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Kinetic and Mechanistic Studies on the Reactions of the Reduced Vitamin B_{12} Complex Cob(I)alamin with Nitrite and Nitrate

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Dedicated to the memory of Dr. Edwin S. Gould[‡]

Keywords: Vitamin B_{12} / Cobalt / Bioinorganic chemistry / Kinetics / Reaction mechanisms

The kinetics of the reactions between cob(I)alamin [Cbl(I)] and nitrite and nitrate have been studied by UV/Vis and stopped-flow spectroscopy. Enzyme-bound Cbl(I) is an important transient species in several B_{12}-catalyzed enzyme reactions. Levels of nitrite and nitrate are elevated during oxidative stress, as a consequence of elevated nitric oxide levels. Although nitrite and nitrate are generally considered to be benign species, our studies show that nitrate and especially nitrite react rapidly with Cbl(I) at neutral pH conditions (k_{app} = 6.5×10^{-3} and 1.7×10^{3} $M^{-1}s^{-1}$, respectively, at pH 7, 25.0 °C). A reaction pathway is postulated for the reaction between Cbl(I) and (H)NO_2 involving a $2e^-$ rate-determining step to form Cbl(III) and HNO. The latter species reacts further with Cbl(I), ultimately resulting in the oxidation of 4Cbl(I) by HNO_2 to yield 4Cbl(II) and NH_2OH. The reaction between Cbl(I) and (H)NO_3 results in the oxidation of 8Cbl(I) by (H)NO_3 to give 8Cbl(II) and NH_4^+ (pH 5–7).

Introduction

The cob(III)alamins methylcobalamin (MeCbl; X = Me, Figure 1) and adenosylcobalamin (AdoCbl; X = 5′-deoxyadenosyl, Figure 1) are essential cofactors for two enzymes in mammals and multiple enzymes in bacteria.[1] Insufficient cobalamin is associated with neurological disorders and megaloblastic anemia.[2]

Reduced cobalamins, cob(II)alamin and cob(I)alamin, are also important intracellular complexes. In the laboratory, free (nonprotein-bound) cob(III)alamins are readily chemically reduced to pentacoordinate cob(II)alamin [H_2OCbl^+/Cbl(II): 0.20 V vs. SHE].[3] Stronger reducing agents such as excess $NaBH_4$ or titanium citrate are required to reduce Cbl(II) to Cbl(I) [redox potential Cbl(II)/Cbl(I): –0.61 V vs. SHE[3]]. Although the X-ray structure of Cbl(I) has not yet been determined, DFT calculations support the claim that this latter complex is tetracoordinate with essentially a square-planar geometry.[4]

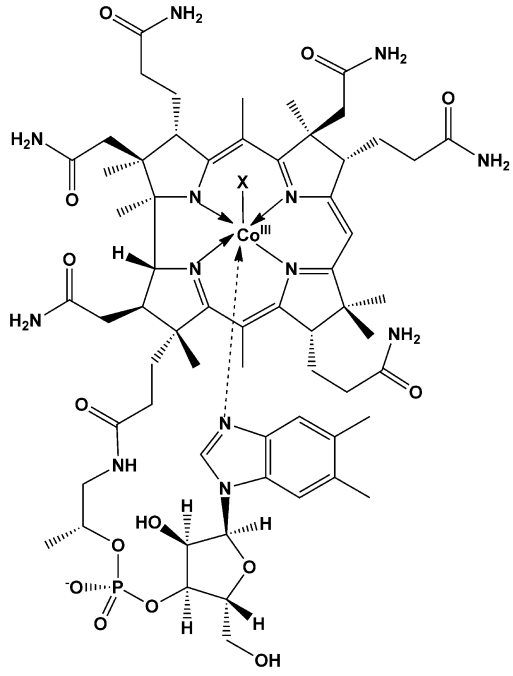

Figure 1. Structure of cob(III)alamins. Biologically relevant Cbl forms include X = CN, CH_3, 5′-deoxyadenosyl, H_2O/HO, NO, or glutathione.[7]

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[‡] In memory of our senior colleague, neighbor, and friend, Dr. Edwin S. Gould, whose deep understanding of inorganic chemistry, and good-humored willingness to share it, will be very much missed.

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In mammals, methionine synthase bound Cbl(I) is a transient complex formed in the MeCbl-dependent methionine synthase catalytic cycle.[1] Synthesis of AdoCbl-bound ATP:cob(I)alamin adenosyltransferase (ATR) also requires transient formation of Cbl(I)-bound ATR.[5] In mammals, AdoCbl is then sequestered by AdoCbl-dependent methylmalonyl-CoA mutase, which catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA.[6] Anaerobic bacterial dehalogenases also utilize cob(I)alamin for the dechlorination of aliphatic and aromatic compounds.[1] There has been much speculation as to how the thermodynamically demanding Cbl(II)/Cbl(I) reduction is achieved within cells given that the redox potential for reduction of free Cbl(II) to Cbl(I) is beyond that achievable by biological reductants.[5c,6,8] Recent spectroscopic and X-ray structural studies have shed considerable light on how, namely, in Cbl(II)-bound ATR, Cbl(II) is a tetracoordinate "base-off" complex, which lacks a ligand (or perhaps has a weakly bound water molecule) at the lower (α) axial site of the Cbl moiety.[5,9] Loss of the lower axial ligand increases the Co^{II}/Co^{I} redox potential, because of the decrease in electron density on the Co center. In Cbl(II)-bound methionine synthase, the α-5,6-dimethylbenzimidazole is replaced by a weakly bound water ligand, which, once again, increases the Co^{II}/Co^{I} redox potential.[10]

Because of the ease of nucleophilic displacement by Cbl(I), Cbl(I) has frequently been referred to as a supernucleophile.[4] Cbl(I) dehalogenates or dealkylates a wide range of organic molecules, including alkanes, alkenes, alkynes, aromatics, and phosphotriesters.[4,11] There is also much interest in using Cbl(I) to trap reactive intermediates such as oxiranes.[12] Kinetic studies have been reported for the dehalogenation of halogenated alkanes,[13] alkenes,[14] and acetic acid[15] and the dealkylation of phosphotriesters.[11a] Rate constants have also been reported for the reduction of functionalized pyridine,[16] transition-metal complexes,[17] esters,[18] organic disulfides,[19] oxyhalogens,[20] hydroxylamine[21] and nitrous oxide[22] by Cbl(I).

