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Studies on the Reaction of Reduced Vitamin B_{12} Derivatives with the Nitrosyl Hydride (HNO) Donor Angeli's Salt: HNO Oxidizes the Transition-Metal Center of Cob(I)alamin

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Although it is well established that nitrosyl hydride (nitroxyl, HNO) reduces transition metals including transition-metal centers of porphyrins and metalloproteins, oxidation of a metal center by HNO has yet to be reported. Kinetic and mechanistic studies on the Co^{II} vitamin B_{12} form, cob(II)alamin [Cbl(II)], with the widely used HNO donor Angeli's salt (AS) have been carried out. The stoichiometry of the reaction is Cbl(II)/AS = 1:2, and AS decomposition to give HNO and nitrite is the rate-determining step. Separate studies on the reaction between cob(I)alamin [$Cbl(I)^-$] and AS and experiments in the presence of excess nitrite or the efficient cob(III)alamin trapping agent cyanide support a mechanism in which HNO reduces Cbl(II) to $Cbl(I)^-$, being itself oxidized to NO·. A second molecule of HNO then oxidizes $Cbl(I)^-$ back to Cbl(II), which reacts rapidly with NO· to form nitroxylcobalamin [nitrosylcobalamin, NO^-–Cbl(III), NOCbl].

Introduction

The importance of the reactive nitrogen species nitrosyl hydride [HNO, nitroxyl, $pK_a(^1HNO/^3NO^-) \approx 11.4$[1]] in biological systems is increasingly being realized. Although it has not yet been unequivocally demonstrated that HNO exists in biological systems,[2] HNO is generated in vitro from L-arginine by nitric oxide synthases in the absence of the tetrahydrobiopterin cofactor[3] or by oxidation of the *N*-hydroxy-L-arginine intermediate,[4] from the reaction of thiols with *S*-nitrosothiols,[5] or by enzyme-catalyzed reduction of NO·.[6] In addition, HNO generation has recently been demonstrated in cells pretreated with *S*-nitrosoglutathione exposed to H_2S using the HNO sensor CuBOT1[7] [a nitrosothiol (HSNO) intermediate is proposed, which reacts further with H_2S to give HNO + H_2S_2].[8] Fe^{3+} heme-catalyzed reduction of nitrite by H_2S also generates HNO, and HNO is detected in cells treated with nitrite and sulfide.[9] Like NO·, HNO is a vasorelaxant[10] and elevated intracellular levels lead to oxidative stress.[11] Recent studies also demonstrate that HNO has a biological activity and chemical reactivity distinct from NO·.[2a,2f,12] However, direct observation and use of HNO is limited by its rapid dimerization and decomposition to N_2O and H_2O [$k = (8 \pm 3) \times 10^6$ M^{-1} s^{-1}, 22 °C[1a]] [Equation (1)].[13] HNO donor molecules are therefore required to generate HNO in situ in HNO studies. Sodium α-oxyhyponitrite or Angeli's salt ($Na_2N_2O_3$, AS) is the most commonly used HNO donor for chemical and biological studies of HNO's reactivity.[14] The monoprotonated form of AS, $HN_2O_3^-$ [$pK_a(H_2N_2O_3) = 2.51$; $pK_a(HN_2O_3^-) = 9.70$[15]] decomposes rapidly to give HNO and NO_2^- in the pH 4–8 region [Equation (2)], with decomposition becoming slower for pH > 8.

$$HNO + HNO \longrightarrow [cis\text{-}N_2O_2H^-] \longrightarrow N_2O + H_2O \quad (1)$$

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

HNO donors show considerable promise in treating alcoholism,[16] cardiovascular disease, ischemia/reperfusion injury and heart failure.[2a,17] The main biological targets of HNO are believed to be thiols, DNA and oxidized metals and oxidized metalloprotein centers.[18] Although it is well established that HNO and HNO donors coordinate to metal centers[19] and/or reduce M^{n+} to $M^{(n-1)+}$ (HNO is oxidized to NO·, and reductive nitrosylation often occurs for porphyrins),[20] to the best of our knowledge the oxidation of a transition-metal center by HNO has not yet been reported. The product of reduction of HNO by a transition-metal complex would be expected to be NH_2OH.[5a,18c,21]

