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Studies on the Formation of Glutathionylcobalamin: Any Free Intracellular Aquacobalamin Is Likely to Be Rapidly and Irreversibly Converted to Glutathionylcobalamin†

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A decade ago Jacobsen and co-workers reported the first evidence for the presence of glutathionylcobalamin (GSCbl) in mammalian cells and suggested that it could in fact be a precursor to the formation of the two coenzyme forms of vitamin B_{12}, adenosylcobalamin and methylcobalamin (Pezacka et al. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 443). It has also recently been proposed by McCaddon and co-workers that GSCbl may be useful for the treatment of Alzheimer's disease (McCaddon et al. *Neurology* **2002**, *58*, 1395). Aquacobalamin is one of the major forms of vitamin B_{12} isolated from mammalian cells, and high concentrations of glutathione (1–10 mM) are also found in cells. We have now determined observed equilibrium constants, K_{obs}(GSCbl), for the formation of GSCbl from aquacobalamin and glutathione in the pH range 4.50–6.00. K_{obs}(GSCbl) increases with increasing pH, and this increase is attributed to increasing amounts of the thiolate forms (RS^-) of glutathione. An estimate for the equilibrium constant for the formation of GSCbl from aquacobalamin and the thiolate forms of glutathione of $\sim 5 \times 10^9$ M^{-1} is obtained from the data. Hence, under biological conditions the formation of GSCbl from aquacobalamin and glutathione is essentially irreversible. The rate of the reaction between aquacobalamin/hydroxycobalamin and glutathione for 4.50 < pH < 11.0 has also been studied and the observed rate constant for the reaction was found to decrease with increasing pH. The data were fitted to a mechanism in which each of the 3 macroscopic forms of glutathione present in this pH region react with aquacobalamin, giving $k_1 = 18.5$ M^{-1} s^{-1}, $k_2 = 28 \pm 10$ M^{-1} s^{-1}, and $k_3 = 163 \pm 8$ M^{-1} s^{-1}. The temperature dependence of the observed rate constant at pH 7.40 ($\sim k_1$) was also studied, and activation parameters were obtained typical of a dissociative process ($\Delta H^{\ddagger} = 81.0 \pm 0.5$ kJ mol^{-1} and $\Delta S^{\ddagger} = 48 \pm 2$ J K^{-1} mol^{-1}). Formation of GSCbl from aquacobalamin is rapid; for example, at ~5 mM concentrations of glutathione and at 37 °C, the half-life for formation of GSCbl from aquacobalamin and glutathione is 2.8 s. On the basis of our equilibrium and rate-constant data we conclude that, upon entering cells, any free (protein-unbound) aquacobalamin could be rapidly and irreversibly converted to GSCbl. GSCbl may indeed play an important role in vitamin B_{12}-dependent processes.

Introduction

The vitamin B_{12} derivatives and B_{12}-dependent enzymes have been active areas of research since vitamin B_{12} (cyanocobalamin, X = CN^-, Figure 1) was first isolated from liver and bacterial broths and the structure was elucidated by Dorothy Hodgkin in the 1950s.[1] A deficiency of B_{12} in humans can lead to either megaloblastic anemia ("pernicious anemia"), which is typically associated with malabsorption of vitamin B_{12} and its derivatives, and/or neurological disorders. Numerous studies have established that the most abundant forms of B_{12} isolated from human tissue and serum are adenosylcobalamin (AdoCbl, X = 5′-deoxyadenosine, Figure 1), methylcobalamin (MeCbl, X = CH_3), aquacobalamin (H_2OCbl^+, X = H_2O),[2] and sulphitocobalamin (SO_3Cbl^-, X = SO_3^{2-}). Vitamin B_{12} itself has also been found in much smaller amounts in humans, and is more prevalent in smokers.[3]

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† Dedicated to Dr. Charles R. Clark, University of Otago, New Zealand, in celebration of the retirement of a true scholar in the field of kinetics and mechanism.

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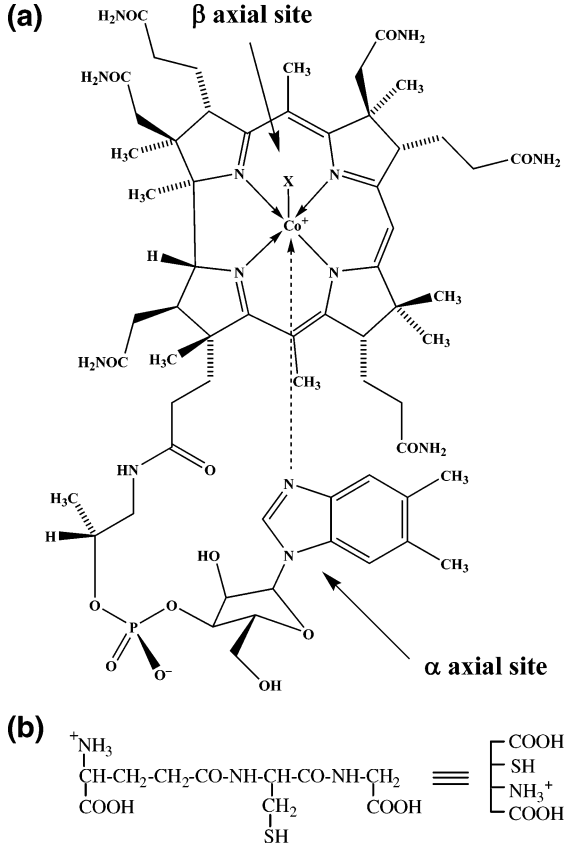

Figure 1. (a) Structure of the cobalamins, showing the two axial sites (upper = β, lower = α) with respect to the corrin ring. (b) The structure of the fully protonated form of glutathione (γ-L-glutamyl-L-cysteinylglycine).

Out of the 15 or so B_{12}-dependent enzyme reactions currently known, two occur in humans−MeCbl-dependent methionine synthase and AdoCbl-dependent methylmalonyl-CoA mutase.[1] In the former reaction, a methyl group is transferred from methyltetrahydrofolate to homocysteine via MeCbl to generate methionine and tetrahydrofolate. This reaction has attracted much attention in the medical literature in the past few years because patients with high levels of serum homocysteine have an increased risk of vascular diseases including myocardial infarction (heart attack) and cerebral strokes.[4] In addition, there is strong evidence that moderately high levels of homocysteine are more prevalent among patients suffering from neurological diseases such as Alzheimer's disease.[5] Furthermore, serum homocysteine levels appear to predict cognitive decline.[5a,6] The National Institute of Aging in the United States is currently recruiting patients as part of the VITAL study to investigate the potential of B_{12} pharmaceuticals to decelerate the progression of Alzheimer's disease, and it is likely that similar studies involving large numbers will be initiated soon in other countries.

Almost a decade ago Jacobsen and co-workers suggested that glutathionylcobalamin, GSCbl[7−9] (X = glutathione, γ-glutamylcysteinylglycine, Figure 1b), should be added to the list of biologically important forms of vitamin B_{12} from the identification of GSCbl in a variety of mammalian cell types.[9] From a chemical point of view this is not especially surprising, since glutathione can be found in concentrations of up to 10 mM in cells of higher animals,[10] and GSCbl is readily formed by reacting excess glutathione with aquacobalamin.[7a,7b,7f,8a] In addition, experiments in which the utilization of various B_{12} derivatives by rabbit spleen extracts were compared led Jacobsen et al to propose that GSCbl (or a closely related thiolatocobalamin adduct) was a precursor of cob(I)alamin and hence the two coenzyme forms of vitamin B_{12}, AdoCbl and MeCbl;[7a,8a,9a] that is, upon removal of the β-axial ligand, H_2OCbl^+ is formed, which reacts with GSH to form GSCbl. It has not yet been established whether these species are free or protein-bound. GSCbl may interact directly with the active sites of methionine synthase or methylmalonyl-coenzyme A mutase, where it undergoes reduction to cob(I)alamin and subsequently reacts with either

