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Kinetic Studies on the Decomposition of Thiolatocobalamins in Acidic Solution

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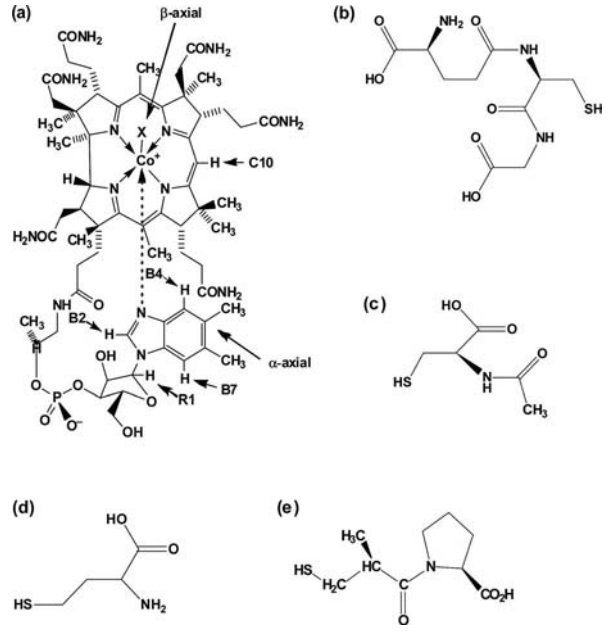
Thiolatocobalamins (RSCbls), or vitamin B_{12} derivatives with a thiolato ligand coordinated at the β-axial site of the cobalamin, have been shown to have superior antioxidant properties relative to the currently available therapeutic forms of cobalamin. It has also recently been shown that small amounts of glutathionylcobalamin are isolable from mammalian cells in the presence of a ligand trap. Kinetic studies are now presented for four representative thiolatocobalamins of glutathione, *N*-acetylcysteine, homocysteine, and captopril, which show that RSCbls spontaneously decompose to aquacobalamin (H_2OCbl^+) at pH < 3. A mechanism is proposed in which rapid protonation at the thiol sulfur of RSCbl precedes rate-determining decomposition. These results indicate that protection of RSCbls from acidic environments such as that found in the stomach is essential if RSCbls are to remain intact.

Introduction

We and others have been interested in the chemistry and biochemistry of thiol derivatives of vitamin B_{12}, thiolatocobalamins (RSCbls, X = SR, Scheme 1), for some time.[1] In the early 1990s, Jacobsen et al. proposed that glutathionylcobalamin (GSCbl) is an intracellular precursor of the two mammalian coenzyme forms of B_{12}, MeCbl (X = Me, Scheme 1) and AdoCbl (X = 5′-deoxyadenosyl, Scheme 1).[2] By using a free-ligand trap to prevent β-axial ligand-exchange reactions that occur between intracellular cobalamins (Cbls) and ligands released from the cell subsequent to cell lysis, in collaboration with the Jacobsen lab we showed that GSCbl (X = glutathione, GS, Scheme 1) is indeed present in mammalian cells, albeit in small amounts.[3] Banerjee et al. demonstrated the requirement for glutathione in the intracellular processing of alkylcobalamins by the protein MMACHC [methylmalonic aciduria (cobalamin deficiency) cblC-type, with homocystinuria].[4] Finally, McCaddon et al. found that patients with cognitive impairment respond better to *N*-acetylcysteine/aquacobalamin (H_2OCbl^+; X = H_2O, Scheme 1) supplementation than H_2OCbl^+ alone.[5] This led them to suggest that RSCbls could be more effective therapeutics than the currently available Cbl forms for the treatment of oxidative stress-associated diseases. Subsequently, Birch et al. showed the superior protection of RSCbls in preventing homocysteine and hydrogen peroxide-induced oxidative stress in a number of cell models relative to H_2OCbl^+, CNCbl, and MeCbl.[1d]



Scheme 1. Structures of (a) thiolatocobalamins (X = SR), (b) glutathione (GSH), (c) *N*-acetyl-L-cysteine (NAC), (d) D,L-homocysteine (Hcy), and (e) captopril (CapSH).