We are interested in the reactions of reduced cobalamins with reactive oxygen and nitrogen species (ROS/RNS).[23] Elevated ROS/RNS levels are implicated in numerous diseases, including septic shock, ischemic/reperfusion injury, atherosclerosis, and asthma.[24] Both mammalian B_{12}-dependent enzyme reactions are inactivated under oxidative/nitrosative stress conditions.[25] The reactive nitrogen species nitric oxide (·NO), a signaling molecule that plays a key role in nerve transmission, vasodilation, and the inflammatory immune response,[26] is oxidized to nitrite, which can be further oxidized to nitrate in biological systems. Indeed, the production of NO_2^- and NO_3^- is used as an indicator of the production of ·NO.[27] Megaloblastic anemia is associated with elevated levels of ·NO, and vitamin B_{12} supplementation restores ·NO concentrations to normal levels.[28] NO_3^- is also reduced to NO_2^- by enzymes possessing nitrate reductase activity, including bacterial nitrate reductases in the intestine and mouth.[26b] Furthermore, it has been suggested that NO_2^- is a biological signaling molecule and may play an important role in vasodilation and intravascular endocrine ·NO transport.[29] NO_2^- concentrations are significant in plasma and tissue, which, under normal conditions, are up to 0.5 and 10 μM, respectively.[26b,30] NO_3^- is present at even higher concentrations.[31]

In this study we focus on the reaction of Cbl(I) with nitrite and nitrate. Although nitrite and nitrate themselves are generally considered to be benign relative to reactive oxygen and nitrogen species, our studies show that the strong reductant cob(I)alamin reacts with both species at biological pH conditions.

Results and Discussion

Studies on the Reaction of Cob(I)alamin with Nitrite

Determination of the Stoichiometry and the Reaction Products

The Cbl product of the reaction of Cbl(I) with NO_2^- was identified by UV/Vis spectrophotometry. Figure 2a gives UV/Vis spectroscopic data as a function of time for the reaction of Cbl(I) (50 μM) with NO_2^- (9.10 mM) (25.0 °C, phosphate buffer, pH 12.00). The initial and final spectra correspond to that of Cbl(I) and Cbl(II), respectively, Figure 2b. From Figure 2a it can be seen that the reaction exhibits clean isosbestic points at 307, 346, 417 and 543 nm, in agreement with literature values for the conversion of Cbl(I) to Cbl(II),[32] which indicates a direct and clean conversion of Cbl(I) to Cbl(II) for the rate-determining step of the reaction. Cbl(II) is also the reaction product at lower pH values.

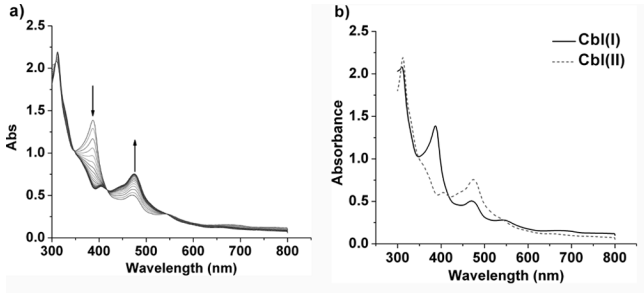

Figure 2. (a) UV/Vis spectra as a function of time for the reaction of Cbl(I) (50 μM, λ_{max} = 388, 464, 547, and 684 nm[33]) with NO_2^- (9.10 mM) at pH 12.00 (25.0 °C, 0.050 M phosphate buffer). Spectra were recorded every 2.5 min for 40 min. (b) Initial and final spectra for the reaction shown in (a). The final spectrum corresponds to Cbl(II) (λ_{max} = 312, 475 nm[33]).

To determine the stoichiometry of the reaction of Cbl(I) with nitrite, UV/Vis spectra of the product solution obtained upon equilibrating Cbl(I) (200 μM) with 0–0.50 molequiv. of NO_2^- under anaerobic conditions were recorded at pH 9.51 (5.0×10^{-3} M CHES buffer, 25.0 °C). The relatively high Cbl(I) concentration was used to enhance its stability in buffered solution. Figure 3 gives a plot of the absorbance at 700 nm vs. molequiv. of nitrite added. From this plot it is clear that the reaction is complete after the addition of ≈0.25 molequiv. of NO_2^-. Analysis of the absorbance data at 700 nm gave a mean value of

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Table 1. Determination of the stoichiometry of the reaction between Cbl(I) and NO_2^- at pH 9.51 (5.0×10^{-3} M CHES buffer). Absorbances were measured at 700 nm.

$10^4[Cbl(I)]_i$ (M)	$10^5[NO_2^-]_i$ (M)	$[NO_2^-]_i/[Cbl(I)]_i$	$Abs_{Cbl(I)}$	$Abs_{Cbl(II)}$	Abs_{obs}	Fraction Cbl(I) reacted[a]	Molequiv. of NO_2^- required[b]
2.00	1.00	0.050	0.388	0.104	0.323	0.229	0.22
2.00	2.00	0.10	0.388	0.104	0.262	0.443	0.23
2.00	3.00	0.15	0.388	0.104	0.203	0.651	0.23
2.00	4.00	0.20	0.388	0.104	0.159	0.806	0.25
2.00	5.00	0.25	0.388	0.104	0.119	0.947	0.26
2.00	6.00	0.30	0.388	0.104	0.110	–	–
2.00	7.00	0.35	0.388	0.104	0.103	–	–
2.00	8.00	0.40	0.388	0.104	0.107	–	–
2.00	9.00	0.45	0.388	0.104	0.099	–	–
2.00	10.0	0.50	0.388	0.104	0.100	–	–

[a] Fraction Cbl(I) reacted = $[Abs_{Cbl(I)} - Abs_{obs}]/[Abs_{Cbl(I)} - Abs_{Cbl(II)}]$. [b] Molequiv. of NO_2^- required = $\{[NO_2^-]_i/[Cbl(I)]_i\}/\{$fraction Cbl(I) reacted$\}$.