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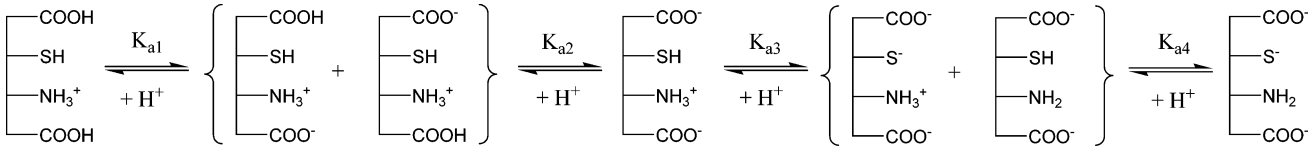
Scheme 1. Macroscopic Acid Dissociation Equilibria for Glutathione



SAM and ATP to form enzyme-bound MeCbl and AdoCbl, respectively. Alternatively, but less likely, GSCbl is reduced to cob(I)alamin, which then binds to the active sites of methionine synthase and methylmalonyl-coenzyme A mutase where it undergoes alkylation.GSCbl has been well characterized—a detailed study of the ^{1}H and ^{13}C NMR spectra has been carried out,[7b] X-ray absorption studies confirm the presence of a Co−S bond,[7c] and the electrochemical behavior has been studied.[7d] So far an X-ray structural determination is not available,[11] although the crystal structure of a closely related analogue, (γ-glutamylcysteinyl)cobalamin, has been reported.[12] Importantly, McCaddon and co-workers have also recently suggested that GSCbl might be more effective than the currently available pharmaceutical forms of B_{12} (CNCbl and H_2OCbl^+) in the treatment of B_{12}-related conditions associated with oxidative stress.[13] Of course, clinical studies will be required to prove or refute this.

For GSCbl to be considered seriously by the wider scientific community as a biologically important form of B_{12}, there is a need to demonstrate that rapid formation of GSCbl from the reaction between aquacobalamin (or hydroxycobalamin; pK_a ~7.8) and glutathione will occur in cells; that is, at neutral pH conditions and at millimolar concentrations of glutathione. The aim of this work was to provide the first detailed study of both the thermodynamics and kinetics of formation of GSCbl from H_2OCbl^+/HOCbl and glutathione. A mechanism for the reaction has also been proposed. In addition to finding that GSCbl is rapidly formed from glutathione and H_2OCbl^+, we have also found that the formation constant for GSCbl is significantly larger than previously believed, and as such, the thiolate forms of glutathione are the first ligands to be identified which approach the remarkably strong binding affinity of cyanide to H_2OCbl^+. Importantly, these studies also demonstrate that, upon entering the cell, it is likely that free (protein-unbound) H_2OCbl^+ is converted within seconds to GSCbl.

Results

Determination of Equilibrium Constants for the Formation of Glutathionylcobalamin. Observed, pH-dependent equilibrium constants, K_{obs}(GSCbl), for the formation of GSCbl from aquacobalamin, H_2OCbl^+, and glutathione were determined at pH 4.50, 5.00, 5.30, and 6.00 under anaerobic conditions using UV−visible spectroscopy

$$H_2OCbl^+ + GSH_T \overset{K_{obs}(GSCbl)}{\rightleftharpoons} GSCbl + H_2O\ (+\ nH^+) \quad (1)$$

where $K_{obs}(GSCbl) = [GSCbl]/[H_2OCbl^+][GSH]_T$, and GSH_T refers to the total glutathione in its various states of protonation (Scheme 1). UV-visible spectroscopy provides an ideal method to determine the proportion of H_2OCbl^+ and GSCbl in solution, since their spectra are so different ($\lambda_{max}(H_2OCbl^+) = 317$, 351, 411, 499, and 527 nm; and λ_{max}-(GSCbl) = 333, 375, 408, 428, 535, and 562 nm).[14] UV−visible and ^{1}H NMR spectroscopy measurements showed that, in agreement with the literature,[7a] the cysteinyl sulfur atom of GSCbl is deprotonated for $4 < pH < 10$; that is, negligible "GS(H)Cbl" exists in this pH region. The details of these experiments are given in the Supporting Information. An acid dissociation constant for the thiol group of cysteinylcobalamin has been reported in the literature, pK_a(Cys-(H)Cbl) = 3.1,[15] which is, as expected,[16] several pH units lower than the pK_a for the ligand itself (pK_a 8.5[17]).

An estimate of K_{obs}(GSCbl) at pH 5.5 is reported in the supporting information of an earlier paper coauthored by one of us (N.E.B; a single experiment was carried out).[7f] Attempts to reproduce this experiment gave scattered data with a poor reproducibility. Cobalamin-catalyzed aerobic oxidation of thiols to their disulfide analogues is well documented in the literature.[18] Experiments confirmed that only the reduced forms of glutathione (not GSSG) react with aquacobalamin.[7e] It therefore seemed reasonable that the presence of O_2 could be the source of the irreproducibility. We carried out control experiments to study how fast GSH is converted to GSSG under aerobic conditions in the presence of GSCbl at a range of pH and GSH concentrations. Details concerning one of these experiments are given below (in the Experimental subsection entitled Determination of the Percentage of Glutathione Oxidation), and results show that even at pH

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Scheme 2

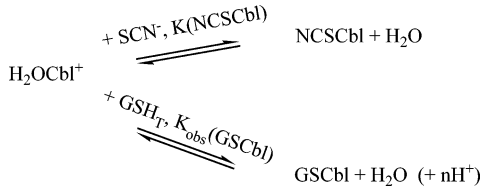

Table 1. Equilibrium Constants for the Formation of NCSCbl and N_3Cbl from Aquacobalamin, Thiocyanate, and Azide (25.0 °C)

electrolyte	I (M)	K(NCSCbl) (M^{-1})[a]	K_{obs}(N_3Cbl) (M^{-1})	reference
$NaCF_3SO_3$	3.0	$(1.4 \pm 0.1) \times 10^3$	$(2.8 \pm 0.3) \times 10^4$	this work
KNO_3	0.50	$(1.04 \pm 0.05) \times 10^3$ [b]	$(2.7 \pm 0.2) \times 10^4$	this work
$NaCF_3SO_3$	0.50	$(1.2 \pm 0.2) \times 10^3$		this work
KNO_3	0.50	1.2×10^3 [c]		ref 19
NaOAc	0.054		2.6×10^4	ref 20

[a] Observed value at pH 5.00 ($pK_a(HN_3) = 4.7$[16d]). [b] Mean value of two experiments. [c] At room temperature.

5.02 (i.e., a pH several units below the pK_a for deprotonation of the thiolate groups of GSH), ~10% of GSH is converted to GSSG within the time frame required to obtain equilibrium in solutions of H_2OCbl^+ and GSH (~3 h).

We therefore attempted to determine K_{obs}(GSCbl) by measuring the absorbances of equilibrated, *anaerobic* solutions containing a fixed concentration of H_2OCbl^+ and varying concentrations of glutathione. A plot of observed absorbance versus glutathione concentration at pH 5.00 is given in Figure B in the Supporting Information, and is linear between 0 and 1 mole equiv GSH_T; hence, under anaerobic conditions the reaction is irreversible and K_{obs}(GSCbl) is too large to be directly determined. Simulation of the data showed that K_{obs}(GSCbl) must be $\geq 2.0 \times 10^6$ M^{-1} for the plot to be linear (see Supporting Information).