An understanding of the stability of thiolatocobalamins in aqueous solution is clearly required to assess their potential as therapeutics and is fundamental to understanding their biochemistry in biological systems. Although many thiolatocobalamins are stable in solutions of neutral pH, when synthesizing RSCbls we observed that RSCbls are unstable in acidic solution.[6] We now wish to report kinetic and mechanistic studies in acidic aqueous solution on the stability of four representative RSCbls: glutathionylcobalamin, *N*-acetylcysteinylcobalamin (NACCbl; X = *N*-acetyl-

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L-cysteine), homocysteinylcobalamin (HcyCbl; X = D,L-homocysteine), and captopril–cobalamin (CapSCbl, X = captopril).

Results and Discussion

The thiolatocobalamins GSCbl, NACCbl, CapSCbl, and HcyCbl were synthesized and characterized according to published procedures.[1b,1c,7] RSCbls were ≥95% pure, as determined by ^{1}H NMR spectroscopy. Kinetic experiments were typically initiated by adding a small aliquot (10–20 μL) of a concentrated RSCbl solution in water to an acidic solution (3.00 mL) at a specific pH condition (I = 0.50 M, KNO_3). All experiments were carried out under aerobic conditions except for studies on the decomposition of HcyCbl, for which anaerobic conditions were necessary due to the air sensitivity of this complex.[1c]

Figure 1 (a) shows UV/Vis spectral changes observed upon adding GSCbl to a solution of pH 3.00 (0.050 M HEPES buffer, 25.0 °C). Similar spectral changes were observed for the decomposition of NACCbl, CapSCbl, and HcyCbl in acidic solution. The sharp isosbestic points at 364, 447, and 536 nm are indicative of a single reaction occurring, in which GSCbl (λ_{max} = 334, 372, 428, and 534 nm) [6] is cleanly converted to H_2OCbl^+ (λ_{max} = 350, 410, and 526 nm).[1c,8] The corresponding plot of absorbance at 350 nm versus time is given in Figure 1 (b). The data fit well to a first-order rate equation, which gives an observed rate constant, k_{obs}, of $(8.76 \pm 0.01) \times 10^{-4}$ s^{-1}. The rate constant in the absence of HEPES buffer was very similar; hence specific acid catalysis applies to this system. Kinetic data were collected in the pH range 0.50–3.50. Measurements below pH 0.50 were not carried out, since this would increase the ionic strength beyond 0.50 M.

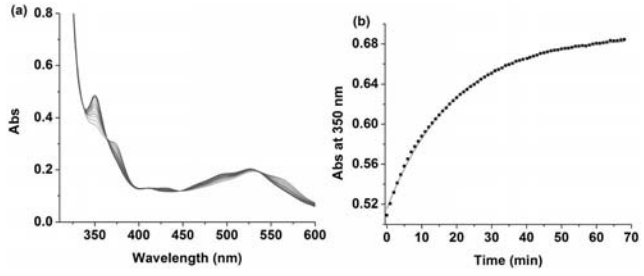


Figure 1. (a) UV/Vis spectra for the acid-catalyzed decomposition of GSCbl (5.00×10^{-5} M) at pH 3.00 [25.0 °C, 0.05 M HEPES buffer, I = 0.5 M (KNO_3)]. Spectra were recorded every 2 min. (b) Plot of absorbance at 350 nm versus time for the same experimental conditions as in (a). The data are fitted to first-order rate equation and give an observed rate constant (k_{obs}) of $(8.76 \pm 0.01) \times 10^{-4}$ s^{-1}.