0.24 ± 0.02, Table 1. A similar analysis for absorbance data at 500 nm gave a value of 0.25 ± 0.03, Table S1, Supporting Information. A duplicate experiment gave similar results (0.26 ± 0.02 molequiv. of NO_2^-, Tables S2 and S3). The Cbl(I)/NO_2^- molar ratio is therefore 4:1.

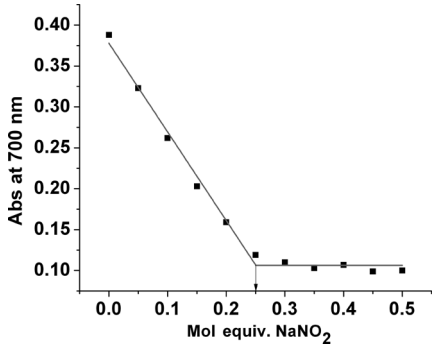

Figure 3. Plot of absorbance at 700 nm vs. molequiv. of $NaNO_2$ for equilibrated solutions of Cbl(I) (200 ± 9 μM) with 0–0.50 molequiv. of NO_2^- (5.0×10^{-3} M CHES buffer, pH 9.51, 25.0 °C).

The stoichiometry of the reaction of Cbl(I) and NO_2^- was also determined at pH 7.40 (5.0×10^{-3} M phosphate buffer, 25.0 °C), in duplicate, which gave a mean NO_2^-/Cbl(I) stoichiometry of (0.25 ± 0.04):1 (Tables S4–S7).

Determination of the Amount of Hydroxylamine Product Formed

A NO_2^-/Cbl(I) reaction stoichiometry of 0.25:1 suggests that the non-Cbl reaction product is NH_2OH, Equation (1).

$$(4)5H^+ + 4Cbl(I)^- + (H)NO_2 \rightarrow 4Cbl(II) + NH_2OH + H_2O \quad (1)$$

Whether the reactive species is NO_2^- and/or HNO_2 will be considered later. The indooxine assay (in which 8-hydroxyquinoline undergoes electrophilic aromatic substitution by NH_2OH and subsequent oxidation by air (O_2) to give 5,8-quinolinequinone-(8-hydroxy-5-quinoyl)-5-imide, "indooxine") is an established colorimetric procedure to quantify NH_2OH.[34] Cbl(I) and NO_2^- were treated in 1:0.27 (near stoichiometric) and 1:0.50 (excess NO_2^-) molar ratios under anaerobic conditions (5.0×10^{-3} M CHES buffer, pH 9.51), and the indooxine test was carried out on the product mixture at completion of the reaction under aerobic conditions (see Experimental Section). Only the indooxine product, not Cbl(II), absorbs in the 650–750 nm region (λ_{max} of indooxine product = 710 nm[34]). The NH_2OH concentration was determined from the absorbance at 710 nm of the product mixture by use of a standard calibration curve of absorbance vs. NH_2OH concentration, Figure S1. Given that 200 μM Cbl(I) was treated with sufficient NO_2^- for the reaction to proceed to completion, 50.0 μM NH_2OH would be expected for both Cbl(I)/NO_2^- ratios of 1:0.27 and 1:0.50. However, only ≈46% of the NH_2OH product (23 ± 2 μM) was obtained.

The low recovery of NH_2OH determined by the indooxine test was probed further. Control experiments showed that NH_2OH is stable for 1 hr in anaerobic water and in anaerobic and aerobic CHES buffer at pH 9.51 (see Supporting Information) and gave the expected concentration with the indooxine test. Interestingly, NH_2OH is not stable in aerobic water. It was also shown that nitrite does not react with 8-hydroxyquinoline to give a positive indooxine test (see Supporting Information). Others have reported that NH_2OH reacts with HNO_2, albeit under strongly acidic conditions;[35] however, experiments also showed that the NH_2OH concentration (77 μM) is unaffected by the presence of $NaNO_2$ (54 μM) (see Supporting Information). As further proof that the indooxine test is functioning reliably, the indooxine test was performed on a Cbl(I) + NO_2^- product sample that was spiked with NH_2OH. By using the calibration curve, it was determined that the unspiked sample contained 12.1 μM NH_2OH and that the spiked sample contained 32.5 μM NH_2OH (see Supporting Information). The spiked sample gave the expected increase in NH_2OH content based on the amount of NH_2OH added to the product solution.

No observable changes occur in the UV/Vis spectrum for a solution of Cbl(I) (50 μM) or Cbl(II) (50 μM) and NH_2OH (50 μM) (see Supporting Information). Gould et al. reported that Cbl(I) reacts with NH_3OH^+ in strongly acidic aqueous solution (pH 0.95–2);[21] however the reaction is slow compared to the reaction between Cbl(I) and nitrite (see later; $k = 4.1\ M^{-1}s^{-1}$, 25.0 °C, $I = 0.11$ M) and would be expected to be orders of magnitude slower under the pH conditions of this study since protonation increases the redox potential