Because the equilibrium constant for formation of GSCbl is so large, it was necessary to determine K_{obs}(GSCbl) by measuring the absorbance of anaerobic solutions which contained a competing nucleophile; that is, another ligand that is known to strongly bind to H_2OCbl^+. For example, K_{obs}(GSCbl) was determined at pH 5.00 by measuring the absorbance (357 nm) of equilibrated anaerobic solutions containing a fixed concentration of H_2OCbl^+ (4.98×10^{-5} M) and GSH_T (1.00×10^{-4} M) and varying concentrations of NaSCN (0, 3.00×10^{-2} to 0.150 M). The reaction is summarized in Scheme 2. Under these conditions, equilibrium is reached within 2.5 h. From Scheme 2 it can be shown that

$$\frac{A_{obs} - A(\text{GSCbl})}{A(\text{NCSCbl}) - A_{obs}} = \frac{[\text{NCSCbl}]}{[\text{GSCbl}]} = \frac{K(\text{NCSCbl})}{K_{obs}(\text{GSCbl})} \times \frac{[\text{SCN}^-]}{[\text{GSH}]_T} \quad (2)$$

where A_{obs} is the observed absorbance and A(GSCbl) and A(NCSCbl) are the absorbances of GSCbl and NCSCbl. The derivation of eq 2 is given in the Supporting Information. Values of K(NCSCbl) (and K(N_3Cbl)) determined in this study are summarized in Table 1, and agree well with values given in the literature.[19,20] Note that in solution "NCSCbl" actually exists as a mixture of NCSCbl and SCNCbl.[21] The ratio of the two linkage isomers should be independent of SCN^- concentration, however, which we confirmed by recording UV−visible spectra of NCSCbl in 0.0100 and 2.00 M SCN^- (see later).

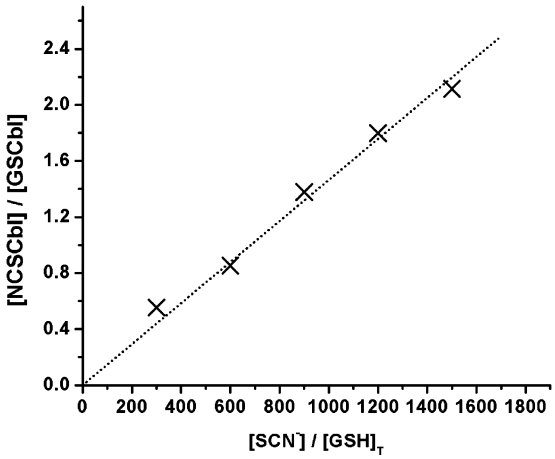

Figure 2. Plot of [NCSCbl]/[GSCbl] versus $[SCN^-]/[GSH]_T$ for anaerobic equilibrated solutions of H_2OCbl^+ (4.98×10^{-5} M), GSH_T (1.00×10^{-4} M), and NaSCN ((0, 3.00×10^{-2} to 0.150 M) (pH 5.00, 25.0 °C, 0.100 M NaOAc, $I = 3.0$ M ($NaCF_3SO_3$)). The data have been fitted to a line passing through the origin (eq 2), which has a slope K(NCSCbl)/K(GSCbl) = $(1.46 \pm 0.04) \times 10^{-3}$. The raw data are given in the Supporting Information.

Figure 2 gives a plot of [NCSCbl]/[GSCbl] versus $[SCN^-]/[GSH]_T$ for the data. From the slope of the line and K(NCSCbl) = $(1.4 \pm 0.1) \times 10^3$ M^{-1} (pH 5.00, 25.0 °C, 0.100 M NaOAc, $I = 3.0$ M ($NaCF_3SO_3$); see the Supporting Information for a description of the procedure used to determine K(NCSCbl)), K_{obs}(GSCbl) = $(9.5 \pm 1.1) \times 10^5$ M^{-1} was obtained. This is, as expected, significantly larger (×2) than the other value obtained under aerobic conditions, as calculation of the latter value failed to take into account the increasing proportion of glutathione oxidized to unreactive GSSG during the experiment. The reason for choosing such a high ionic strength for some of the measurements will become apparent later.

Because it has been previously suggested that acetate, which was used as the buffer in these experiments, can react with H_2OCbl^+ to form (CH_3COO)Cbl, experiments were also carried out which confirm that appreciable amounts of (CH_3COO)Cbl are not formed under the conditions used to determine K_{obs}(GSCbl) (and K_{obs}(NCSCbl)). For further details see the section entitled Acetate Binding to Aquacobalamin in the Supporting Information.

The observed formation constant for GSCbl at pH 5.00 was also determined under the same electrolyte conditions ($I = 3.0$ M ($NaCF_3SO_3$)) with N_3^- (NaN_3) rather than SCN^- as the competing nucleophile. This result is given in Table 2, along with other K_{obs}(GSCbl) data at a total ionic strength

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Table 2. Observed Equilibrium Constants for the Formation of GSCbl from Aquacobalamin and Glutathione at pH 5.00 (25.0 °C)

competing anion	electrolyte	I (M)	K_{obs}(GSCbl) (M^{-1})
SCN^-	$NaCF_3SO_3$	3.0	$(9.5 \pm 1.1) \times 10^5$
SCN^-	KNO_3	0.50	$(1.8 \pm 0.2) \times 10^6$ [a]
SCN^-	$NaCF_3SO_3$	0.50	$(1.6 \pm 0.3) \times 10^6$
N_3^-	$NaCF_3SO_3$	3.0	$(8.9 \pm 1.0) \times 10^5$
N_3^-	KNO_3	0.50	$(3.0 \pm 0.4) \times 10^6$ [a]

[a] Mean value of two experiments.

of 0.50 M, with either SCN^- or N_3^- as the competing nucleophile. Evidence for the formation of $(N_3)_2Cbl^-$ at high N_3^- concentrations was also checked. The UV−visible spectra of equilibrated solutions of H_2OCbl^+ and either 0.0100 or 2.00 M N_3^- at pH 5.00 (i.e., at an N_3^- concentration over an order of magnitude larger than those used) were very similar ($\Delta\lambda_{max} \leq 1$ nm). There is, therefore, no evidence for the formation of significant amounts of any species other than N_3Cbl^-. From the data in Table 2 it can be seen that K_{obs}(GSCbl) is independent of the choice of the competing anion within experimental error, validating the method used. K_{obs}(GSCbl) was found to be somewhat larger (~2 times) at $I = 0.50$ M compared with 3.0 M and appears relatively insensitive to the choice of electrolyte (i.e., KNO_3 or $NaCF_3SO_3$).

The pH dependence of K_{obs}(GSCbl) was also determined with SCN^- as the competing nucleophile. It was necessary to have a total ionic strength of 3.0 M, since at pH 6.00 up to 2.00 M NaSCN was required to obtain a substantial proportion of SCNCbl at equilibrium (~50% SCNCbl in the equilibrium mixture).[22] Control experiments in which UV−visible spectra of equilibrated solutions of H_2OCbl^+ and SCN^- at $[SCN^-] = 0.0300$ or 2.00 M at pH 6.00 were recorded showed that under the conditions chosen to determine K_{obs}(GSCbl), only NCSCbl is present ($\Delta\lambda_{max} \leq 1$ nm). Hence, formation of significant amounts of species such as $(NCS)_2Cbl^-$, which would need to be taken into account in the data analyses, does not occur up to $[SCN^-] = 2.00$ M. Thiocyanate was chosen since, at higher pH conditions, N_3^- was found to accelerate the reduction of GSCbl compared with SCN^-. We were unable to determine values of K_{obs}(GSCbl) above pH 6.00, since in the presence of SCN^- (or N_3^-) and $GluS^-$, GSCbl is unstable, and is partially reduced. It is well-known that RS^- can reductively cleave the Co−S bond of thiolatocobalamins.[15,18] $NaCF_3SO_3$ was the preferred choice of electrolyte at these high ionic strength conditions ($I = 3.0$ M), since NO_3^- is a mild reducing agent.