Plots of k_{obs} versus pH are given in Figure 2 (a–d). It is clear from these plots that the rate of RSCbl decomposition increases with decreasing pH to reach a limiting value at low pH, which is consistent with protonation occurring prior to decomposition. For GSCbl, NACCbl, and CapSCbl, k_{obs} approaches 0 as the pH is increased, which is as expected since control experiments showed that these complexes are stable in aqueous solution at pH > 5. The experimental data were therefore fit to Equation (1), where $K_{a(RSCbl+H)}$ is the acid dissociation constant of the protonated cobalamin. Fitting the rate data for these three RSCbls to Equation (1) gives k and pK_a values that range from $(3.24–10.2) \times 10^{-2}$ s^{-1} and 1.14–1.31, respectively (Table 1). For HcyCbl, significant decomposition was observed even at pH 4. The data for this system were therefore fit to allow for spontaneous decomposition of RSCbl (k*) in addition to the protonated thiolatocobalamin (k) [Equation (2)] to give $k = 5.43 \times 10^{-2}$ s^{-1}, $k^* = 1.60 \times 10^{-3}$ s^{-1}, and pK_a(RSH) = 1.34 (Table 1).

$$k_{obs} = \frac{10^{-pH}\, k}{10^{-pH} + K_{a(RSCbl+H)}} \quad (1)$$

$$k_{obs} = \frac{10^{-pH}\, k + k^* K_{a(RSCbl+H)}}{10^{-pH} + K_{a(RSCbl+H)}} \quad (2)$$

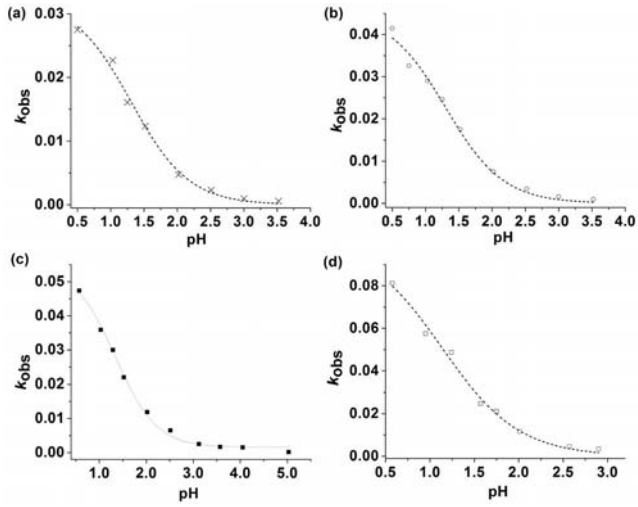


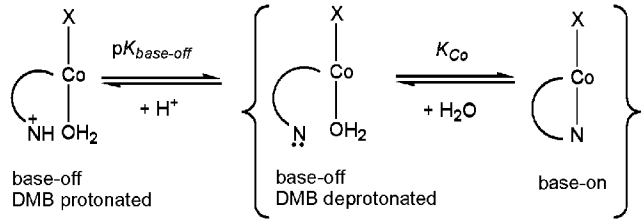
Figure 2. Plots of k_{obs} versus pH for the H^+-catalyzed decomposition of (a) GSCbl, (b) NACCbl, (c) HcyCbl, and (d) CapSCbl. The data has been fit to Equation (1) in the text (values of k and pK_a summarized in Table 1).

Table 1. Rate constants (k) and pK_a values for the decomposition of thiolatocobalamins [25.0 °C, I = 0.50 M (KNO_3)]. pK_a(RSH) values for the sulfhydryl group of the thiols are also given.

Complex	10^2k [s^{-1}]	10^3k^* [s^{-1}]	pK_a	pK_a(RSH)
GSCbl	3.24 ± 0.14		1.31 ± 0.07	9.63[9]
NACCbl	4.54 ± 0.21		1.31 ± 0.09	9.55[10]
HcyCbl	5.43 ± 0.17	1.60 ± 0.51	1.34 ± 0.05	10.0[10]
CapSCbl	10.2 ± 0.57		1.14 ± 0.08	9.80[11]