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of oxyanions [pK_b(NH_2OH) = 7.97].[36] Cbl(I) is also stable in the presence of Cbl(III) (hydroxycobalamin) (see Supporting Information). However, in the presence of Cbl(II) (200 μM), only ≈70% NH_2OH was observed by the indooxine test (see Supporting Information). The experiments also showed that ≈30% of the original NH_2OH is absent from samples containing Cbl(II) regardless of how long the solution was allowed to react (0, 30, or 60 min), while NH_2OH was completely recovered in solutions not containing Cbl(II) (Table S8). Additionally, since the final step in the indooxine test requires oxygen, control experiments were carried out to determine the effect of bubbling air through the Cbl(I) + NO_2^- product solution prior to performing the indooxine test. The bubbling of air for 3 min recovered about 80% of the original NH_2OH concentration, but further bubbling for 10 min was unsuccessful at recovering the remaining NH_2OH (Table S9). Transition-metal-catalyzed disproportionation of NH_2OH has been reported by others.[37] It is likely that the aqueous reduced products of the disproportionated NH_2OH are re-oxidized to NH_2OH in the presence of oxygen, but the gaseous and oxidized products are not retrieved. Under anaerobic conditions in the presence of transition metals, NH_2OH disproportionates to give N_2, N_2O, and NO^+.[37a]

Finally, an experiment was performed to check that significant amounts of NH_3 are not formed in the reaction. Cbl(I) (200 μM) was treated with NO_2^- (50 μM) under anaerobic conditions (0.050 M CHES buffer, pH 9.51), and the Nessler reagent was added under aerobic conditions to the product solution. The color change indicative of the presence of NH_3 was not observed, which confirms that NH_3 is not produced in the reaction of Cbl(I) with nitrite.

Kinetic Studies on the Reaction of Cbl(I) with Nitrite

The kinetics of the reaction between Cbl(I) and (H)NO_2 were studied in the pH range 6.50–10.80, at 25.0 °C. Data were collected under pseudo-first-order conditions, with the NO_2^- concentration in excess (≥10×). Figure 4 gives a typical plot of absorbance vs. time for the reaction of Cbl(I) (50 μM) with NO_2^- (5.00 mM) at pH 10.80. The inset to Figure 4 gives a plot of absorbance vs. time for the identical Cbl(I) solution in the absence of NO_2^- at pH 10.80. Cbl(I) is slowly oxidized to Cbl(II) even under strictly anaerobic conditions;[38] however, this reaction is significantly slower (ΔAbs = 0.02 at 388 nm) than the reaction of interest (ΔAbs = 0.45 at 388 nm for the same time period) at pH 10.80. This was the case under all the pH conditions of this study. Data was collected from 1.00 to 35.0 mM NO_2^- for the reaction between Cbl(I) and NO_2^- at pH 10.80, and the results are summarized in Figure 5. Fitting of the data to a straight line passing through the origin gives an apparent second-order rate constant, k_{app} = 0.49 ± 0.02 $M^{-1}s^{-1}$. Data collected at other pH conditions is shown in Figure S2. Under acidic pH conditions (pH 6.50 and 6.92), Cbl(I) was not sufficiently stable in buffer to mix a buffered solution of Cbl(I) with a buffered solution of nitrite. Therefore, the Cbl(I) solution was diluted in water and mixed with buffer within the stopped-flow spectrophotometer's tubing itself (see Experimental Section).

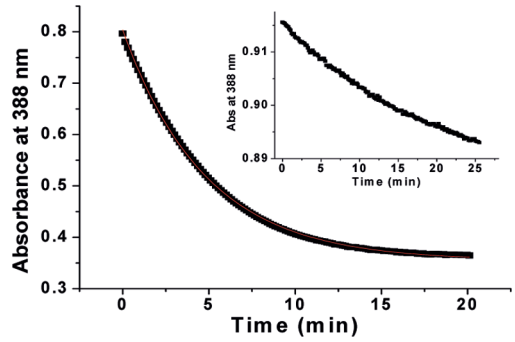

Figure 4. Plot of absorbance at 388 nm vs. time for the reaction of Cbl(I) (50 μM) with NO_2^- (5.00 mM) at pH 10.80 [25.0 °C, 0.050 M CAPS buffer, I = 0.50 M (Na_2HPO_4)]. Fitting of the data to a first-order rate equation gives k_{obs} = 0.209 ± 0.001 s^{-1}. Inset: Plot of absorbance vs. time for the spontaneous decomposition of Cbl(I) (50 μM) to Cbl(II) under the same conditions and time period.

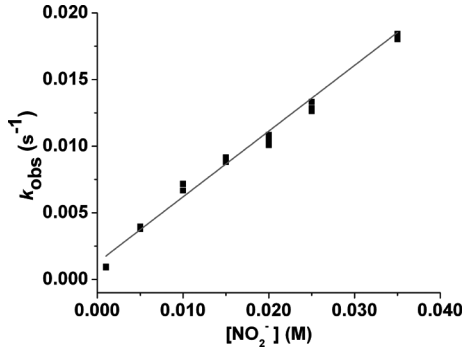

Figure 5. Plot of k_{obs} vs. NO_2^- concentration for the reaction between Cbl(I) and NO_2^- at pH 10.80 (25.0 °C, 0.050 M CAPS, I = 0.50 M phosphate buffer). Fitting of the data to a straight line gives a slope, k_{app} = 0.49 ± 0.02 $M^{-1}s^{-1}$ and an intercept of (1.3 ± 0.4) × 10^{-3} s^{-1}.

Figure 6 and Table S10 summarize the dependence of the k_{app} value on pH. Below pH 6.5, the reaction was too rapid to be followed by stopped-flow spectrophotometry. Under acidic conditions, significant intercepts were observed in the plots of k_{obs} vs. (H)NO_2 concentration, and control experiments showed that this arises from partial spontaneous oxidation of Cbl(I) to Cbl(II) [see Table S10 and the accompanying discussion, Supporting Information; the reaction equilibrium lies towards Cbl(I)]. At high pH conditions k_{app} approaches 0; hence, HNO_2, and not NO_2^- (pK_a(HNO_2) = 3.16[39]), reacts with Cbl(I). It is not unexpected that HNO_2 oxidizes Cbl(I) more rapidly than NO_2^-, since HNO_2 is a stronger oxidant [E(HNO_2/N_2) = 1.45 V, pH 0; E(NO_2^-/N_2) = 0.41 V, pH 14 vs. SHE[24d]] because of the electron-withdrawing effects of the acidic proton. By assuming that only HNO_2 reacts with Cbl(I), the corresponding rate Equation (2) is:

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$$k_{app} = k_{obs}/[Cbl(I)]_T = (k \times 10^{-pH})/[10^{-pH} + K_a(HNO_2)] \quad (2)$$

Fitting of the data in Figure 6 to Equation (2) and by fixing $K_a(HNO_2) = 6.9 \times 10^{-4}$ gives $k = (1.15 \pm 0.08) \times 10^7$ M^{-1} s^{-1}. At pH 7, $k_{app} = 1.7 \times 10^3$ M^{-1} s^{-1}.

