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Kinetic and Mechanistic Studies on the Reaction of the Vitamin B_{12} Complex Aquacobalamin with the HNO Donor Angeli's Salt: Angeli's Salt and HNO React with Aquacobalamin

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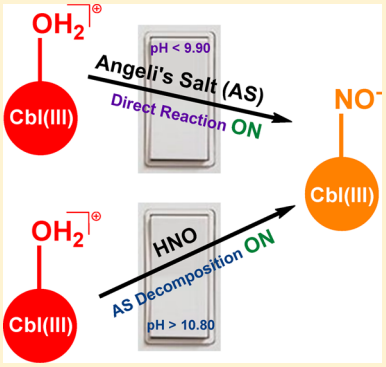
Supporting Information

ABSTRACT: We report the first studies on the reaction between an HNO donor compound and vitamin B_{12} complexes. Kinetic and mechanistic studies have been carried out on the reaction between the vitamin B_{12} derivative aquacobalamin (H_2OCbl^+/HOCbl; pK_a = 7.8) and the HNO donor Angeli's salt. Studies were carried out with aquacobalamin in excess, since nitrite also reacts with aquacobalamin to form nitrocobalamin (NO_2Cbl). At pH <9.90 aquacobalamin reacts directly with the monoprotonated form of Angeli's salt, $HN_2O_3^-$, to form nitroxylcobalamin (NO^--Cbl(III); NOCbl) and nitrite. At pH >10.80 the reaction instead switches predominantly to a mechanism in which spontaneous decomposition of Angeli's salt to give HNO and nitrite becomes the rate-determining step, followed by the rapid reaction between aquacobalamin and HNO/NO^- to again give NOCbl. Both reactions proceed with a 1:1 stoichiometry and formation of nitrite is confirmed using the Griess assay.



INTRODUCTION

There is currently much interest in the chemical and biochemical reactivity of the reactive nitrogen species nitrosyl hydride (nitroxyl, HNO).[1−8] HNO is generated in vivo by nitric oxide synthases from L-arginine in the absence of the tetrahydrobiopterin cofactor or by the oxidation of *N*-hydroxy-L-arginine.[9−11] Reduction of NO by enzymes such as ferrocytochrome *c*[12] and superoxide dismutase[13] leads to HNO generation. Spontaneous decomposition of *S*-nitrosothiols or their reactions with thiols may also generate HNO.[10,11,14] Like NO, elevated HNO levels are associated with damage to cellular components leading to nitrosative stress.[15] Thus far HNO formation in biological systems has not been unequivocally demonstrated, and multiple groups are currently focused on designing molecules which efficiently trap HNO.[16−21]

An interesting aspect of HNO-associated chemistry is the spin state change which occurs upon deprotonation of HNO in alkaline solution (the pK_a for deprotonation of ^{1}HNO to give $^3NO^-$ ($+H^+$) is ∼11.4).[10,22−24] Furthermore, HNO has a unique chemical and biological reactivity distinct from NO.[9,25−27] For example, HNO reacts directly with thiols while NO does not.[9] HNO inhibits aldehyde dehydrogenase, and as such may be useful in treating alcoholism.[28] Like NO, HNO is also a vasorelaxant[29] and shows potential in treating cardiovascular disease and preventing ischemia/reperfusion injury and congestive heart failure.[9,25,30] However, one major challenge in elucidating the chemical and biochemical reactivity of HNO stems from its rapid, spontaneous dimerization and subsequent decomposition to nitrous oxide and water in aqueous solution, eq 1.[31]

$$\mathrm{HNO + HNO \rightarrow H_2N_2O_2 \xrightarrow{k=8\times10^6\,M^{-1}s^{-1}} N_2O + H_2O} \quad (1)$$

Owing to its inherent instability, HNO donor compounds are therefore required to generate HNO in situ, and indeed the design of molecules that release HNO within a useful time frame is an active area of research.[32−34] Currently, sodium trioxodinitrate or Angeli's salt ($Na_2N_2O_3$, AS), first synthesized by Angeli in 1903,[35] is the most widely used HNO donor in chemical and biochemical studies of HNO reactivity.[36] AS ($HN_2O_3^-$, pK_a($HN_2O_3^-$) = 9.70; pK_a($H_2N_2O_3$) = 2.51[37]) decomposes rapidly to give HNO and NO_2^- at pH 4−8, eq 2, whereas at pH <4, AS is primarily a NO donor.[36,38]

$$\mathrm{N_2O_3^{2-} + H^+ \underset{pK_a(HN_2O_3^-)}{\rightleftharpoons} HN_2O_3^- \xrightarrow{k_L} HNO + NO_2^-} \quad (2)$$

Vitamin B_{12} derivatives (also known as cobalamins; Cbls) are a class of redox active cobalt containing complexes belonging to the corrinoid family which are synthesized by a number of microorganisms.[39] Cobalamins are essential coenzymes in all mammalian cells. The two B_{12}-dependent enzyme reactions in humans require either adenosylcobalamin (AdoCbl, X = 5′-deoxy-5′-adenosyl (Ado), Figure 1) or methylcobalamin

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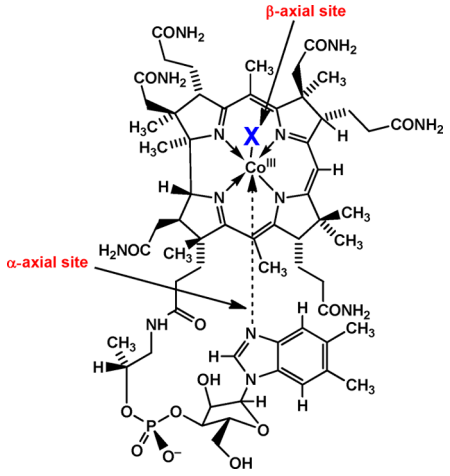

Figure 1. Structure of vitamin B_{12} derivatives (cobalamins): X = CN^-, CH_3, Ado, H_2O, OH^-, NO^-, NO_2^-, etc.

