is roughly 0.1 Å shorter (2.049(6) Å) than that predicted for glutathionylcobalamin by EXAFS. This same EXAFS Co−N(DMB) bond length discrepancy was also observed in the crystallographic examination of $(NH_2)_2$CSCbl, where a 2.01(1) Å Co−N(DMB) bond length is reported.[24] In our partially refined structure of glutathionylcobalamin[19] (see footnote 17 therein), a Co−N(DMB) bond length of approximately 2.05 Å is seen, in agreement with the value found herein for γ-GluCys-Cbl.

The upward fold angle of the corrin ring is 24.2°.[35] *This is the largest fold angle yet observed in a cobalamin*; previous fold angles ranged from 10.9 to 20.7 Å.[25,36] With a Co−N(DMB) distance of 2.049(6) Å, the upward fold falls outside the correlation previously observed between these two parameters, where as the dimethylbenzimidazole base is pulled closer to the cobalt by the β ligand, the corrin is pushed upward by the steric bulk of the dimethylbenzimidazole group.[25,26] This large fold is presumably in part due to the long Co−S bond of 2.267 Å (cf. typical Co−C bonds of 1.92−2.08 Å), but the smaller, only 14.9°, fold angle in $(NH_2)_2$CSCbl with its even longer, 2.300(1) Å, Co−S bond (and a shorter Co−N(DMB) bond),[24] Table 3, indicates that other factors are at work here. Such large corrin folds, especially at larger Co−X (X = C, S, other) axial distances, are of general interest concerning the still much debated mechanism of Co−C cleavage in AdoCbl-dependent enzymes (for lead references, see elsewhere[37−39]).

(31) Savage, H. F. J.; Lindley, P. F.; Finney, J. L. *Acta Crystallogr.* **1987**, *B43*, 280−295.
(32) Bouquiere, J. P.; Finney, J. L.; Savage, H. F. J. *Acta Crystallogr.* **1994**, *B50*, 566−578.
(33) Scheuring, E. M.; Sagi, I.; Chance, M. R. *Biochemistry* **1994**, *33*, 6310−6315.
(34) Randaccio, L. Private communication of unpublished results.
(35) The dihedral angle is defined as the angle between the planes defined by atoms of plane 1 (atoms N21, C4, C5, C6, N22, C9, C10) and plane 2 (atoms C10, C11, N23, C14, C15, C16, N24), Figures 2 and 3. Note that the atoms themselves deviate from their planes with mean deviations from plane 1 = 0.0737 Å and those from plane 2 = 0.0360 Å.
(36) See also the essentially equivalent fold angle vs Co−NB3 plot in Figure 6 in Randaccio's recent paper, ref 27. Of interest is that Coβ-cyanoimidazolylcobamide also falls off the rough correlation in that figure, but with an unusually *small* fold angle.
(37) Sirovatka, J. M.; Rappé, A. K.; Finke, R. G. *Inorg. Chim. Acta* **2000**, *300−302*, 545−555.

Additionally, N40 is hydrogen bonded to the sulfur atom (the S−N40 distance is 3.413 Å), another probable contributor to the upward fold of the corrin. This type of intramolecular hydrogen bond between N40 and the β axial ligand has also been observed in the LiCl salts of chloro- and azidocobalamin and in SO_3−Cbl.[24,40] In aquocobalamin perchlorate, an intramolecular hydrogen bond occurred between the axial water ligand and O39.[25]

The 1:1 molar complex of glutathione and cobalamin is neutral at pH 2.5, has one negative charge from pH 4 to pH 7, and has two negative charges at pH 11.[4] Glutathione is zwitterionic like all peptides, but the glutamyl carboxyl group ($pK_a = 2.3$) has a lower pK_a than the main chain carboxyl group ($pK_a = 3.3$).[29,41] Therefore, the Na^+ counterion is expected to be localized on the glutamate carboxylate group, given the pH of our synthesis and crystallization, pH 5.5. However, while a Na^+ countercation was definitely located for only one conformation of the γ-glutamylcysteine moiety, the other countercation was unresolvable in the water network due to additional disorder.

Toward an Understanding of the Stability of γ-Glutamylcysteinylcobalamin and γ-Glutamylcysteinylglycinylcobalamin. A full product and kinetic study of the thermolysis of γ-GluCys-Cbl, γ-GluCysGly-Cbl, and the less stable CysGly-Cbl and Cys-Cbl, all under controlled pH, ionic strength, and other conditions (and with attention to whether the products are Co−S homolysis, heterolysis, or both, as well as attention to the already demonstrated sizable role of the axial base in Co−SR complex stability[13]—that is, a full study), will be required to completely understand the stability difference of the γ-glutamyl- vs cysteinyl-containing cobalamins. In particular it should be noted that the relative stabilities that we have determined, while quite useful, are kinetic composites and not relative rate constants for individual elementary steps, as will ultimately prove most valuable. However, it is useful to summarize briefly here both some possible contributing factors to Co−SR bond stability or instability and factors that can be ruled out at this time.