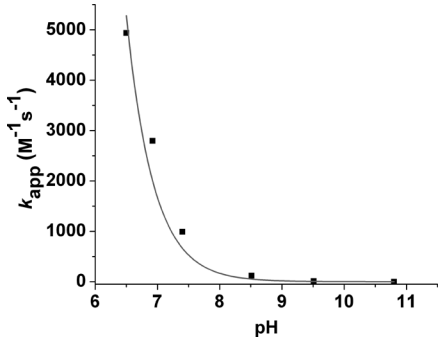
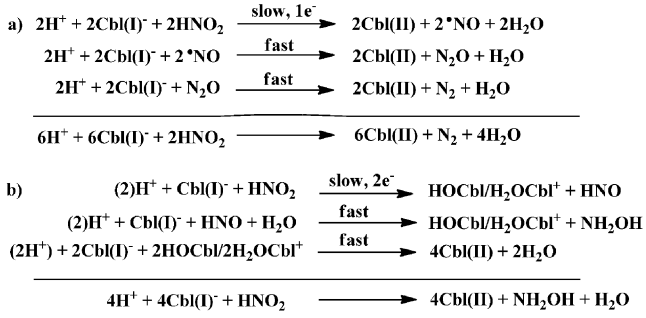


Figure 6. Plot of the second-order rate constant k_{app} vs. pH for the reaction between Cbl(I) and (H)NO_2. The data was fitted to Equation (2) in the text, which gives $k = (1.15 \pm 0.08) \times 10^7$ M^{-1} s^{-1}.

The two most likely reaction pathways are outlined in Scheme 1a and b. Importantly, complete conversion of Cbl(I) to Cbl(II) is observed in the rate-determining step under all conditions; hence, the first step is slow compared with subsequent reactions. In Scheme 1a, Cbl(I) is oxidized by HNO_2 in a 1e$^-$ process to form Cbl(II) and $^{\bullet}NO$. The subsequent reaction between Cbl(I) and $^{\bullet}NO$ has been studied by others,[41] and yields Cbl(II) and N_2O. The latter species oxidizes Cbl(I) to give Cbl(II) and N_2.[22] Given that NH_2OH is experimentally observed as the reaction product, this reaction pathway is unlikely. In Scheme 1b, Cbl(I) is oxidized by HNO_2 in a 2e$^-$ process to form Cbl(III) (hydroxycobalamin/aquacobalamin) and HNO in the rate-determining step. Although to the best of our knowledge the reaction between HNO and Cbl(I) has not yet been studied (HNO is a demanding species to work with as it rapidly and spontaneously dimerizes to ultimately form N_2O and H_2O, and requires the use of HNO donor molecules[42]), there is precedent for HNO acting as a 2e$^-$ oxidant, being itself reduced to NH_2OH.[43] The redox potential of (HNO, $2H^+/NH_2OH$) is 0.3 V vs. SHE at pH 7,[44] which makes it a moderate oxidizing agent capable of oxidizing Cbl(I) {$E°$[Cbl(II)/Cbl(I)] = –0.61 V vs. SHE.[3]}. Alternatively, N_2O rather than HNO could be produced in the first step of this pathway. However, others have shown that N_2O reacts with Cbl(I) to give N_2.[22] Finally, the first step in Scheme 1b must be the rate-determining step since there is no observed accumulation of the HOCbl intermediate. The rate constant for the reaction of Cbl(I) with Cbl(III) is 3.2×10^7 M^{-1} s^{-1}.[22]

To summarize, the stoichiometry of the reaction between Cbl(I) and NO_2^- was found to be 4:1 Cbl(I)/NO_2^-. Cbl(II) is the Cbl product (UV/Vis spectroscopy). On the basis of the stoichiometry, the expected non-Cbl product is NH_2OH. The indooxine test for the presence of NH_2OH was positive, albeit nonquantitative. It is likely that partial disproportionation of NH_2OH occurs.



Scheme 1. Possible reaction pathways for the reaction between Cbl(I) and HNO_2. (a) 1e$^-$ rate-determining step. (b) 2e$^-$ rate-determining step. pK_a(H-^{3}NO) ≈ 11.6.[40]

Studies on the Reaction of Cob(I)alamin with Nitrate

Determination of the Stoichiometry and the Reaction Products

Studies on the reaction between Cbl(I) and nitrate in strongly acidic solution (pH 1.5–2.5) have been reported;[32] however it was of interest to determine whether this mechanism is still in operation in the biological pH range. Figure 7 gives typical UV/Vis spectra as a function of time for the reaction between Cbl(I) (50 μM) and NO_3^- (0.200 M) under anaerobic conditions at pH 6.02 (25.0 °C, 0.10 M phosphate buffer, I = 1.5 M). Cbl(I) (λ_{max} = 388, 464, 547, and 684 nm[33]) is again cleanly oxidized to Cbl(II) (λ_{max} = 312, 475 nm[33]), with sharp isosbestic points observed at 356, 416 and 539 nm, in agreement with literature values for the oxidation of Cbl(I) to Cbl(II).[32]