(MeCbl, X = CH_3, Figure 1).[40] Cbl deficiency in humans is associated with hyperhomocysteinemia and/or methylmalonic acidemia leading to megaloblastic anemia and/or neurological disorders.[41] Cyanocobalamin (CNCbl, X = CN^-, Figure 1) is the most common form of vitamin B_{12} used in vitamin supplements.[42]

Multiple studies have recently been published on the chemistry of HNO donor molecules with porphyrin complexes.[1,21,43−46] Thus far no studies have been reported on the reactivity of HNO or HNO donor molecules with structurally similar vitamin B_{12} complexes, although Cbls are well-known to modulate NO-associated events in biological systems.[47−51] We have carried out kinetic and mechanistic studies on the reaction of the cob(III)alamin aquacobalamin/hydroxycobalamin (H_2OCbl^+/HOCbl; X = H_2O/OH^-, Figure 1) with the HNO donor Angeli's salt. Our studies show that H_2OCbl^+/HOCbl reacts either directly with AS and/or with its decomposition product, HNO, depending upon the pH conditions used.

EXPERIMENTAL SECTION

Reagents. Hydroxocobalamin hydrochloride (HOCbl·HCl, 98% stated purity by the manufacturer) was purchased from Fluka. The percentage of water in HOCbl·HCl (·nH_2O) (10−15% water, batch dependent) was determined by converting HOCbl·HCl into dicyanocobalamin, $(CN)_2Cbl^-$ (0.10 M KCN, pH 11.0, $\varepsilon_{368\ nm}$ = 3.04 × 10^4 M^{-1} cm^{-1}).[52,53] Angeli's salt (AS) was purchased from Cayman Chemical and used without further purification. $NaBH_4$ (≥98%), $Na_2S_2O_4$ (≥85%), $NaNO_2$ (99.6%), NH_2OH·HCl (≥97%), KCN (≥99.1%), 8-hydroxyquinoline (≥99%), diethylenetriaminepentaacetic acid (DTPA; ≥98%), D_2O (99.8 atom % D), acetone, triflic acid, NaOH, biological buffers (MES, TES, TAPS, and CHES), and phosphate buffers (NaH_2PO_4 and Na_2HPO_4) were obtained from either Fisher Scientific or Acros Organics. TSP (3-(trimethylsilyl)-propionic 2,2,3,3-d_4 acid, sodium salt) and the Griess reagent were obtained from Sigma Aldrich. Nessler's reagent was purchased from RICCA Chemical. Water was purified using a Barnstead Nanopure Diamond water purification system.

General Methods. All solutions were prepared using standard biological buffers and phosphate buffers (0.05−0.30 M), and a constant ionic strength was maintained using sodium triflate ($NaCF_3SO_3$; I = 1.0 M). Anaerobic solutions were prepared by bubbling argon through the solutions for ∼24 h. Stock solutions were stored in the MBRAUN Labmaster 130 (1250/78) glovebox filled with argon, equipped with O_2 and H_2O sensors and a freezer at −24 °C. Temperature-sensitive solutions were stored in the freezer. All pH measurements were carried out at room temperature using an Orion Model 710A pH meter equipped with Mettler-Toledo Inlab 423 or 421 electrodes. An electrode was filled with 3 M KCl/saturated AgCl solution (pH 7). The electrodes were calibrated with standard buffer solutions at pH 4.00, 7.00, 10.00, and 12.45. Air-free UV−vis spectrometric measurements were carried out in Schlenk cuvettes (cuvettes fitted with a J-Young or an equivalent stopcock) on a Cary 5000 spectrophotometer equipped with a thermostatted (25.0 ± 0.1 °C) cell changer operating with WinUV Bio software (version 3.00). Freshly prepared solutions were used for kinetic measurements. For rapid reactions, kinetic data were collected under strictly anaerobic conditions at 25.0 ± 0.2 °C using an Applied Photophysics SX20 stopped-flow instrument equipped with a photodiode array detector, operating with Pro-Data SX (version 2.1.4) and Pro-Data Viewer (version 4.1.10) software, using either a 2 or 10 mm path length cell. The instrument was continuously purged with nitrogen gas during data collection and pretreated with anaerobic sodium dithionite (at least for 1 h) to remove oxygen and thoroughly washed with plenty of anaerobic water. Hamilton gastight syringes filled with the anaerobic reactant solutions in the glovebox were used to introduce the reactant solutions into the reservoir syringes of the stopped-flow instrument. Data were fitted using the program Microcal Origin (version 8.0). Data simulations were carried out using Pro-Kineticist software (Applied Photophysics). ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer equipped with a 5 mm probe at 23 ± 1 °C. TSP was used as internal reference. For ^{1}H NMR experiments under anaerobic conditions, airtight J-Young NMR tubes (Wilmad, 535-JY-7) were used. Reaction mixtures were equilibrated for 15 min prior to measurements.