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Vitamin B_{12} derivatives (also known as cobalamins, Cbls; Figure 1) are essential cobalt-containing micronutrients synthesized by microorganisms.[22] Methylcobalamin (MeCbl; X = CH_3; Figure 1) and adenosylcobalamin (AdoCbl; X = Ado; Figure 1) serve as cofactors for two mammalian enzymatic reactions.[23] Cob(III)alamins (Co^{3+}) are reduced to cob(II)alamin [Cbl(II)$^{\cdot}$, Cbl(II)] upon cellular uptake,[24] and cob(I)alamin [Cbl(I)$^{-}$] is a short-lived precursor of the two B_{12} enzyme cofactors MeCbl and AdoCbl.[24] Severe cobalamin deficiency is associated with oxidative stress, which has been linked to various cardiovascular and neurodegenerative diseases.[25] Both enzymes are inactivated by nitric oxide.[26]

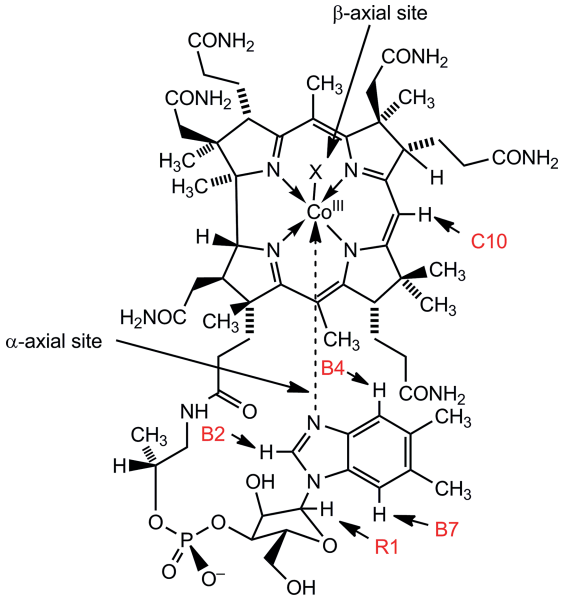


Figure 1. The structure of vitamin B_{12}: X = CN^{-}, CH_3, Ado, H_2O, NO etc.

Several studies report the reactivity of HNO and HNO donors with transition-metal complexes including porphyrins.[19,20] We recently demonstrated that cob(III)alamin (H_2OCbl^{+}/HOCbl) reacts with AS to form nitroxylcobalamin [nitrosylcobalamin, NO^{-}–Cbl(III)].[27] In this paper we present detailed mechanistic studies on the reaction between reduced cobalamins, cob(II)alamin and cob(I)alamin, with AS. These systems provide, to the best of our knowledge, the first example of oxidation of a transition-metal center by HNO.

Results

Kinetic Studies on the Reaction between Cob(II)alamin [Cbl(II)] and Angeli's Salt (AS)

Kinetic studies on the reaction between Cbl(II) and AS were carried out using UV/Vis spectroscopy. The rate of AS ($HN_2O_3^{-}$) decomposition to give HNO and NO_2^{-} is pH-independent in the pH 4–8 range ($t_{1/2}$ = 17 min at 25 °C[28]) and becomes increasingly slower for pH > 8.[28] Upon the addition of excess AS (4.00×10^{-4} M) to Cbl(II) (4.00×10^{-5} M) in buffer (pH 8.00) under strictly anaerobic conditions, Cbl(II) is cleanly converted into nitroxylcobalamin [nitrosylcobalamin, NOCbl, NO^{-}–Cbl(III);[29] λ_{max} = 256, 280 (shoulder), 289, 315 and 478 nm[29]]. The isosbestic points at 330, 387, 484 and 542 nm (Figure 2) are in agreement with literature values for the Cbl(II)/NOCbl conversion.[29] The corresponding plot of absorbance at 308 nm vs. time is given in Figure 2. The linear trend (0–250 s, inset to Figure 2) indicates that the rate of reaction is independent of the Cbl(II) concentration and suggests that the rate-determining step is decomposition of AS to give HNO (and NO_2^{-}), followed by a rapid reaction between HNO and Cbl(II).

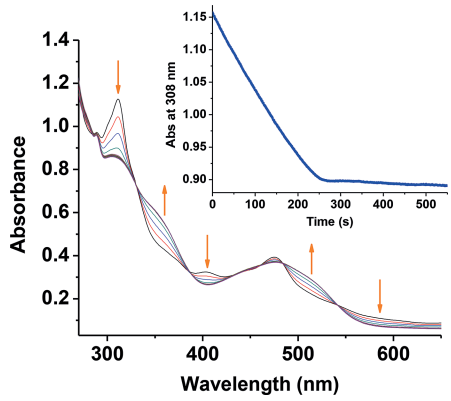


Figure 2. Selected UV/Vis spectra for the reaction between Cbl(II) (4.00×10^{-5} M) and excess AS (4.00×10^{-4} M) at pH 8.00 ± 0.03 [25.0 °C, 0.30 M TAPS buffer, I = 1.0 M ($NaCF_3SO_3$)] under anaerobic conditions. Spectra are shown for every 0.5 min. Inset: Plot of absorbance at 308 nm vs. time for the same reaction.