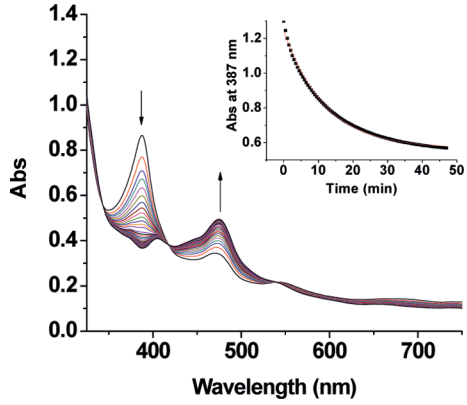


Figure 7. UV/Vis spectra as a function of time for the reaction of Cbl(I) (50 μM) with NO_3^- (0.200 M) at pH 6.02 (25.0 °C, 0.10 M phosphate buffer, I = 1.5 M). Selected spectra are shown every 1.0 min. Cbl(I) (λ_{max} = 388, 464, 547, and 684 nm[33]) is converted to Cbl(II) (λ_{max} = 312, 475 nm[33]). Inset: Plot of absorbance at 387 nm vs. time for the data. The data has been fitted to a first-order rate equation, which gives $k_{obs} = (7.13 \pm 0.07) \times 10^{-2}$ min^{-1}.

The non-Cbl reaction product was identified as ammonia by using the Nessler's test, consistent with a reaction stoichiometry of 8:1 Cbl(I)/NO_3^- (see Experimental Section). NH_4^+ is also produced when Cbl(I) reacts with nitrate under strongly acidic pH conditions.[32] The overall reaction can therefore be written as Equation (3).

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$$8Cbl(I)^- + NO_3^- + 10H^+ \rightarrow 8Cbl(II) + NH_4^+ + 3H_2O \quad (3)$$

Attempts to confirm this reaction stoichiometry by recording UV/Vis spectra of equilibrated solutions of Cbl(I) containing varying concentrations of nitrate were unsuccessful since considerable Cbl(I) decomposition occurred during the time frame of the reaction even at the lowest pH value of this study (pH 5.02), where the reaction between Cbl(I) and nitrate is the most rapid. The reaction rate was also too slow compared with the rate of Cbl(I) decomposition to accurately determine the stoichiometry at higher pH values.

Kinetic Studies on the Reaction of Cbl(I) with Nitrate

The inset in Figure 7 gives the best fit of the absorbance data at 387 nm vs. time for the reaction between Cbl(I) and nitrate at pH 6.02 to a first-order rate equation, which gives $k_{obs} = (7.13 \pm 0.07) \times 10^{-2}$ min^{-1}. The rate of decomposition of Cbl(I) to Cbl(II) is significant under acidic pH conditions and was therefore also independently studied. This latter reaction was found to be over an order of magnitude slower than that of Cbl(I) with nitrate at each pH condition, Table 2.

Table 2. Second-order rate constants (k_{app}) for the reaction of Cbl(I) with NO_3^-, and approximate first-order rate constants (k_{decomp}) for the spontaneous decomposition of Cbl(I) at different pH values. Note that the latter reaction is not strictly first-order, does not proceed to completion, and exhibits considerable variability between experiments.

pH (± 0.02)	k_{app} (M^{-1} min^{-1})	10^3 k_{decomp} (min^{-1})
5.02	14.92 ± 0.96	17.1 ± 0.2, 26.2 ± 0.7
6.02	3.15 ± 0.05	2.84 ± 0.01, 2.14 ± 0.02
7.00	0.39 ± 0.01	0.65 ± 0.02, 0.68 ± 0.06

The dependence of k_{obs} on the NO_3^- concentration (0.0500–0.500 M) was studied at pH 6.02, and the results are summarized in Figure 8. Fitting of the data to a straight line that passes through the origin gives an apparent second-order rate constant, $k_{app} = 3.15 \pm 0.05$ M^{-1} min^{-1}. Data under other pH conditions is shown in Figures S3 and S4, and k_{app} values are summarized in Table 2. From Table 2, it is clear that the rate of the reaction increases with decreasing pH. At pH 8.00, the reaction was too slow relative to the spontaneous decomposition of Cbl(I) to be determined.

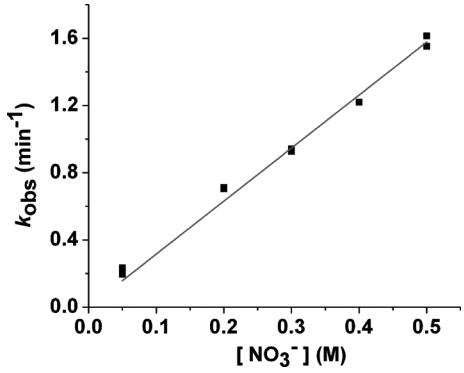

Figure 8. Plot of k_{obs} vs. NO_3^- concentration for the reaction between Cbl(I) and NO_3^- at pH 6.02 ± 0.02 (25.0 °C, 0.10 M phosphate buffer, I = 1.5 M). The data was fitted to a straight line passing through the origin, which gives a slope of 3.15 ± 0.05 M^{-1} min^{-1}.

The data in Table 2 were fitted to the rate Equation (4):

$$k_{app} = k_{obs}/[Cbl(I)]_T = k^* [NO_3^-] \, 10^{-pH} \quad (4)$$

Fitting of the data to Equation (4) gives $k^* = (2.53 \pm 0.12) \times 10^4$ M^{-2} s^{-1}. This agrees well with the value obtained elsewhere at pH 1.5–2.5 ($k^* = 2.1 \times 10^4$ M^{-2} s^{-1}, 25.0 °C, 0.01 M $HClO_4$, I = 0.11 M).[32] Hence, the mechanism of the oxidation of Cbl(I) by (H)NO_3 is unchanged in the pH range 1.5–7.0. Given that the overall reaction is an 8e$^-$ process and therefore involves multiple steps, we are reluctant to propose a reaction scheme for this reaction without carrying out further extensive experimentation. Since NH_3 rather than NH_2OH is produced, this suggests that NO_2^- is unlikely to be an intermediate, and the first step of the reaction is therefore most likely a 1e$^-$ redox reaction. The first-order dependence of the rate of the reaction on [H^+] is consistent with the reaction of HNO_3 with Cbl(I).