Determination of Cobalamin (Cbl) Concentrations. The Cbl concentrations were determined by converting Cbl's to dicyanocobalamin, $(CN)_2Cbl^-$. Cobalamins were allowed to react with KCN (0.10 M, pH 11.50) to produce $(CN)_2Cbl^-$. The concentration of the final product was determined using UV−vis spectrometry ($\varepsilon_{368\ nm}$ = 3.04 × 10^4 M^{-1} cm^{-1}).[52,53]

Study of the Spontaneneous Decomposition of AS. Rate constants for the spontaneous decomposition of AS as a function of pH under anaerobic conditions were measured using UV−vis spectrophotometry by following the decay in the Angeli's salt absorbance at 245 nm. The reaction was initiated by adding an aliquot (0.050 mL, 6.00 mM) of a stock solution of AS in 10.0 mM NaOH to a cuvette containing buffer (2.95 mL) which had been thermostatted in the cell holder of the Cary 5000 spectrophotometer. CAPS, CHES, and TAPS buffers (0.30 M) were used, and the total ionic strength was maintained at 1.0 M ($NaCF_3SO_3$). The absorbance at 245 nm versus time data were fitted to a first-order rate equation.

Griess assay for the Quantification of Nitrite. The amount of nitrite in the product solutions was determined using the Griess assay[54] under strictly anaerobic conditions. In a typical experiment, an aliquot of Griess reagent (1.50 mL) was added to an equal volume of buffer (0.1 M, 1.50 mL) containing varying concentrations of nitrite (0, 20.0, 40.0, 60.0, 80.0, and 100.0 μM) and NOCbl (1.00 × 10^{-4} M) and the absorbance at 586 nm was determined. The resulting calibration curve is shown in Figure S13 in the Supporting Information (note that the calibration curves were found to be pH independent). An aliquot of the product of the reaction between HOCbl (1.0 × 10^{-4} M) and AS (1.0 equiv) was subjected to the identical procedure. The concentration of nitrite was estimated from the calibration plot.

Determination of Percentage of Nitrite Impurity in Commercially Available AS. The percentage nitrite impurities in commercially available AS was determined using the Griess assay[54] under strictly anaerobic conditions. A typical procedure is as follows: Griess reagent (1.50 mL) was added to a solution of AS (1.17 and 1.43 mM; carried out in duplicate) in 0.10 M CAPS buffer, pH 10.80, and the absorbance was measured at 586 nm. The concentration of nitrite was obtained from the calibration curve (as described elsewhere). The percentage of nitrite impurity was found to be 4.8 ± 0.1%.

Sample Preparation for Kinetic Measurements on the Reaction of Cbl(III) (H_2OCbl^+/HOCbl) with AS. All samples were prepared under strictly anaerobic conditions inside the glovebox. Stock

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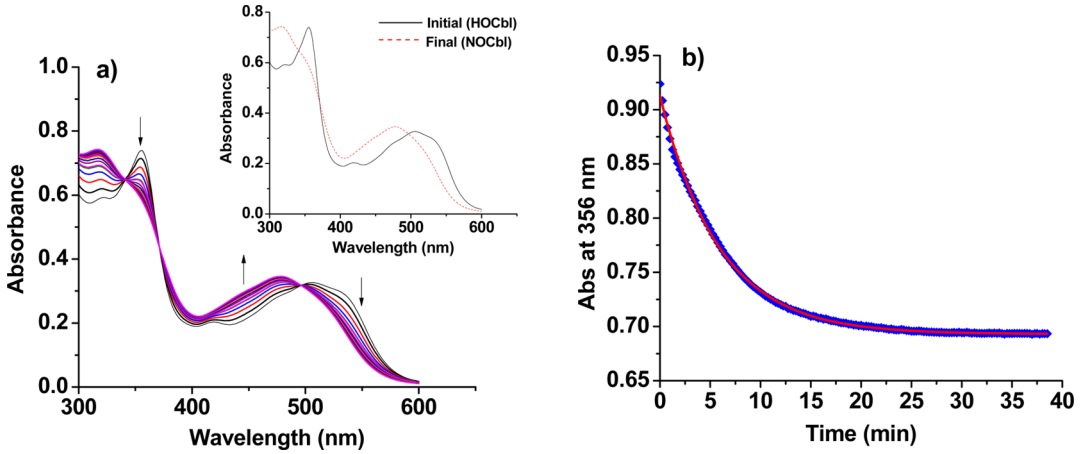

Figure 2. (a) UV−vis spectra for the reaction between HOCbl (5.00×10^{-5} M) and excess AS (2.50×10^{-3} M) at pH 9.80 (25.0 °C, 0.30 M CHES buffer, I = 1.0 M ($NaCF_3SO_3$)). Isosbestic points occur at 341, 370, and 498 nm. **Inset**: First and last spectra. (b) Fit of absorbance data at 356 nm versus time to a first-order rate equation, giving k_{obs} = (2.85 ± 0.02) × 10^{-3} s^{-1}.

Cbl(III) solutions were prepared by dissolving solid HOCbl·HCl in the appropriate buffer. Stock AS solutions were prepared by dissolving solid AS in NaOH (10.0 mM). Stock solutions were stored in the freezer (−24 °C) inside the glovebox and used within 24 h. Typically a small aliquot (~0.10 mL) of the AS solution was added to a solution of Cbl(III) (~2.90 mL) in buffer.

RESULTS AND DISCUSSION

Studies on the Reaction between H_2OCbl^+/HOCbl and Angeli's salt (AS) at pH ≤9.90. Upon the addition of AS to a solution of aquacobalamin/hydroxycobalamin (H_2OCbl^+/HOCbl; pK_a = 7.8[55]) under strictly anaerobic conditions, significant changes in the UV−vis spectrum were observed and the reaction solution changed from red to orange. Figure 2a shows typical UV−vis spectra for the reaction between HOCbl (5.00×10^{-5} M) and excess AS (2.50×10^{-3} M) as a function of time under anaerobic conditions at pH 9.80 (25.0 °C, 0.30 M CHES buffer, I = 1.0 M, $NaCF_3SO_3$). The inset to Figure 2a shows a comparison between the initial spectrum and final spectrum, and it is clear that the product is nitroxylcobalamin, NO^--Cbl(III) (λ_{max} = 256, 278 (shoulder), 289, 315, and 478 nm), with sharp isosbestic points observed at 341, 370, and 498 nm, in agreement with literature values for the HOCbl to NOCbl conversion.[56] Figure 2b gives the best fit of the absorbance data at 356 nm versus time to a first-order rate equation, giving an observed rate constant (k_{obs}) = (2.85 ± 0.02) × 10^{-3} s^{-1} ($t_{1/2}$ = 4.1 min).