Table 3 gives the observed equilibrium constant, K_{obs}(GSCbl), as a function of pH for $4.50 < pH < 6.00$. For this pH region, insignificant amounts of HOCbl are present ($pK_a(H_2OCbl^+) = 7.76$ (25.0 °C), see Experimental section). It has been established by many authors that the hydroxy ligand of HOCbl is inert to substitution by other ligands.[20,23] The acidity constants for the glutamate amino group and the thiol group of glutathione overlap, as do the acidity constants for the glutamate and glycine carboxylic acid groups. As a consequence of this, only macroscopic (mixed) values of the acidity constants are obtainable by potentiometric titration. From the data in Table 3 it can be seen that K_{obs}(GSCbl) increases by approximately 1 order of magnitude for each incremental increase in pH. The most reasonable explanation for this is that small amounts of the thiolate forms (GS^-) of glutathione react with H_2OCbl^+; that is

Table 3. Equilibrium Constants for the Formation of GSCbl from Aquacobalamin and Glutathione (25.0 °C) as a Function of pH

pH	K_{obs}(GSCbl) (M^{-1})	K(GSCbl) (M^{-1})[a]
4.50	$(5.0 \pm 0.4) \times 10^5$	$(8.3 \pm 0.6) \times 10^9$
5.00	$(9.5 \pm 0.9) \times 10^5$	$(5.0 \pm 0.5) \times 10^9$
5.30	$(1.8 \pm 0.1) \times 10^6$	$(4.7 \pm 0.3) \times 10^9$
6.00	$(1.4 \pm 0.2) \times 10^7$	$(7.4 \pm 1.1) \times 10^9$

[a] $K(GSCbl) = K_{obs}(GSCbl)(K_{a3} + [H^+])/K_{a3}$.

$$H_2OCbl^+ + GS^- \overset{K(GSCbl)}{\rightleftharpoons} GSCbl + H_2O \qquad (3)$$

where $K(GSCbl) = [GSCbl]/[H_2OCbl^+][GS^-]$.

The third macroscopic deprotonation process (K_{a3} in Scheme 1; $pK_{a3} = 8.72$[24b]) corresponds mainly to deprotonation at the thiol site of glutathione, and using this value one obtains an estimate for $K(GSCbl) \sim 5 \times 10^9$ M^{-1}, since

$$K(GSCbl) = K_{obs}(GSCbl)(K_{a3} + [H^+])/K_{a3} \qquad (4)$$

(a derivation of this equation is given in the Supporting Information).[24] Hence $K(GSCbl)$ is in the order of 10^9 M^{-1}, and as such, the thiolate forms of glutathione have a remarkably strong binding affinity to H_2OCbl^+.

Determination of Rate Constants and a Mechanism for the Formation of GSCbl. To probe the mechanism for the formation of GSCbl from H_2OCbl^+/HOCbl and glutathione, rate constants were determined as a function of glutathione concentration for a range of pH conditions (pH 4.50−11.0) at 25.0 °C.

$$HOCbl/H_2OCbl^+ + GSH_T \overset{k_{obs}}{\rightleftharpoons} GSCbl + H_2O\ (+ nH^+) \qquad (5)$$

Figure 3 shows a plot of observed rate constant, k_{obs}, versus total glutathione concentration, GSH_T, at pH 5.00, which is typical of the results we obtained. The data can be fitted to

(22) To obtain pH dependence data at a lower total ionic strength, a nucleophile is required which (i) binds stronger to H_2OCbl^+ compared with SCN^- and (ii) which is not capable of reducing GSCbl. Despite an extensive search, other nucleophiles could not be identified with these properties.

(23) (a) Marques, H. M. *S.-Afr. Tydskr. Chem.* **1991**, *44*, 114. (b) Marques, H. M.; Bradley, J. C.; Campbell, L. A. *J. Chem. Soc., Dalton Trans.* **1992**, 2019. (c) Reenstra, W. W.; Jencks, W. P. *J. Am. Chem. Soc.* **1979**, *101*, 5780. (d) Marques, H. M.; Brown, K. L.; Jacobsen, D. W. *J. Biol. Chem.* **1988**, *263*, 12378. (e) Marques, H. M.; Egan, T. J.; Marsh, J. H.; Mellor, J. R.; Munro, O. Q. *Inorg. Chim. Acta* **1989**, *166*, 249. (f) Conn, J. B.; Wartman, T. G. *Science* **1952**, *115*, 72.

(24) (a) Eight microscopic acid dissociation constants for glutathione have been reported in the literature,[24b,c] and are calculated from NMR spectroscopy data assuming that a change in the chemical shift of a specific carbon-bonded proton is due only to ionization of the adjacent acidic group. If microscopic rather than macroscopic values of the acid dissociation constants were used to fit our data, the resulting formation constants for GSCbl from aquacobalamin and the thiolate forms of glutathione would be somewhat higher than the macroscopic estimate of $\sim 5 \times 10^9$ M^{-1}. (b) Rabenstein, D. L. *J. Am. Chem. Soc.* **1973**, *95*, 2797. (c) Fujiwara, S.; Formicka-Kozlowska, G.; Kozlowski, H. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 3131.

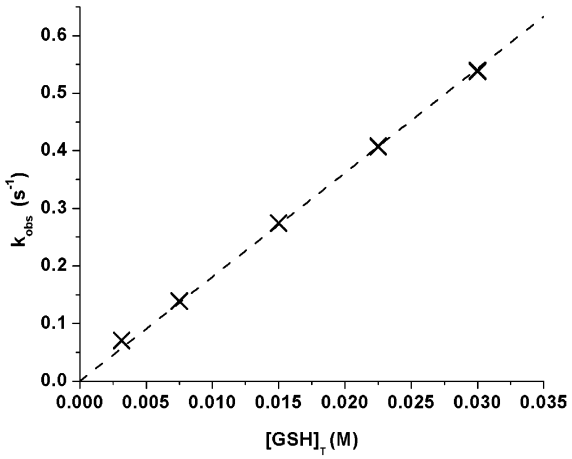


Figure 3. Plot of observed rate constant, k_{obs}, versus total glutathione concentration for the reaction $H_2OCbl^+ + GSH_T \rightarrow GSCbl$ (+ nH^+) at pH 5.00 (0.100 M NaOAc, 25.0 °C, I = 0.50 M (KNO_3)). Data have been fitted to a line passing through the origin, giving $k_{obs}/[GSH]_T = 18.09 \pm 0.08\ M^{-1}\ s^{-1}$.

a line which passes through the origin; hence, eq 5 is irreversible under the conditions used in this study as expected, since K(GSCbl) is so large. From the slope of the line, $k_{obs}/[GSH]_T = 18.09 \pm 0.08\ M^{-1}\ s^{-1}$ was obtained. Similar plots were obtained for $4.50 \leq pH \leq 8.01$, with two further plots of the data at pH 6.01 and 8.01 being given in Figures D and E in the Supporting Information. All the data under aerobic conditions ($pH \leq 8.01$) was obtained using a stopped-flow UV−visible spectrophotometer. Note that B_{12}-catalyzed aerial oxidation of glutathione was negligible within the time frame of the experiment under these conditions.

Rate constants for the reaction between $HOCbl/H_2OCbl^+$ and glutathione at pH 8.31−11.0 were determined under anaerobic conditions using either a stopped-flow spectrophotometer (pH 8.31) or a conventional Cary 1E UV−vis spectrophotometer ($pH \geq 8.71$). Anaerobic conditions were necessary for $pH \geq 8.31$ since the concentrations of the thiolate forms of glutathione become significant in alkaline solution. As a consequence, the rate of reduction of GSCbl to cob(II)alamin by the thiolate forms of glutathione becomes rapid enough to interfere with determination of the rate of formation of GSCbl from $HOCbl/H_2OCbl^+$ and glutathione. The reaction (which is again irreversible) was therefore monitored by UV−visible spectroscopy at an isosbestic wavelength (e.g., 498 nm at pH 9.00; 495 nm at pH 10.11) for the reduction of GSCbl to cob(II)alamin. Typical plots of k_{obs} versus $[GSH]_T$ under anaerobic conditions at pH 9.00 and 10.11 are given in Figures F and G in the Supporting Information. The plots are linear, intercept the origin, and once again $k_{obs}/[GSH]_T$ can be determined from the slope.