Summary

The reactions of the strong reductant Cbl(I) with nitrite and nitrate have been studied by UV/Vis spectroscopy and/or stopped-flow spectroscopy. At pH 7, $k_{app} = 1.7 \times 10^3$ M^{-1} s^{-1} for the reaction between Cbl(I) and (H)NO_2, whereas the apparent rate constant for the reaction between Cbl(I) and (H)NO_3 is five orders of magnitude smaller ($k_{app} = 6.5 \times 10^{-3}$ M^{-1} s^{-1}). The total Cbl concentration in cells is 30–700 nM in humans,[6] whereas tissue NO_2^- concentrations are as high as 10 μM[26b,30] under normal conditions and may be significantly elevated during periods of cellular oxidative and nitrosative stress. At 10 μM nitrite, the half-life for oxidation of Cbl(I) by nitrite is ≈40 s. A reaction pathway is postulated for the reaction between Cbl(I) and (H)NO_2 that involves a 2e$^-$ rate-determining step to form Cbl(III) and HNO. The latter species reacts further with Cbl(I), which ultimately results in the oxidation of 4Cbl(I) by HNO_2 to yield 4Cbl(II) and NH_2OH. Detailed kinetic and mechanistic studies on the reaction between Cbl(I) and HNO are required, however, to probe this further. The reaction between Cbl(I) and (H)NO_3 results in the oxidation of 8Cbl(I) by (H)NO_3 to give 8Cbl(II) and NH_4^+.

Experimental Section

Chemicals: Hydroxycobalamin hydrochloride, HOCbl·HCl (·$n$$H_2O$) (≥96%, 10–15% water, batch dependent[45]) was purchased from Fluka. BIS-TRIS and TAPS buffers were purchased from Sigma. Sodium borohydride (≥98%), CAPS, CHES, and MES buffer, sodium nitrite (99.6%), sodium nitrate (≥99%), and hydroxylamine hydrochloride (NH_2OH·HCl, ≥97%) were purchased from Acros Organics. Disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium carbonate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, potassium cyanide (≥99.1%), and 8-hydroxyquinoline (≥99%) were purchased from Fisher Scientific.

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Nessler's reagent was purchased from RICCA Chemical. Water was purified by using a Barnstead Nanopure Diamond water purification system.

Instrumental and Procedures: For Cbl(I) experiments, anaerobic solutions were degassed by four freeze-pump-thaw cycles under argon gas by using standard Schlenk techniques. Solutions prepared for Cbl(II) work were degassed by bubbling through argon for at least 2 h. Preparation of all anaerobic solutions was carried out in an MBRAUN Labmaster 130 (1250/78) glove box filled with argon, equipped with O_2 and H_2O sensors and a freezer at –24 °C. The pH measurements were carried out at room temperature by using an Orion model 710A pH meter equipped with Mettler-Toledo Inlab 423 or 421 pH electrodes. The electrode was filled with a 3 M KCl/saturated AgCl solution (pH 7.0) and standardized with standard buffer solutions at pH 4.00, 7.00, 10.00, and 12.45. The solution pH was adjusted with H_3PO_4, NaOH, or KOH solutions as necessary.

UV/Vis spectroscopy data were recorded on a Cary 5000 spectrophotometer equipped with a thermostatted (25.0 ± 0.1 °C) cell changer operating with WinUV Bio software (version 3.00). Kinetic data for rapid reactions were collected under strictly anaerobic conditions at 25.0 ± 0.1 °C by using an Applied Photophysics SX20 stopped-flow spectrophotometer equipped with a photodiode array detector in addition to a single wavelength detector, with sequential mixing capabilities. A continuous flow of nitrogen was used to maintain anaerobic conditions. Data were collected with Pro-Data SX (version 2.1.4) and Pro-Data Viewer (version 4.1.10) software, and a 1.0 cm pathlength cell was utilized. All data was analyzed by using Microcal Origin version 8.0.

Synthesis of Cbl(I): Cbl(I) was synthesized under anaerobic conditions according to a modified published procedure.[46] For a typical synthesis, HOCbl·HCl [≈25 mg, 1.6×10^{-5} mol (10–15% H_2O)] was dissolved in anaerobic water (0.75 mL) in a vial. An aqueous, anaerobic stock solution of $NaBH_4$ (≈10 mg, 2.6×10^{-4} mol, 1.00 mL) was prepared, and $NaBH_4$ (6.0 mol equiv.) was added to HOCbl·HCl. The vial was shaken vigorously for ≈1 min, and the reaction was allowed to occur for an additional 15–30 min. After the reaction was complete, the excess $NaBH_4$ was quenched by addition of acetone (0.200 mL), except when noted otherwise. Note that for all experiments performed with the stopped-flow instrument, the excess $NaBH_4$ was not quenched in order to provide added stability to the Cbl(I) reagent solution. The product was characterized by UV/Vis spectrophotometry (λ_{max} = 388, 464, 547, and 684 nm[33]) and stored under anaerobic conditions at –24 °C. Cbl(I) solutions were used within 1 week of preparation.

Synthesis of Cbl(II): Cbl(II) was synthesized under anaerobic conditions according to a modified published procedure analogous to the synthesis of Cbl(I), except that 1.1 equiv. $NaBH_4$ was used.[46] Cbl(II) was characterized by UV/Vis spectrophotometry (λ_{max} = 267, 285, 312, 475 nm[33]), and solutions were stored under anaerobic conditions at –24 °C.

Determining Cobalamin Concentrations: The concentration of cobalamin in stock solutions was determined by converting to dicyanocobalamin, $(CN)_2Cbl^-$, with 0.1 M KCN [ε_{368} = 3.04×10^4 M^{-1} cm^{-1} for $(CN)_2Cbl^-$[47]].