In order to be able to directly compare rate constants for the reaction between AS and H_2OCbl^+/HOCbl with rate constants for spontaneous AS decomposition, observed rate constants for AS decomposition (k_L) were determined under the identical experimental conditions (25.0 °C, I = 1.0 M ($NaCF_3SO_3$)) in the pH 7.00−11.40 range by following the decay of AS at 245 nm. The resulting plot of k_L versus pH is shown in Figure S1 in the Supporting Information, giving a first-order rate constant for $HN_2O_3^-$ decomposition, k_{AS} = (5.16 ± 0.23) × 10^{-4} s^{-1} and pK_a($HN_2O_3^-$) = 9.48 ± 0.16. These values are in very good agreement with literature values (pK_a($HN_2O_3^-$) = 9.70[37] and k_{AS} = 6.7 × 10^{-4} s^{-1} (25 °C, I = 0.25 M)[57,58]).

The rate constant for the spontaneous acid-catalyzed decomposition of AS at pH 9.80 (k_L = (1.34 ± 0.01) × 10^{-4} s^{-1}; Supporting Information, Figure S1) was found to be more than 1 order of magnitude (~20 times) slower than the reaction of AS (2.50×10^{-3} M) with HOCbl (5.00×10^{-5} M) at pH 9.80 (k_{obs} = (2.85 ± 0.02) × 10^{-3} s^{-1}). Therefore, decomposition of AS is not important under these reaction conditions and a direct reaction occurs between H_2OCbl^+/HOCbl and AS. The dependence of k_{obs} on AS concentration (2.50−10.0 mM) at pH 9.80 was determined, and the data are summarized in Figure 3. Fitting the data to a straight line passing through the origin gives a slope of 1.18 ± 0.01 M^{-1} s^{-1}.

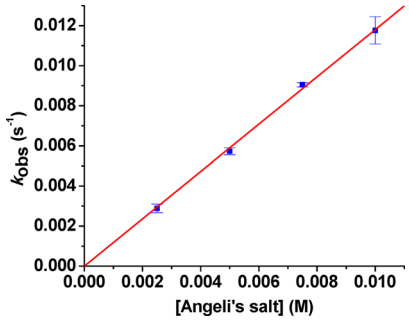

Figure 3. Plot of k_{obs} versus AS concentration at pH 9.80 (25.0 °C, 0.30 M CHES buffer, I = 1.0 M ($NaCF_3SO_3$)). The data have been fitted to a straight line passing through the origin, giving a second-order rate constant, k_{app} = 1.18 ± 0.01 M^{-1} s^{-1}.

It occurred to us that nitrite could interfere with the determination of the rate constant for the reaction of interest, since H_2OCbl^+ reacts rapidly with nitrite to form nitrocobalamin, NO_2Cbl.[59] There are several possible sources of nitrite. Nitrite is produced in the reaction between H_2OCbl^+/HOCbl and AS (see below) and is also a product of AS decomposition (eq 2). Furthermore, a control experiment showed that commercial AS can contain ~5% nitrite impurity (Experimental Section). Importantly, the UV−vis spectra of H_2OCbl^+ and NO_2Cbl are practically identical;[60] hence if H_2OCbl^+ is partially converted to NO_2Cbl by reacting with nitrite as the reaction proceeds prior to being converted to NOCbl, little or no change in the isosbestic points would be observed. Values of K = 2.2 × 10^5 M^{-1} and k_1 = 99.82 M^{-1} s^{-1} at 25 °C have been reported for the formation of NO_2Cbl from the reaction of H_2OCbl^+ with nitrite, eq 3.[59,61,62]

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$$H_2OCbl^+ + NO_2^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} NO_2Cbl + H_2O; \qquad K = k_1/k_{-1} \tag{3}$$

Control experiments showed that the rate of the reaction between H_2OCbl^+/HOCbl and nitrite is still rapid even in alkaline solution despite the reaction proceeding through H_2OCbl^+ ($pK_a(H_2OCbl^+)$ = 7.8[55]). Specifically, Figures S2a and S2b in the Supporting Information show that k_{app} = 5.95 ± 0.04 M^{-1} s^{-1} and k_{app} = 41.5 ± 0.2 M^{-1} s^{-1} at pH 9.80 and 8.80, respectively, where k_{app} is the apparent rate constant for the reaction between H_2OCbl^+/HOCbl and nitrite (25.0 °C, 0.30 M CAPS or TAPS buffer, I = 1.0 M ($NaCF_3SO_3$)). Note that the hydroxo ligand of HOCbl is inert to substitution.[63] It therefore is possible that NO_2Cbl is formed as an intermediate in the reaction. To determine whether forming NO_2Cbl would have an effect on the observed rate constant, the rate of the reaction between the authentic NO_2Cbl and AS was determined at pH 9.80 and 9.09 (25.0 °C, 0.30 M CHES buffer, I = 1.0 M ($NaCF_3SO_3$)). Figure S3 in the Supporting Information shows the plots of absorbance at 356 nm versus time for the reaction of HOCbl or NO_2Cbl (5.00 × 10^{-5} M) with excess AS (5.00 × 10^{-3} M) at pH 9.80 (25.0 °C, 0.30 M CHES buffer, I = 1.0 M ($NaCF_3SO_3$)). The data fit well to first-order rate equations giving k_{obs} = (8.29 ± 0.01) × 10^{-3} and (5.79 ± 0.04) × 10^{-3} s^{-1} for the reaction of AS with HOCbl and NO_2Cbl, respectively. Substituting HOCbl by NO_2Cbl still results in the complete formation of NOCbl, but leads to a slight decrease in the rate of the reaction. It is likely that NO_2Cbl does not itself react directly with AS, but that NO_2Cbl is in equilibrium with H_2OCbl^+ and the latter species reacts with AS to form NOCbl. However, experiments were not carried out to probe this mechanism further.