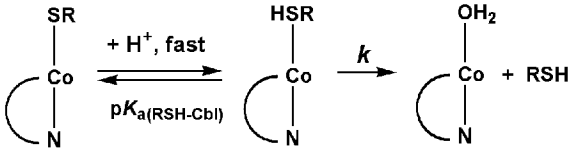
There are two likely possibilities for the site of protonation. In acidic solution, Cbls protonate at the imino-N of the α-5,6-dimethylbenzimidazole (α-DMB) to form "base-off" Cbls (Scheme 2) with an associated apparent pK_a, or

p$K_{base-off}$; hence one possibility is that protonation at the α-DMB occurs. Values of p$K_{base-off}$ have been reported for several Cbls with inorganic ligands at the β-axial site, including CNCbl (0.10[12]), H_2OCbl^+ (–2.13[12]), NO_2Cbl (≤ –0.15[13]), and NOCbl (5.1[14]), with p$K_{base-off}$ increasing with the electronic σ-donor strength of the β-axial ligand.[13] Given that cyanide is a stronger σ-donor ligand than RS^-, it is likely that p$K_{base-off} < 0.1$ for RSCbls, which is lower than the experimentally observed values of 1.14–1.41, Table 1. Furthermore, if rapid protonation of the α-DMB of RSCbl occurs to form base-off RSCbl-NH^+ prior to rate-determining cleavage to ultimately give H_2OCbl^+, this should be reflected in the isosbestic wavelengths observed during the decomposition reaction since large spectral shifts are observed for the base-on to base-off conversion of Cbls.[15] However, the isosbestic wavelengths were unchanged as the pH decreased for each system.



Scheme 2. Cobalamin conformations in aqueous solution.

It is therefore most likely that the sulfur atom of the thiol ligand itself is the site of protonation [Equation (3)]. Values of pK_a for the sulfhydryl group of CapSH, GSH, Hcy, and NAC are given in Table 1. It is well established that the pK_a of ligands drops several pH units upon binding to Cbl.[1a] Furthermore, protonating the hydroxy ligand of HOCbl to give H_2OCbl^+ [pK_a(H_2OCbl^+) = 7.8[1a]] does not cause significant changes in the isosbestic wavelengths of the HOCbl/GSCbl interconversion (344, 368, 457, and 551 nm; pH 9.7) relative to the H_2OCbl^+/GSCbl interconversion (341, 366, 460, and 548 nm; pH 1.4), and the same is probably true for the protonation of the thiolato ligand of RSCbl, which is consistent with the observation of insignificant changes in the isosbestic wavelengths despite rapid protonation that occurs prior to the rate-determining step.



(3)

Attempts to obtain further information on the protonated reaction intermediate for the acid-catalyzed decomposition of GSCbl by NMR spectroscopy were unsuccessful, with complete decomposition of GSCbl to H_2OCbl^+ occurring by the time the first spectrum was collected under the acidic pH conditions of this study. To probe p$K_{base-off}$ for GSCbl, the NMR spectrum of GSCbl was investigated as a function of time in the presence of an excess amount of glutathione. An excess amount of glutathione was added to shift the GSCbl ↔ H_2OCbl^+ + GS^- equilibrium to the left-hand side and hence prevent decomposition to H_2OCbl^+. After 6 min, approximately 1:1 (base-on GSCbl)/(base-off GSCbl) was observed at pD 1.00 (see the Supporting Information), with a slow increase in the percentage of base-off GSCbl to about 70% over a period of several hours. It was, however, subsequently realized that adding glutathione may favor the formation of base-off Cbl species due to the formation of base-off $(GS)_2Cbl^-$; hence this approach cannot be used to obtain an estimate for p$K_{base-off}$(GSCbl).