The first possibility is that the availability of an adjacent γ-NH_3^+ group in CysGly-Cbl and Cys-Cbl (but not glutathionylCbl or GluCys-Cbl, see Figure 1) could form a five-membered-ring, H-bonded intermediate with S of the Co−S bond, thereby leading to more rapid Co−S heterolysis (or, conceivably, more rapid homolysis) via, presumably, primarily transition state stabilization. Note also that such a species would be electron withdrawing from Co and thus should promote Co−SR cleavage-enhancing axial 5,6-dimethylbenzimidazole base coordination.[13] Second, it is possible that the presence of a γ-glutamate group has a heretofore unrecognized stabilizing effect on the Co−S bond; however, this is not obvious chemically and is not seen in the otherwise normal Co−S bond length, so that this second possibility is deemed less likely. Third, solvent cage effects[42,43] likely contribute at least somewhat to the higher apparent stability of γ-CysGly-Cbl vs simple Cys-Cbl (i.e., relative abilities of the larger, more massive γ-CysGly$^\bullet$, vs the smaller, less heavy Cys$^\bullet$, to escape $Co^{II}Cbl^\bullet$ within the solvent cage). However, the predicted fractional cage recombination efficiency, F, as a function of the mass and effective radius of the departing fragment has recently been shown by Tyler and co-workers[44] to be $F\alpha(\text{mass})^{1/2}/\text{radius}^2$, just as Noyes predicted long ago,[45−48] so that this effect is predicted to be relatively small vs the observed $>6 \times 10^4$ stability difference.

A fourth possibility is the one suggested by Brown and co-workers[49] based on NMR spectroscopic studies of glutathionylcobalamin. Specifically, based on NOE observations, these authors suggested that the glutamate α-amino group may be hydrogen bonded to the f side-chain carbonyl oxygen (O58).[49] Although the γ-glutamylcysteinyl moiety is disordered in two conformations in the crystal structure reported above, no intramolecular hydrogen-bonding interaction is observed between the glutamate residue and the f side chain, so that this type of interaction is not seen *in the solid state*. Of course in solution the glutathionyl group will have a higher degree of conformational flexibility, so the possibility still remains that this NMR-observed intramolecular interaction could be contributing to the stability difference documented as part of the present work. Last, a fifth possibility is that the α-carboxylate group of the glutamate residue could cause stabilization through interaction with the corrin ring or its appendages. However, as with the α-amino group, no such interaction is observed in at least the crystal structure; hence, this possibility, too, is deemed less likely. The possibilities above form a concise basis from which to launch the needed Co−S cobalamin complex thermolysis product and kinetic studies, and to be certain that the apparent stabilities documented herein hold up under more rigorous kinetic examination.

Biological Implications. It is useful to reflect for a moment on the natural product glutathionylcobalamin. A conceivable function of the relatively stable intermediate, glutathionylcobalamin, could be to prevent H_2OCbl^+ from reacting with adventitious protein cysteine groups. Such a process is known to generate protein-cobalamin adducts.[51] If formed, such Co−S adducts could block the function of the enzyme contributing the cysteinyl group or, if such adducts were unstable as is the case of simple Cys-Cbl, the process would lead to the formation of a reactive cysteine radical and cob(II)alamin radical pair. Interestingly, this function would parallel that of some of the other known functions of glutathione, namely, that of serving as a detoxification agent.[52]

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(39) Brown has recently hypothesized that, at the elongated [Co- - -C]‡ distance of the Co−C cleavage transition state, a significant upward fold of the corrin can be part of a "transition-state mechanochemical" effect enhancing cleavage of the Co−C bond, ref 38.
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(52) The conversion of CNCbl to glutathionylcobalamin is a reaction reminiscent of the reaction effected by the family of glutathione S-transferases, ref 10, enzymes which catalyze the nucleophilic attack of glutathione on a broad range of target molecules possessing electrophilic centers; the results are thioether products, ref 1. This process generally serves as a detoxification mechanism for electrophilic metabolites, forming less toxic compounds that are more water soluble than the original compound, ref 3. However, glutathione S-transferases are also involved in forming glutathione adducts in the biosynthesis of compounds such as leukotrienes and estrogens, ref 2.
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Conclusions

The primary conclusions of this work are as follows: (i) the apparent stability series is γ-GluCysGly-Cbl $\sim$ γ-GluCys-Cbl $\gg$ CysGly-Cbl $\gg$ Cys-Cbl and spans a range of rates of decomposition of $>6 \times 10^4$; (ii) the extra stability of glutathionylcobalamin and related cobalamins, compared to cysteinyl and its related cobalamins, appears to derive from destabilization by the γ-NH_3^+ group in cysteinyl cobalamin, or possibly stabilization by the γ-NHC(=O)− amide linkage in glutathionylcobalamin, or both; (iii) the crystal structure of γ-GluCys-Cbl was reported, the first structure of a true thiolatocobalamin; and (iv) the key results from crystallographic investigation are the Co−SR distance of 2.267(2) Å, a Co−N(axial base) distance of 2.049(6) Å, and a record 24.2° corrin fold angle.

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Supporting Information Available: Tables of crystal data, atomic coordinates, bond lengths and angles, and anisotropic thermal parameters for γ-glutamylcysteinylcobalamin. UV−visible, ^{1}H NMR, and FAB-MS spectra for γ-glutamylcysteinylcobalamin. Spectral changes during the formation of the thiolatocobalamins. This material is available free of charge via the Internet at http://pubs.acs.org.

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