It was, therefore, likely that nitrite interferes with the reaction of interest. Given that NO_2^- is the non-cobalamin product of the reaction between H_2OCbl^+/HOCbl and AS (see below), the maximum concentration of nitrite produced during the reaction is ~[AS]. *In order to minimize the amount of NO_2^- produced during the reaction of H_2OCbl^+/HOCbl with AS, the kinetics of the reaction between H_2OCbl^+ and AS were instead investigated with excess H_2OCbl^+* (at least 7 times compared to the concentration of AS), rather than with AS in excess. Importantly, control experiments demonstrated that the addition of 1.0 mol equiv NO_2^- (5.00 × 10^{-5} M) decreases the rate constant by only ~10% for the reaction between AS (5.00 × 10^{-5} M) and excess H_2OCbl^+ (5.00 × 10^{-4} M) at pH 4.15 ± 0.03 (k_{obs} = (4.91 ± 0.03) × 10^{-2} and (4.40 ± 0.02) × 10^{-2} s^{-1} in the absence and presence of 5.00 × 10^{-5} M $NaNO_2$, respectively), whereas there was no difference observed at pH 7.20 ± 0.03 (k_{obs} = 6.78 ± 0.03 and 6.86 × 10^{-2} s^{-1}) in the absence and presence of 5.00 × 10^{-5} M $NaNO_2$, respectively). Hence since only ~0.14 mol equiv NO_2^- (=7 times excess H_2OCbl^+/HOCbl) is produced in our experiments, NO_2^- production will not significantly alter the observed rate of the reaction. Control experiments also demonstrated that the rate constant is unaffected by the presence of the metal chelator DTPA (k_{obs} = (4.91 ± 0.03) × 10^{-2} and (4.94 ± 0.05) × 10^{-2} s^{-1} in the absence and presence of 50.0 μM DTPA, respectively, at pH 4.15 ± 0.03), showing that free metal ions are not involved in the reaction.

It was established that spontaneous decomposition of AS is not significant on the time scale of the experiment. Typical UV–vis spectra as a function of time for the reaction of AS (6.50 × 10^{-5} M) with excess H_2OCbl^+/HOCbl (3.25 × 10^{-4} M) at pH 5.00 (25.0 °C, 0.30 M acetate buffer, I = 1.0 M ($NaCF_3SO_3$)) under anaerobic conditions are shown in Figure S4, Supporting Information. The isosbestic points were the same as those observed for the conversion of H_2OCbl^+/HOCbl to NOCbl at pH 7.40 within the limitations of the small absorbance changes due to an incomplete reaction.[56] Kinetic data were collected at a range of H_2OCbl^+ concentrations in order to determine the apparent second-order rate constant for the reaction at pH 5.00, maintaining a H_2OCbl^+:AS ratio of ~7:1. The data are summarized in Figure 4, which shows a plot of k_{obs} versus the concentration of H_2OCbl^+ at pH 5.00 ± 0.03. The data fit to a straight line passing through the origin, giving a slope (second-order rate constant, k_{app}) = 129.4 ± 1.4 M^{-1} s^{-1}.

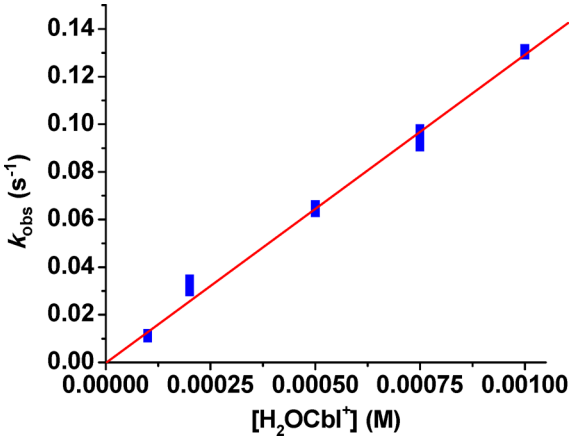

Figure 4. Plot of k_{obs} versus concentration of H_2OCbl^+ at pH 5.00 ± 0.03 (25.0 °C, 0.30 M acetate buffer, I = 1.0 M ($NaCF_3SO_3$)). The data have been fitted to a straight line passing through the origin, giving a slope (k_{app}) = 129.4 ± 1.4 M^{-1} s^{-1}.