Conclusion

The decomposition of four thiolatocobalamins to aquacobalamin in acidic aqueous solution has been investigated. Decomposition occurs at pH < 3 and is acid-catalyzed. A mechanism is proposed in which rapid protonation at the sulfur atom of the RS^- ligand of RSCbl occurs prior to rate-determining decomposition. HcyCbl also appears to undergo slow decomposition in acidic solution in the absence of protonation. Given that the pH of the gastric juice of the stomach is less than 2,[16] it is clear that protection from this acidic environment is necessary to prevent decomposition of orally ingested RSCbls. It is unclear to us whether or not the binding of RSCbl to the B_{12} transport protein haptocorrin (HC) will be sufficient to prevent RSCbl decomposition. Photolysis of the β-axial Co–C bond of alkylcobalamins is considerably slower upon binding of the alkylcobalamin to B_{12} transport proteins,[17] and HC could also conceivably protect RSCbls from acid hydrolysis in the stomach.

Experimental Section

General: Hydroxycobalamin hydrochloride (HOCbl·HCl, 98% stated purity by manufacturer) was purchased from Fluka. The percentage of water in HOCbl·HCl (·nH_2O) (batch-dependent, typically 10–15%), was determined by converting HOCbl·HCl to dicyanocobalamin, $(CN)_2Cbl^-$ (0.10 M KCN, pH 10.5, ε_{368} = 30.4 $mM^{-1}cm^{-1}$[18]). Glutathione (98%) and captopril (98%) were purchased from Acros. D,L-Homocysteine (95%) was purchased from Fluka. *N*-Acetyl-L-cysteine (99%), HEPES buffer, and MES buffer were purchased from Sigma. KNO_3 (99%) was purchased from Acros. HPLC-grade H_2O was used without further purification.

pH measurements were carried out at room temperature with an Orion Model 710A pH meter equipped with a Mettler–Toledo Inlab 423 or 421 electrodes. The electrode was filled with a KCl/saturated AgCl solution (3 M, pH 7.0). The electrodes were standardized with standard BDH buffer solutions at pH 2.01, 4.01, and 6.98. Solution pH was adjusted using HCl, HNO_3, acetic acid, or NaOH solutions as necessary.

^{1}H NMR spectra were recorded with a Bruker 400 MHz spectrometer equipped with a 5 mm probe at room temperature (22 ± 1 °C). Solutions were prepared in D_2O or buffer (MES, TES) in D_2O and TSP {2,2,3,3-[D_4]3-(trimethylsilyl)propionic acid, sodium salt} was used as an internal standard.

UV/Vis spectra were recorded with a Cary 5000 spectrophotometer equipped with a thermostatted cell changer (25.0 ± 0.1 °C) and operated with WinUV Bio software (version 3.00). Reactant solutions were thermostatted for 15 min prior to beginning measurements. Kinetic data were fit with Origin 7 or 8 software. Errors are reported as one standard deviation.

For experiments under anaerobic conditions, solutions were degassed using freeze–pump–thaw cycles under argon by using standard Schlenk techniques. Air-free experiments were carried out on a Schlenk line or in a MBRAUN Labmaster 130 (1250/78) glovebox.

Kinetic Measurements: Solutions of pH < 1.5 were prepared by diluting a standardized HNO_3 solution. Solutions in the pH range 1.5–2.5 were prepared from aqueous HNO_3, and the pH was adjusted using concentrated HNO_3/NaOH in conjunction with a pH meter. For the pH range 3.0–3.5, the solutions were prepared in 0.050 M HEPES buffer (pK_{a1} = 3.05) and once again the pH of the solutions was adjusted as necessary. The total ionic strength was maintained at 0.50 M (KNO_3) for all solutions.

Thiolatocobalamin solutions (ca. 0.01 M, in water) were used within 1 h of preparation. The rates of the reactions for the decomposition of the thiolatocobalamins were determined under pseudo-first-order conditions in an excess amount of $[H^+]$. A small aliquot of the thiolatocobalamin solution was added ($[RSCbl]_{final} = 5.0 \times 10^{-5}$ M) to a solution of a specific pH, and the absorbance at 350 nm followed as a function of time with a Cary 5000 UV/Vis spectrophotometer. Experiments with HcyCbl were carried out under strictly anaerobic conditions.

Supporting Information (see footnote on the first page of this article): NMR spectrum of GSCbl in the presence of excess GSH at pD 1.00.

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