Figure 4 summarizes the dependence of the second-order rate constant for formation of GSCbl, $k_{obs}/[GSH]_T$, as a function of pH. As mentioned earlier, it has previously been established that only H_2OCbl^+, not HOCbl, undergoes ligand substitution reactions at the β axial site.[20,23] The proposed mechanism is given in Scheme 3, which has three pathways involving the different macroscopic forms of glutathione with respect to its protonation state existing in solution for $4.5 < pH < 11.0$.[25] An alternative plot of the experimental data, $k_{obs}[GSH]_T \times 1/\text{fraction } H_2OCbl^+$, which in essence corrects for the fraction of unreactive HOCbl in the solution (or, in other words, a plot which shows the dependence of the rate of the reaction on the protonation state of glutathione only), is given in the inset to Figure 4. From Scheme 3 it can be shown that

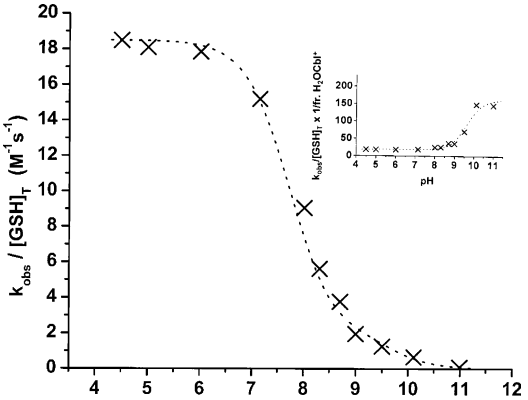
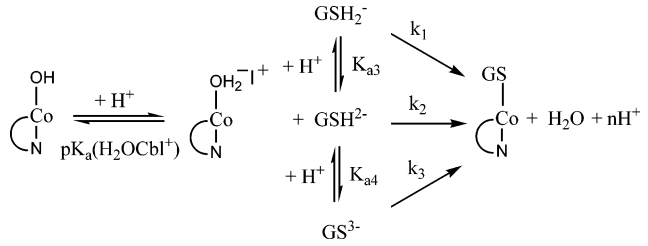


Figure 4. Plot of $k_{obs}/[GSH]_T$ versus pH for the reaction $HOCbl/H_2OCbl^+ + GSH_T \rightarrow GSCbl$ (+ nH^+) (0.100 M buffer, 25.0 °C, I = 0.50 M (KNO_3)). The inset gives a plot of $k_{obs}/[GSH]_T \times 1/\text{fraction } H_2OCbl^+$ versus pH for the same data. Fitting the data shown in the inset to eq 6 in the text while fixing $pK_{a3} = 8.72$, $pK_{a4} = 9.63$, $pK_a(H_2OCbl^+) = 7.76$, and $k_1 = 18.5\ M^{-1}\ s^{-1}$ gives a best fit with $k_2 = 28 \pm 10\ M^{-1}\ s^{-1}$ and $k_3 = 163 \pm 8\ M^{-1}\ s^{-1}$ as shown by the dotted curve. The dotted curve on the main figure also corresponds to a fit using these same values.

Scheme 3



$$k_{obs}/[GSH]_T \times 1/\text{fraction } H_2OCbl^+ = k_1(\text{fraction } GSH_2^-) + k_2(\text{fraction } GSH^{2-}) + k_3(\text{fraction } GS^{3-}) = (k_1[H^+]^2 + k_2K_{a3}[H^+] + k_3K_{a3}K_{a4})/([H^+]^2 + K_{a3}K_{a4} + K_{a3}[H^+]) \quad (6)$$

where pK_{a3} and pK_{a4} are macroscopic acid dissociation constants corresponding to the equilibria shown in Scheme 1. Fixing $pK_{a3} = 8.72$,[24b] $pK_{a4} = 9.63$,[24b] $pK_a(H_2OCbl^+) = 7.76$ (see Experimental Section), and $k_1 = 18.5\ M^{-1}\ s^{-1}$ (i.e., the limiting value of $k_{obs}/[GSH]_T \times 1/\text{fraction } H_2OCbl^+$ at low pH) gave a best fit with $k_2 = 28 \pm 10\ M^{-1}\ s^{-1}$ and $k_3 = 163 \pm 8\ M^{-1}\ s^{-1}$ as shown by the dotted line on the inset figure in Figure 4. The fit shown on the main plot of Figure 4 was also generated using these values. Given that the rate of the reaction between H_2OCbl^+ and glutathione should be relatively unaffected by the protonation state of the glutamate amine since it is 10 bonds away from the cobalt of GSCbl,

(25) See ref 24. Since $k_{obs}/[GSH]_T$ is rapidly diminishing in magnitude for pH values > 8.5 in addition to significant scatter in the data, it is not meaningful to attempt to fit our data to pathways involving the four microscopic forms of glutathione present in the pH region of this study.

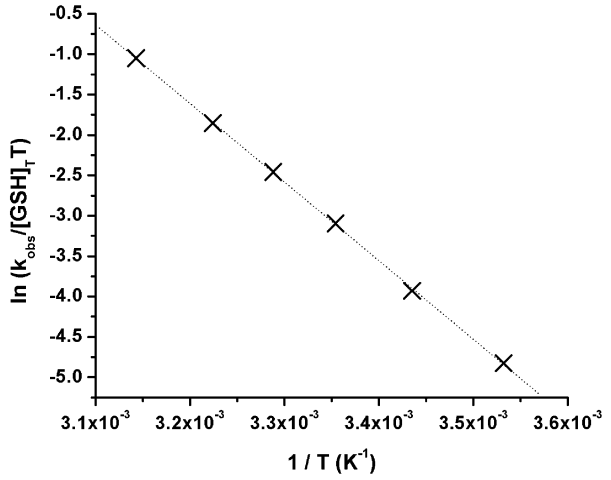


Figure 5. Plot of $\ln(k_{obs}/[GSH]_T T)$ versus 1/temperature for the reaction given in eq 5 at pH 7.40 ($I = 0.50$ M (KNO_3)). Best fit of the data to the Eyring equation gives $\Delta H^{\ddagger} = 81.0 \pm 0.5$ kJ mol^{-1} and $\Delta S^{\ddagger} = 48 \pm 2$ J K^{-1} mol^{-1}.

and that the K_{a3} ionization process mainly corresponds to deprotonation of the thiol of glutathione, one would expect k_2 to be similar in magnitude to k_3, rather than k_1. This is not the case, and simply reflects the fact that k_2 is not accurately determined in the data fit, hence the large experimental error associated with this value. This is a direct result of a combination of factors, including the fact that (i) the k_2 term of eq 6 only becomes significant for pH > 8.2 ($pK_{a3} = 8.72$), and under these conditions, $k_{obs}/[GSH]_T$ is already small since the proportion of H_2OCbl^+ is rapidly decreasing with increasing pH ($pK_a(H_2OCbl^+) = 7.76$), (ii) the relative insensitivity of the observed rate constant to the protonation state of both the thiol and amine of glutathione, in addition to (iii) the added complication that the thiol and amine acid dissociation constants overlap significantly.

Determination of Activation Parameters at pH 7.40. The temperature dependence of the second-order rate constant, $k_{obs}/[GSH]_T$, for the reaction given in eq 5 was studied by measuring the observed rate constant at a series of glutathione concentrations at 6 temperatures (10.0−45.0 °C). The results are summarized in Figure 5, which gives an Eyring plot[26] of $\ln(k_{obs}/[GSH]_T T)$ versus $1/T$, where T = temperature. The data were fitted to a line, and values of $\Delta H^{\ddagger} = 81.0 \pm 0.5$ kJ mol^{-1} and $\Delta S^{\ddagger} = 48 \pm 2$ J K^{-1} mol^{-1} were calculated from the slope and intercept, respectively.

Discussion

Although strictly anaerobic conditions are not physiologically relevant, measurement of equilibrium and rate constants under anaerobic conditions is simply a means to accurately determine these values without other reactions which involve O_2 interfering; that is, the reaction between aquacobalamin and glutathione proceeds regardless of the presence or absence of air. Glutathione exists predominately in its reduced forms in cells, with the ratio of GSH/GSSG being determined by factors other than the B_{12}-catalyzed oxidation of GSH. (It should also be kept in mind that the concentration of GSH in cells is in vast excess (over 6 orders of magnitude) compared with the concentration of the vitamin B_{12} derivatives.)