Values of k_{app} were similarly determined in the pH range 4.15–9.90 (individual plots given in Figures S5–S11 in the Supporting Information), and the results are summarized in Figure 5. Plots of k_{obs} versus [H_2OCbl^+/HOCbl] at pH 9.05, 9.40, and 9.90 had a small but significant y-intercept. This arises because under these pH conditions decomposition of AS is no

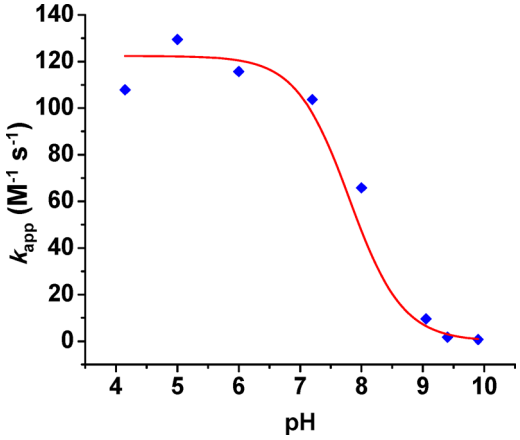

Figure 5. Plot of k_{app} versus pH for the reaction between H_2OCbl^+/HOCbl and AS. The best fit of the data to eq 5 fixing $pK_a(H_2OCbl^+)$ = 7.8 and $pK_a(HN_2O_3^-)$ = 9.48 gives $k_{Cbl(III)}$ = 122.6 ± 5.3 M^{-1} s^{-1}.

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longer insignificant compared to the reaction of interest. In this case the data were fitted to eq 4.

$$k_{obs} = k_{app}[H_2OCbl^+/HOCbl] + k_L \quad (4)$$

The stoichiometry of the reaction between H_2OCbl^+ and AS was determined using UV−vis spectroscopy. UV−vis spectra were taken of equilibrated solutions of H_2OCbl^+ (5.00×10^{-5} M) and AS (0, 0.25−2.5 equiv) at pH 6.00. The isosbestic points obtained (Figure 6) are in agreement with the literature

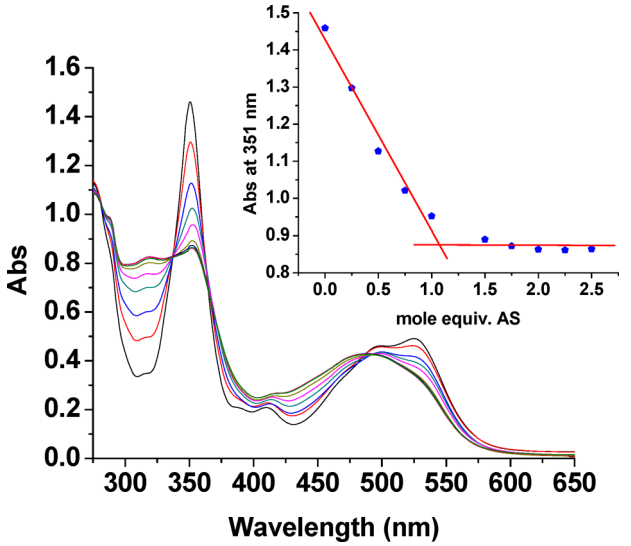


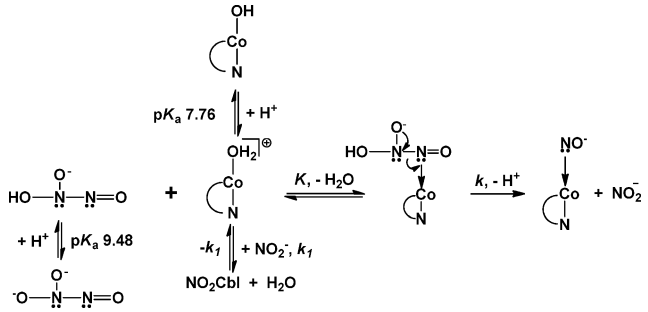
Figure 6. UV−vis spectra obtained from titration for the reaction of H_2OCbl^+ (5.00×10^{-5} M) with AS (0, 0.25−2.5 mol equiv) at pH 6.00 under anaerobic conditions. Isosbestic points occur at 337, 365, and 490 nm. **Inset**: Plot of absorbance at 351 nm versus mol equiv of AS at pH 6.00 (0.30 M MES buffer).

values for the conversion of H_2OCbl^+/HOCbl to NOCbl at pH 7.4.[56] The plot of absorbance at 351 nm versus mol equiv of AS (inset to Figure 6) gives a stoichiometry of 1:1 H_2OCbl^+:AS at pH 6.00. The stoichiometry and the Cbl product(s) were also determined at pD 9.86 using ^{1}H NMR spectroscopy. HOCbl is completely converted to NOCbl upon reacting HOCbl with 1.1 mol equiv AS (pD 9.86, 24 °C, 0.30 M CHES buffer, Supporting Information, Figure S12).

The non-cobalamin product was also identified. Nitrite was shown to be the non-cobalamin product using the Griess assay.[54] A calibration curve of absorbance versus nitrite concentration was generated (see the Experimental Section for details). From the Griess test, it was found that 0.79 equiv NO_2^- was produced in the reaction between HOCbl (1.00×10^{-4} M) and AS (1.0 mol equiv) at pH 9.80, using a calibration curve of absorbance versus nitrite concentration (Figure S13, Supporting Information). Interestingly, others have reported significantly less NO_2^- formation than expected from AS decomposition based on the stoichiometry of the reaction.[64−66] This has been attributed to N−N bond homolysis (resulting in formation of $2^{\bullet}NO$ and H_2O) in addition to N−N heterolysis of $HN_2O_3^-$ (HNO and NO_2^- formed).[38] We have noticed in our studies with AS that a slight excess of AS (~1.1 mol equiv) is always required for reactions to proceed to completion.