Determining the Stoichiometry of the Reaction between Cbl(I) and Nitrite: The stoichiometry of the Cbl(I) + NO_2^- reaction was determined in buffer (5.0×10^{-3} M; CHES buffer at pH 9.51 and phosphate buffer at pH 7.40) under strictly anaerobic conditions. In a typical experiment, Cbl(I) (200 ± 9 μM, in water) was added to solutions containing varying mol equiv. of NO_2^- (2.96×10^{-4} M in CHES buffer; 0.050, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, and 0.50 mol equiv. of NO_2^-). After 30 min (the minimal time required for the slowest reaction to proceed to completion as observed by UV/Vis spectroscopy), the UV/Vis spectrum was recorded. The experiment was completed in duplicate at pH 9.51 and 7.40. At pH 7.40, the reaction was complete in less than 1.5 min (the time required to remove the sample from the glove box to record a UV/Vis spectrum).

Determining the Amount of Hydroxylamine Product Formed in the Reaction between Cbl(I) and Nitrite by using the Indooxine Test: A modified literature procedure[34] was used to determine the amount of NH_2OH produced by the reaction of Cbl(I) and NO_2^-. Calibration standards were prepared by the addition of 8-hydroxyquinoline (1.00 mL, 4.0% w/v in ethanol) to a NH_2OH solution (1.00 mL) of a specific concentration in CHES buffer (5.0×10^{-3} M), pH 9.51, followed by the addition of Na_2CO_3 (1.00 mL, 1.00 M) under aerobic conditions. The reaction was allowed to proceed for 25 min (room temperature), and UV/Vis spectra recorded. The calibration curve is shown in Figure S1.

The reaction of Cbl(I) (200 ± 7 μM) and $NaNO_2$ (2.96×10^{-4} M) in 5.0×10^{-3} M CHES buffer, pH 9.51, was allowed to proceed to completion (1 h) under strictly anaerobic conditions inside the glove box for Cbl(I)/NO_2^- ratios of 1:0.27 and 1:0.50, each in triplicate. The indooxine test was then performed on each sample by the addition of aerobic 8-hydroxyquinoline solution (1.00 mL, 4.0% w/v in ethanol) and aqueous aerobic Na_2CO_3 solution (1.00 mL, 1.00 M) to the reaction product (1.00 mL) outside the glove box. After standing for 30 min at room temperature, the UV/Vis spectrum was recorded.

Determining whether Ammonia is a Product of the Reaction between Cbl(I) and Nitrite: A Nessler test for the presence of NH_3 was performed on a solution containing the reaction products of 200 μM Cbl(I) with 50 μM NO_2^- (0.050 M CHES buffer, pH 9.51) under aerobic conditions. Approximately 8 drops of the Nessler reagent were added to the product solution. A positive result for NH_3 is indicated by a yellow or, at high concentrations, brown coloring in the reaction solution.[48] In this case, no brown or yellow coloring was observed above the pink color of the HOCbl species.

Determining whether Ammonia is a Product of the Reaction between Cbl(I) and Nitrate: Nessler's reagent (2–3 drops) was added to the product mixture (3.00 mL) of the reaction between Cbl(I) (50.0 μM) and NO_3^- (0.500 M) at pH 5.02 [25.0 °C, 0.01 M acetate buffer, I = 1.5 M (K_2HPO_4)] immediately upon the completion of the reaction. The immediate formation of a brown-colored precipitate at the bottom of the vial occurred. In order to check that the formation of the brown precipitate is not due to the reaction of Cbl(II) with Nessler's reagent, a control experiment was carried out in which 2–3 drops of Nessler's reagent was added to Cbl(II) solution (50.0 μM) at pH 5.02 [25.0 °C, 0.01 M acetate buffer, I = 1.5 M (K_2HPO_4)]. A cloudy red-colored solution was instead formed.

Kinetic Studies on the Reaction between Cbl(I) and Nitrite: All kinetic experiments for the reaction of Cbl(I) with NO_2^- were performed by using at least ten times excess NO_2^- to ensure pseudo-first-order kinetics at a total ionic strength of 0.50 M (Na_2HPO_4/NaH_2PO_4). Buffers (0.050 M; CAPS, CHES, TAPS, phosphate, MES, and BIS-TRIS) were used to maintain the solution pH. Data collected by using the UV/Vis spectrophotometer were obtained by using Schlenk cuvettes equipped with a bulb, which was filled with a concentrated Cbl(I) solution in CHES buffer, to maximize its stability. The solutions were thermostatted at 25.0 °C for at least 10 min before initiating the reaction. Data for the reaction of Cbl(I) with NO_2^- at pH < 10.80 were collected by using the stopped-flow

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spectrophotometer. Experiments at pH < 7.40 required that the Cbl(I) solution (300 μM, to maximize its stability) be diluted in H_2O and mixed with the nitrite solution in buffer within the tubing of the instrument. Unless otherwise specified, data were analyzed at 388 nm. At pH 6.92 and 6.50, data were collected and analyzed at 500 nm to accommodate the higher Cbl concentrations.

Kinetic Studies on the Reaction between Cbl(I) and Nitrate: All kinetic experiments for the reaction of Cbl(I) with NO_3^- were performed by using at least ten times excess NO_3^- at a total ionic strength of 1.50 M (K_2HPO_4/KH_2PO_4). Buffers (0.10 M, phosphate or acetate, 25.0 °C) were used to maintain the solution pH. Data were collected by using the Cary 5000 UV/Vis spectrophotometer. All experiments were carried out by using Schlenk cuvettes equipped with a bulb, which was filled with a concentrated Cbl(I) solution in H_2O. The solutions were thermostatted at 25.0 °C for at least 10 min before mixing the reagents and initiating the reaction. The data were analyzed at 387 nm.

Supporting Information (see footnote on the first page of this article): Determination of the stoichiometry of the reaction between Cbl(I) and NO_2^- under different conditions, details of the indooxine test experiments, and kinetic plots and details are presented.

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