Our values of K_{obs}(GSCbl) at pH 5.00 ($(0.9-3) \times 10^6$ M^{-1}, Table 2) are considerably larger than earlier reported estimates of K_{obs}(GSCbl) ($\sim 5 \times 10^5$ M^{-1} (pH 4.7, temperature not specified)[7e] and $(1.1 \pm 0.3) \times 10^5$ M^{-1} (pH 5.5, 25 °C)[7f]). These latter two values were determined under conditions where significant amounts of glutathione are oxidized to its disulfide form within the time required to reach equilibrium ($\sim$3 h). They are therefore less reliable, and, as anticipated, smaller than the values obtained in this study. From the pH dependence of K_{obs}(GSCbl) in the region 4.50−6.00, an estimate of K(GSCbl) in the order of 5×10^9 M^{-1} was obtained. The formation constant for GSCbl is remarkably large, and as such the thiolate forms of glutathione are the first ligands to be identified which even approach the extremely high binding affinity of cyanide for H_2OCbl^+ (K(cyanocobalamin) $\approx 10^{14}$ M^{-1};[27] the next largest reported formation constant is K(sulfitocobalamin) $= 2.2 \times 10^7$ M^{-1} [28]). The large formation constants of cyanocobalamin and thiolate−vitamin B_{12} model complexes are attributed to π-bonding from the ligand to the cobalt.[23c,29]

Fendler and Nome studied the thermodynamics and kinetics of the reaction between H_2OCbl^+/HOCbl and cysteine,[15] and determined an observed equilibrium constant for the formation of cysteinylcobalamin, K_{obs}(CysCbl) = $(2.4 \pm 0.2) \times 10^5$ M^{-1} at pH 5.5 (25.0 °C), which is within an order of magnitude of our value of K_{obs}(GSCbl) at similar pH conditions. Their value was determined by a method similar to that used in this study; that is, by measuring UV−visible spectra of equilibrated solutions containing H_2OCbl^+ and cysteine in the presence of a competing nucleophile. However, their studies were carried out under aerobic rather than anaerobic conditions, and within the time frame of their experiments it is quite likely that a significant amount of cysteine was converted to its disulfide form as a result of (i) B_{12}-catalyzed oxidation of cysteine (as we observed for glutathione—see Experimental Section), and (ii) homolysis of the Co−S bond of CysCbl, generating cob(II)alamin (which is rapidly converted to H_2OCbl^+ in the presence of air) and CysS-SCys.[7f] Indeed, the authors noted that for approximately equimolar concentrations of the H_2OCbl^+ and cysteine reactants, the resulting CysCbl rapidly decomposed. Considering both these decomposition reactions consume reduced cysteine, we regard their value of K_{obs}(CysCbl) as an estimate only. (Note that both B_{12}-catalyzed oxidation of cysteine and decomposition of CysCbl as a result of Co−S bond homolysis are effectively masked in the presence of air and excess cysteine, since under these conditions aerial oxidation of cob(I)alamin and/or cobalamin(II) products to give H_2OCbl^+ is rapid, as is the subsequent reaction between

(26) Espenson, J. H. *Chemical Kinetics and Reaction Mechanisms*, 2nd ed.; McGraw-Hill: New York, 1995; p 156.

(27) Baldwin, D. A.; Betterton, E. A.; Pratt, J. M. *S. Afr. J. Chem.* **1982**, *35*, 173.

(28) Firth, R. A.; Hill, H. A. O.; Pratt, J. M.; Thorp, R. G.; Williams, R. J. P. *J. Chem. Soc. A* **1969**, 381.

(29) (a) Brown, K. L.; Kallen, R. G. *J. Am. Chem. Soc.* **1972**, *94*, 1894. (b) Brown, K. L.; Gupta, B. D. *Inorg. Chem.* **1990**, *29*, 3854.

H_2OCbl^+ and cysteine to regenerate CysCbl. The observation that several thiolate derivatives of B_{12} appear to be stable only in the presence of air and excess thiol has been noted by a number of authors.)

Aquacobalamin is one of the most abundant forms of vitamin B_{12} isolated from human tissues. The formation constant of GSCbl, clearly large enough to ensure formation of GSCbl from aquacobalamin and millimolar concentrations of intracellular glutathione, is, in practical terms, irreversible. An important part of this study was also to determine if the glutathione concentration in cells is sufficient to *rapidly* convert aquacobalamin to GSCbl; that is, are the kinetics favorable for GSCbl formation? The observed rate constant for formation of GSCbl was found to increase with decreasing pH, reaching a limiting value for pH < 6. The decrease in $k_{obs}/[GSH]_T$ with increasing pH is accounted for by only H_2OCbl^+ (not HOCbl) reacting with glutathione. Fitting the data to the rate law for the mechanism shown in Scheme 3 with k_1 fixed at 18.5 M^{-1} s^{-1} gave $k_2 = 28 \pm 10$ M^{-1} s^{-1} and $k_3 = 163 \pm 8$ M^{-1} s^{-1}. Hence, by comparing k_1 and k_3 it is apparent that deprotonating both the glutamate amine and the cysteine thiol protons of glutathione results in only a modest ($\sim 5\times$) increase in the rate of the reaction between H_2OCbl^+ and glutathione.

The temperature dependence of the second-order rate constant at pH 7.40 ($\sim k_1$) was also investigated, allowing activation parameters to be determined ($\Delta H^{\ddagger} = 81.0 \pm 0.5$ kJ mol^{-1} and $\Delta S^{\ddagger} = 48 \pm 2$ J K^{-1} mol^{-1}). A significantly positive value for $\Delta S^{\ddagger}$ suggests that the reaction is occurring via a dissociative (*D* or I_d) type of mechanism.[30] At pH 7.40 and 37.0 °C, $k_{obs}/[GSH]_T = 48.7 \pm 0.1$ M^{-1} s^{-1}. From these results it can be concluded that even at low intracellular glutathione concentrations, the conversion of H_2OCbl^+/HOCbl to GSCbl will occur within seconds in biological systems; for example, at 5 mM GSH at 37.0 °C, the half-life for the reaction is 2.8 s.

Brown and co-workers examined the kinetics of the reaction between (β-methyl)(α-aqua)cobaloxime and a variety of thiols (mercaptoacetate, 2-mercaptoethanol, and methyl mercaptoacetate) and proposed a mechanism similar to that shown in Scheme 3 in which (β-methyl)(α-aqua)-cobaloxime reacts with both the RSH (k_1) and RS^- (k_2) forms of the thiols.[29a] Accurate values of both k_1 and k_2 could be obtained from this study since, unlike the reaction between H_2OCbl^+ and GSH, the pK_a of the aqua ligand of (β-methyl)-(α-aqua)cobaloxime is at least 2.8 pH units higher than the pK_a of the three thiols. Interestingly, although k_1 and k_2 are once again within the same order of magnitude, the values of k_1 (50.4, 49.6, and 55.2 M^{-1} s^{-1} for mercaptoacetate, 2-mercaptoethanol, and methyl mercaptoacetate, respectively) are larger than those for k_2 (12.8, 27.3, and 24.2 M^{-1} s^{-1} for mercaptoacetate, 2-mercaptoethanol, and methyl mercaptoacetate, respectively), which is unexpected since the -1 charge on RS^- would be expected to enhance its reactivity with neutral (β-methyl)(α-aqua)cobaloxime compared with neutral RSH. This result has been discussed in depth,[29a] and it was concluded that ligand-to-metal π effects may be important in stabilizing the transition state.

Fendler and Nome also investigated the kinetics of the reaction between H_2OCbl^+/HOCbl and cysteine.[15] There are problems associated with their data treatment, however (see the Supporting Information for further discussion). The rate constant for the reaction between H_2OCbl^+ and $HSCH_2CH(NH_3^+)CO_2^-$, which is the only species present in mildly acidic solution, is 11.5 M^{-1} s^{-1}. This value is, as expected, similar in magnitude to the k_1 pathway for the reaction between H_2OCbl^+ and glutathione, 18.5 M^{-1} s^{-1}.