From Figure 5 it is clear that the reaction of HOCbl/H_2OCbl^+ with AS becomes faster with decreasing pH, becoming pH independent at pH ≤6. Scheme 1 gives the proposed reaction pathways involved for pH ≤9.90. The

Scheme 1. Proposed Mechanism for the Reaction of H_2OCbl^+/HOCbl with AS at pH ≤9.90



equilibrium between H_2OCbl^+, nitrite, and NO_2Cbl is shown for completeness; however, the effect of this equilibrium under the conditions of the kinetic experiments is insignificant. It is likely that rapid substitution of the aqua ligand of H_2OCbl^+ by AS occurs prior to AS undergoing N−N bond cleavage. It is well established that β-axial ligand substitution reactions for cobalamins occur via a dissociative interchange mechanism.[67] Furthermore, only H_2OCbl^+, not HOCbl, undergoes these reactions, since HOCbl is inert to β-axial ligand substitution;[58] hence in Scheme 1 only H_2OCbl^+ ($pK_a = 7.8$[55]), not HOCbl, reacts with AS. The corresponding rate equation is given in eq 5.

$$k_{app} = \frac{kK[H^+]^2}{([H^+] + K_a(H_2OCbl^+)) + ([H^+] + K_a(HN_2O_3^-))} \quad (5)$$

The best fit of the data in Figure 5 to eq 5 with $pK_a(H_2OCbl^+) = 7.8$[55] and $pK_a(HN_2O_3^-) = 9.48$ gives $kK = 123 \pm 5$ M^{-1} s^{-1}. This value is of a comparable magnitude to values reported for the reactions of H_2OCbl^+ with a wide variety of ligands.[62,68] By fitting the data to this eq 5, we assume that the reaction between H_2OCbl^+ and $N_2O_3^{2-}$ is not important under the pH conditions of our study. Indeed, if the data are instead fitted to a model in which both $HN_2O_3^-$ and $N_2O_3^{2-}$ react with H_2OCbl^+, kK is the same within experimental error as expected ($kK = 121 \pm 5$ M^{-1} s^{-1}; see Supporting Information, Figure S14 and Scheme S1), since practically no Cbl remains in the reactive H_2OCbl^+ form at pH conditions where the concentration of $N_2O_3^{2-}$ is significant (pH >9).

Two mechanisms have been proposed for the reaction of AS with porphyrins and heme proteins. AS reacts directly with Mn^{III} porphyrins (Mn^{III}TEPyP[69]) and Fe^{III} porphyrins (Fe^{III}TEPyP[70]) to form an AS-bound intermediate (rate-determining step, RDS) followed by rapid decomposition of intermediate to form the final nitrosyl products, Mn^{II}TEPyP-(NO) and Fe^{II}TEPyP(NO), respectively. Alternatively, the reaction may occur via a mechanism in which HNO, not AS, reacts. This mechanism has been proposed for the reaction of AS with Mn^{III}TPPS,[69] microperoxidase-11 (Fe^{III}MP11),[70] Fe^{III}(TPPS),[71] methemoglobin (metHb) and metmyoglobin (metMb),[72,73] and horseradish peroxidase,[74] to form the corresponding Mn^{II}(NO) and Fe^{II}(NO) complexes. Indeed, evidence for this latter mechanism occurring in our system was obtained at pH 10.80 and 11.40 (see below).

Studies on the Reaction between HOCbl and AS at High pH Conditions (pH 10.80, 11.40). Figure 7a gives a

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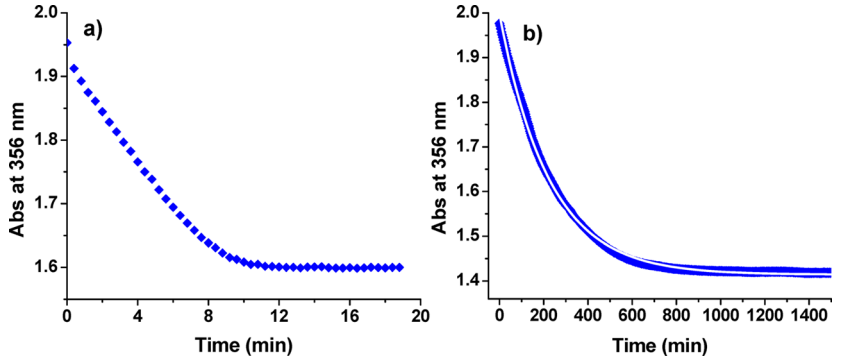


Figure 7. (a) Plot of absorbance at 356 nm versus time for reaction of HOCbl (1.00×10^{-4} M) with excess AS (1.00×10^{-2} M) at pH 10.80 (25.0 °C, 0.30 M CAPS buffer, I = 1.0 M ($NaCF_3SO_3$)). The linear dependence suggests that the reaction is independent of HOCbl concentration. (b) Plot of absorbance at 356 nm versus time for reaction of HOCbl (1.00×10^{-4} M) with 1.0 equiv AS at pH 10.80 (25.0 °C, 0.30 M CAPS buffer, I = 1.0 M ($NaCF_3SO_3$)). The best fit of the data to a first-order reaction gave k_{obs} = (7.45 ± 0.01) × 10^{-5} s^{-1}.