Given that both our thermodynamic and kinetic data for the formation of GSCbl from H_2OCbl^+/HOCbl and glutathione indicate that aquacobalamin will be rapidly converted to GSCbl when mM concentrations of glutathione are present, why has GSCbl only recently been isolated from human cells? Jacobsen and co-workers have suggested that GSCbl is not stable under the conditions used to extract and separate the B_{12} derivatives in the majority of studies. In addition, to detect GSCbl (or its decomposition product sulfitocobalamin[8d]), it was necessary for them to develop new HPLC procedures for characterizing cobalamins from cells.[8c] After working with GSCbl for several years, we also believe this is quite plausible. In solution, H_2OCbl^+ and glutathione exist in equilibrium with GSCbl, albeit in very small quantities. Since the aerial oxidation of glutathione to its disulfide form is catalyzed by B_{12} derivatives,[18] an aerobic solution of GSCbl could potentially eventually be converted to H_2OCbl^+ and GSSG. Additionally, in the presence of excess glutathione under alkaline conditions, GSCbl is reductively cleaved to give Cbl(I) (which is rapidly oxidized to Cbl(II)) and GSSG. Indeed, the relative ease in which GSCbl is reductively cleaved by nucleophilic attack of the thiolato forms of glutathione at the β-axial site of GSCbl to form Cbl(I) led Jacobsen and co-workers to propose that, upon entering the cell, B_{12} derivatives are initially converted to GSCbl prior to being reduced to Cbl(I), assisted by protein-bound or free thiols. The reduced, enzyme-bound cobalamin can then react with either *S*-adenosylmethionine or ATP to form enzyme-bound vitamin B_{12} coenzymes MeCbl or AdoCbl, respectively. This seems a much more feasible way of generating cob(I)alamin compared with simple outer-sphere reduction of cobalamins by a reductant to generate cob(I)alamin, given the low (for biological systems) redox potential of the Cbl(II)/(I) redox couple (-490 mV versus NHE for methionine synthase-bound cob(II)alamin/cob(I)-alamin).[31] Facts supporting this hypothesis include finding that compared with CNCbl and H_2OCbl^+, (i) methionine synthase utilizes GSCbl more readily as a cofactor in the methylation of homocysteine to methionine,[8a] (ii) the conversion of apo-methionine synthase to holo-methionine synthase is faster with GSCbl,[9a] and (iii) the yield of AdoCbl is higher when GSCbl is added to the cell culture.[8a] In addition, model studies have shown that GSCbl is readily converted to MeCbl

(30) Given that H_2OCbl^+ and GSCbl have different charges, as do the entering and leaving groups in the substitution process, caution must be used when inferring the type of mechanism from the activation parameters.

(31) Jarrett, J. T.; Choi, C. Y.; Matthews, R. G. *Biochemistry* **1997**, *36*, 15739.

in the presence of a thiol and *S*-adenosylmethionine.[8b] Of course, direct observation of GSCbl in in vivo rather than in vitro lysed cell extracts would unequivocally demonstrate the importance of GSCbl in biology.

Recently McCaddon and co-workers proposed that GSCbl could be a more effective therapeutic in the treatment of neurological disorders associated with oxidative stress such as Alzheimer's disease.[13] They have suggested that such disorders are associated with the development of a "functional" B_{12} deficiency (where "functional" refers to derivatives of vitamin B_{12} which can be directly utilized by the B_{12}-dependent enzymes), in contrast to "classical" clinical deficiencies arising from reduced intake or malabsorption of the vitamin. They postulate that oxidative stress will impair the B_{12}-dependent methionine synthase reaction and also compromise the intracellular reduction of B_{12} to its active co-enzyme forms due to a scarcity of glutathione in its reduced form. However, it is not yet established whether administered GSCbl would provide any benefits compared with H_2OCbl^+, although it may well be absorbed better and/or retained longer in the body. Our studies show that upon entering the cell, free H_2OCbl^+ will be quickly converted to GSCbl by intracellular glutathione, provided glutathione is available in its reduced form.

Note that it is currently unclear whether significant amounts of free B_{12} derivatives exist in cells, or whether B_{12} derivatives are always protein-bound. Further studies are also required to establish whether intracellular glutathione concentrations are sufficient to readily convert protein-bound aquacobalamin to protein-bound GSCbl, or indeed, whether aquacobalamin can readily react with the cysteine thiol groups of proteins to form protein-S-Cbls.

To summarize, in this study we have carried out a detailed investigation of both the thermodynamics and kinetics of formation of GSCbl from H_2OCbl^+/HOCbl and glutathione, and found (i) the formation constant of GSCbl is much larger than previously believed and (ii) aquacobalamin reacts rapidly with glutathione to form GSCbl. Reliable values of K_{obs}(GSCbl) are reported for the first time under anaerobic conditions, avoiding unwanted side reactions involving the aerial autoxidation of glutathione by B_{12}. In the course of this work we have found that the equilibrium between H_2OCbl^+ and glutathione is rapidly shifted, and the relative amounts of H_2OCbl^+ to GSCbl can be perturbed by factors such as pH, introduction of air to an anaerobic system, and the glutathione concentration. Investigations on the stability of GSCbl in the presence of glutathione as a function of pH, air, and other factors are currently underway in our laboratory. From our studies we conclude that GSCbl is a likely form of protein-unbound vitamin B_{12} in mammalian cells and that GSCbl could very well play an important role in the biochemistry of B_{12}-dependent processes. Identification of GSCbl in whole cells in vivo would unequivocally demonstrate the importance of GSCbl in biology.

Experimental Section

Hydroxycobalamin hydrochloride (HOCbl·HCl, 98% stated purity by manufacturer[32]) was purchased from Fluka. Glutathione (GSH, 98%; i.e., in its reduced form) was purchased from Aldrich, NaN_3 (99%) and potassium hydrogenphthalate (≥ 99.9%) were obtained from Labchem, MES, TES, TAPS, and CAPS[33] were from Sigma, and KNO_3 (99%), NaOAc (99%), and NaSCN (>98%) were from BDH. Sodium trifluoromethanesulfonate ($NaCF_3SO_3$) was prepared by neutralizing a concentrated, aqueous solution of trifluoromethanesulfonic acid with NaOH, reducing it to dryness and drying overnight under vaccum at 70 °C. Glutathione solutions were prepared directly before use.

The percentage of water in HOCbl·HCl(·$n$$H_2O$) (10 ± 3%, 15 ± 2%, two different batches) was determined by converting HOCbl·HCl to $(CN)_2Cbl^-$ (0.10 M KCN, pH 10.0, $\epsilon_{368} = 3.04 \times 10^4$ M^{-1} cm^{-1} [34]).

All pH measurements were made at 25.0 °C with an Orion model 710A pH meter equipped with an Orion 9101 BN glass electrode and an Orion 900200 double junction reference electrode. The outer chamber of the reference electrode was filled with 1.60 M NH_4NO_3/0.200 M $NaNO_3$, pH 7.0, which has been shown to have the same performance characteristics as the usual KCl filling solution. The electrodes were standardized with BDH buffer solutions at pH 4.01, 6.98, and 10.00. Measurements in alkaline solution were carried out under a nitrogen or argon atmosphere. Solution pH was adjusted using concentrated HNO_3, CF_3SO_3H, or KOH solutions as necessary. The error in individual pH measurements was ≤0.02.

^{1}H NMR spectra were recorded on an Inova 500 MHz spectrometer equipped with a 5-mm thermostated (25.0 ± 0.2 °C) probe. All solutions were prepared in D_2O, and TSP (3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid, sodium salt) was used as an internal standard.

UV−visible spectra were recorded on a Cary 1E spectrophotometer equipped with a thermostated cell changer (25.0 ± 0.1 °C), operating with WinUV Bio software (version 2.00). Rate constants were obtained using the fitting program Olis KINFIT. For reactions with half-lives ($t_{1/2}$) < 18 s, data were obtained and analyzed using an Applied Photophysics SF 17 MV stopped-flow spectrophotometer (25.0 ± 0.2 °C) and kinetic analysis software. For experiments conducted under anaerobic conditions, solutions were degassed using three freeze−pump−thaw cycles and transferred to air-free Schlenk cuvettes[35] under N_2.