plot of absorbance versus time for the reaction of HOCbl (1.00×10^{-4} M) with excess AS (1.00×10^{-2} M) at pH 10.80 (25.0 °C, 0.30 M CAPS buffer, I = 1.0 M ($NaCF_3SO_3$)). An almost linear dependence is observed, with HOCbl reacting with AS to again give NOCbl. A similar result was obtained at 5.00×10^{-4} M HOCbl. The linear dependence suggests that the reaction is independent of HOCbl concentration (i.e., zero order in [HOCbl]), and that the decomposition of AS is instead the rate-determining step at these pH conditions. Therefore, the same experiment was repeated using 1.0 mol equiv AS at pH 10.80 (0.30 M CAPS buffer, I = 1.0 M, $NaCF_3SO_3$). Under these conditions the absorbance at 356 nm versus time data fit well to a first-order rate equation, with an observed rate constant (k_{obs}) of (7.45 ± 0.01) × 10^{-5} s^{-1} (see Figure 7b). A similar rate constant was observed with 0.5 equiv AS at the same conditions ((8.00 ± 0.05) × 10^{-5} s^{-1}). Given that the reaction is stoichiometric (see below), k_{obs} would be expected to be independent of HOCbl for [HOCbl]:[AS] ≥ 1.0 (also see data simulations in the Supporting Information). The reaction of excess HOCbl (1.00×10^{-4} M) with AS (1.40×10^{-5} M) at pH 10.80 (25.0 °C, 0.30 M CAPS buffer, I = 1.0 M, $NaCF_3SO_3$) gives k_{obs} = (5.32 ± 0.06) × 10^{-5} s^{-1}; see Figure S15 in the Supporting Information. The observed rate constant for the spontaneous decomposition of AS at the same pH is (3.17 ± 0.01) × 10^{-5} s^{-1}. The observed rate constants for the reaction between HOCbl and AS are therefore up to 2.5 times larger than one would expect based solely on a pathway involving rate-determining AS decomposition. Possible explanations for this discrepancy are that there is a small contribution to the observed reaction from the direct reaction (Scheme 1) or that weak association of HOCbl with AS in aqueous solution promotes AS decomposition.

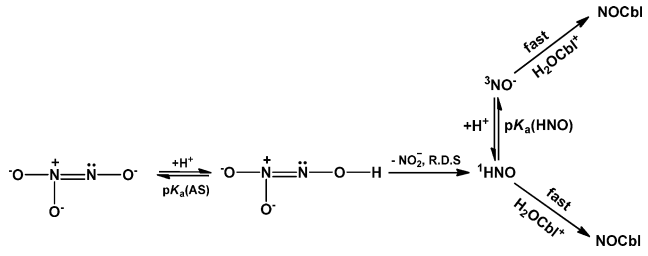
The reaction between HOCbl and AS was also studied at pH 11.40 (0.40 M phosphate buffer), using 0.50 and 1.0 mol equiv AS. The best fit of the absorbance versus time data to a first-order equation gives k_{obs} = (1.10 ± 0.01) × 10^{-5} and (1.15 ± 0.01) × 10^{-5} s^{-1}, respectively. At pH 11.40 the observed rate constant for the spontaneous decomposition of AS is (4.70 ± 0.02) × 10^{-6} s^{-1}; hence once again the rate of the reaction is slightly faster than one would expect based purely on AS decomposition.

Since the reaction of AS with HOCbl at higher pH conditions occurs via a different mechanism, the stoichiometry of the reaction of HOCbl with 0.55, 1.1, and 2.2 mol equiv AS was investigated by ^{1}H NMR spectroscopy at pD 10.86 (0.50 M CAPS). A minimum of 1.1 mol equiv AS is required for the reaction to proceed to completion (Figure S16, Supporting Information), with NOCbl once again formed as the Cbl product. Reacting 0.50 mol equiv AS yielded approximately ∼50% NOCbl and ∼50% unreacted starting material (HOCbl). An attempt was also made to determine the stoichiometry of the reaction at pD 11.40. However, since the reaction at this pH condition is very slow and Cbls are not stable in alkaline solution,[75,76] considerable cobalamin decomposition to Co(II) corrinoid species was observed at this pH value.

Finally, the amount of NO_2^- produced at pH 10.80 was determined using the Griess assay[54] under strictly anaerobic conditions. From the calibration curve of absorbance versus nitrite concentration, the resulting absorbance corresponded to 0.81 and 0.78 mol equiv nitrite produced (see Experimental Section). Hence nitrite is the non-Cbl product.

The proposed major reaction pathway for the reaction between H_2OCbl^+/HOCbl and AS at pH ≥10.80 is summarized in Scheme 2. Decomposition of monoprotonated

Scheme 2. Proposed Major Mechanism for the Reaction of H_2OCbl^+/HOCbl with AS at pH ≥10.80



form of AS ($HN_2O_3^-$; pK_a 9.48) to give HNO/NO^- is the rate-limiting step of the reaction followed by fast substitution of aqua ligand of H_2OCbl^+ by HNO/NO^- to form NOCbl. Since decomposition of $HN_2O_3^-$ is rate-limiting, our experimental data do not allow us to determine whether $^3NO^-$ in addition to ^{1}HNO (pK_a(^{1}HNO) ∼ 11.4;[23] ^{1}HNO ⇌ $^3NO^-$ + H^+) reacts with H_2OCbl^+ and/or HOCbl to form NOCbl. Furthermore, no information is obtained on the mechanism of this reaction.

CONCLUSIONS

Kinetic and mechanistic studies have been carried out on the reaction of aquacobalamin with the HNO donor Angeli's salt using UV−vis and ^{1}H NMR spectroscopies. At lower pH

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conditions (≤9.90), H_2OCbl^+ reacts directly with AS with a 1:1 stoichiometry, giving NOCbl and NO_2^-. A direct transfer of a nitroxyl group to the cobalt(III) center of H_2OCbl^+ from the ligand was also observed for the reaction of R_2N-NONOates with H_2OCbl^+.[77] Under strongly alkaline conditions (pH ≥10.80) the rate-determining step instead involves AS decomposition to give HNO/NO^- and NO_2^- (RDS), with HNO/NO^- subsequently rapidly reacting with H_2OCbl^+ to give NOCbl.

■ ASSOCIATED CONTENT

Supporting Information

Additional kinetic data, ^1H NMR spectra, calibration plot for nitrite detection, and data simulations; Figures S1−S16 and Scheme S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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