Determination of the Acidity Constant for H_2OCbl^+. A HOCbl·HCl solution (30.00 mL, 5.02×10^{-3} M,[35] I = 0.50 M (KNO_3)) was titrated with aliquots of a NaOH solution (8.00 × 10^{-2} to 0.240 mL, 0.107 M, I = 0.50 M (KNO_3)), giving pK_a(H_2OCbl^+) = 7.76 ± 0.02 (25.0 °C, I = 0.50 M (KNO_3)).

Determination of K(NCSCbl). A series of solutions with $H_2OCbl^+ = 5.53 \times 10^{-5}$ M[36] and varying concentrations of NaSCN (0, 3.33×10^{-5} to 2.50×10^{-2} M) were prepared in vials (pH

(32) (a) It has been demonstrated by ^{1}H NMR spectroscopy that commercial HOCbl·HCl is not as pure as claimed by the manufacturer.[32b] (b) Brasch, N. E.; Finke, R. G. *J. Inorg. Biochem.* **1999**, *73*, 215.

(33) Abbreviations: NaOAc = Na($OOCCH_3$), MES = (2-[*N*-morpholino]-ethanesulfonic acid), TES = (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), TAPS = (*N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid), CAPS = (3-[cyclohexyl-amino]-1-propanesulfonic acid).

(34) Barker, H. A.; Smyth, R. D.; Weissbach, H.; Toohey, J. I.; Ladd, J. N.; Volcani, B. E. *J. Biol. Chem.* **1960**, *235*, 480.

(35) Hay, B. P.; Finke, R. G. *Polyhedron* **1988**, *7*, 1469.

(36) All reported mole amounts and concentrations of HOCbl·HCl are corrected for the percentage of water present (see Experimental Section).

5.00, 0.100 M NaOAc, $I = 3.0$ M ($NaCF_3SO_3$)), capped, and left to equilibrate for 1 h at 25.0 °C (water bath). The absorbance of each solution was measured at 315 nm by visible spectroscopy using the same cuvette (1 cm) for all measurements. *K*(NCSCbl) was also determined using the same procedure at a total ionic strength of 0.50 M with either $NaCF_3SO_3$ or KNO_3 as the supporting electrolyte. *K*(NCSCbl) could also be determined from absorbance data collected at 351 nm.

Determination of *K*(N_3Cbl). A series of solutions with $H_2OCbl^+ = 9.29 \times 10^{-5}$ M[36] and varying concentrations of NaN_3 (0, 3.33×10^{-6} to 5.00×10^{-3} M) were prepared in vials (pH 5.00, 0.100 M NaOAc, $I = 3.0$ M ($NaCF_3SO_3$)), capped, and left to equilibrate for 1 h at 25.0 °C (water bath). The absorbance of each solution was measured at 315 nm by visible spectroscopy using the same cuvette (1 cm) for all measurements. *K*(N_3Cbl) was also determined using the same procedure at $I = 0.50$ M (KNO_3). *K*(N_3Cbl) could also be determined from absorbance data collected at 351 nm.

Determination of K_{obs}(GSCbl). A series of solutions with $[H_2OCbl^+] = 4.98 \times 10^{-5}$ M,[36] $[GSH]_T = 1.00 \times 10^{-4}$ M, and varying concentrations of NaSCN (0, 3.00×10^{-2} to 0.150 M) at pH 5.00 (0.100 M NaOAc, $I = 3.0$ M ($NaCF_3SO_3$)) were prepared in vials in the absence of air (glovebox) by the addition of an air-free solution of HOCbl·HCl to air-free NaSCN/GSH solutions. A small volume of several of the samples were transferred to a Schlenk cuvette for monitoring the extent of reaction by visible spectroscopy (300−800 nm), and the vials were capped and left to equilibrate in a glovebox. Immediately upon reaching equilibrium (~2.5 h later), the solution from each vial was transferred to a cuvette (one cuvette used for all measurements), and its absorbance (25.0 °C) was determined at 357 nm.

The same procedure was used to obtain absorbance data for the determination of K_{obs}(GSCbl) (a) with NaSCN ($I = 0.50$ M ($NaCF_3SO_3$ or KNO_3)) or NaN_3 ($I = 3.0$ or 0.50 M ($NaCF_3SO_3$ or KNO_3)) as a competing nucleophile at pH 5.00 and (b) at pH 4.50, 5.30, and 6.00 in the presence of NaSCN ($I = 3.0$ M ($NaCF_3SO_3$)). For solutions containing NaN_3 the absorbance was recorded at 367 nm. The time required for solutions to reach equilibrium lengthened with increasing pH (from minutes at pH 4.50 to 6 h at pH 6.00).

Kinetic Measurements. All solutions were prepared in 5.00×10^{-2} or 0.100 M buffer at a total ionic strength of 0.50 M (KNO_3) and the reaction was monitored using a Cary 1E spectrophotometer or an Applied Photophysics stopped-flow spectrophotometer. The rates of the reaction at pH 4.50−8.01 were determined under aerobic conditions and the reaction was monitored at 350 nm. The rates of the reactions at pH 8.31−11.0 were measured under anaerobic conditions. In a typical experiment, 4.0 mL of freshly prepared anaerobic GSH solution of the appropriate pH and ionic strength (pre-thermostated to 25.0 °C in a circulating bath) was quickly added via a syringe into an air-free Schlenk cuvette under positive nitrogen pressure which contained a small amount of solid HOCbl·HCl, and the cuvette was shaken and transferred to the cell holder of the Cary 1E spectrophotometer. The absorbance at 498 nm (pH 8.71−9.51), 495 nm (pH 10.11), or 388 nm (pH 11.0) was monitored. The kinetic data at pH 8.31 was collected at 498 nm under anaerobic conditions using the Applied Photophysics stopped-flow spectrophotometer. Glutathione solutions were freshly prepared and control experiments showed that ≤2% oxidation of glutathione occurred between preparing the solutions and data collection at the pH conditions and glutathione concentrations employed for the measurements.

Data fits to the equations given in the text were carried out using the program Microcal Origin version 3.5. All experimental errors are reported as one standard deviation of the mean value.

Determination of the Percentage of Glutathione Oxidation. A typical experiment was carried out as follows. An aerobic solution of GSH (6.25×10^{-5} M) and GSCbl (6.25×10^{-5} M) was freshly prepared in pH 5.02 buffer (1.0×10^{-3} M NaOAc). At periodic intervals a freshly prepared Ellmans solution (0.400 mL, 1.27×10^{-3} M in 0.10 M phosphate buffer, pH 8.0) was added to aliquots of the GSH/GSCbl solution (1.60 mL) and the absorbance at 412 nm was measured. From the known extinction coefficient of the reduced Ellman's reagent ($\epsilon = 1.36 \times 10^4$ M^{-1} cm^{-1} at 412 nm,[37] the percentage of glutathione oxidized to its disulfide form, GSSG, at times ~1, 3, 5, 7, 23, and 25 h after preparing the solution were calculated as 4, 10, 13, 16, 35, and 40%, respectively.

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Supporting Information Available: Evidence that GSCbl exists as GSCbl between pH 4.01 and 10.11; plot of A_{obs} versus mol equiv GSH_T for equilibrated anaerobic solutions of H_2OCbl^+ and GSH_T at pH 5.00 and details of the simulation of these data (Figure B); description of the procedure used to determine *K*(NCSCbl) at pH 5.00, 25.0 °C, $I = 3.0$ M ($NaCF_3SO_3$); evidence that acetate binding to H_2OCbl^+ is minimal; plots of k_{obs} versus $[GSH]_T$ for the reaction $HOCbl/H_2OCbl^+ + GSH_T \rightarrow GSCbl (+ nH^+)$ at pH 6.01, 8.01, 9.01, and 10.11; discussion on ref 15; Abs_{obs} data from which Figure 2 was generated; derivation of eqs 2 and 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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