In order to test this further, kinetic data were collected for the reaction of Cbl(II) (4.00×10^{-5} M) with 1.0 mol-equiv. AS at pH 8.00. The absorbance at 308 nm vs. time data were fitted to a first-order rate equation giving an observed rate constant (k_{obs}) of $(3.58\pm0.01)\times10^{-2}$ min^{-1} (Figure S1, Supporting Information). This rate constant is similar to the rate of spontaneous decomposition of AS at pH 8.00 [k_L = $(3.06\pm0.01)\times10^{-2}$ min^{-1}; L = ligand; Table 1]; hence, decomposition of AS is the rate-determining step of the reaction between Cbl(II) and AS.

Table 1. Observed rate constants for the reaction between Cbl(II) and AS (k_{obs}) and the spontaneous decomposition of AS (k_L) as a function of pH [25.0 °C, I = 1.0 M ($NaCF_3SO_3$)] under anaerobic conditions.

pH (± 0.03)	$k_{obs}\times10^2$ [min^{-1}][a]	$k_L\times10^2$ [min^{-1}]
9.00	2.43 ± 0.01	2.40 ± 0.01
8.00	3.58 ± 0.01	3.06 ± 0.01
7.00	3.58 ± 0.02	3.25 ± 0.01

[a] Determined at [Cbl(II)] = [AS] = 4.00×10^{-5} M.

Control experiments showed that the rate constant is unchanged upon addition of the free metal scavenger DTPA (5.00×10^{-5} M) to the reaction mixture {k_{obs} =

3.52×10^{-2} min^{-1} and 3.69×10^{-2} min^{-1} in the presence and absence of DTPA, respectively [1.0 mol-equiv. AS, pH 7.40, 0.10 M phosphate buffer, 25.0 °C, I = 1.0 M ($NaCF_3SO_3$)]}; hence, free metals are not involved in the reaction. Studies were also carried out on the reaction between Cbl(II) and AS at pH 7.00 and 9.00. NOCbl is formed under all conditions, and the rate of the reaction was again found to be essentially the same as that for AS salt decomposition at each pH (Table 1).

Determination of the Stoichiometry and Identification of the Products of the Reaction between Cob(II)alamin and AS

In order to probe the mechanism of the reaction, the stoichiometry of the reaction between Cbl(II) and AS at pH 8.00 was determined. From UV/Vis spectra of anaerobic equilibrated solutions of Cbl(II) (50.0 μM) with AS (0, 0.25–3.5 mol-equiv.), a plot of absorbance at 316 nm vs. mol-equiv. AS was generated (Figure 3). The absorbance at 316 nm decreases linearly up to 2.0 mol-equiv. AS and is unchanged upon further addition of AS (Figure 3, inset). The stoichiometry of the reaction of Cbl(II) with AS at pH 8.00 is therefore Cbl(II)/AS = 1:2.

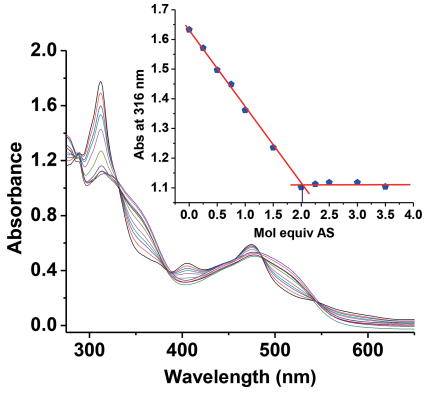


Figure 3. UV/Vis spectra for anaerobic equilibrated solutions of Cbl(II) (50.0 μM) with AS (0, 0.25–3.5 mol-equiv.) at pH 8.00 (25.0 °C, 0.30 M TAPS buffer). Inset: corresponding plot of absorbance at 316 nm vs. mol-equiv. AS.

To confirm that NOCbl is the cobalamin product, the ^{1}H NMR spectrum of the products of the reaction of Cbl(II) with 2.2 mol-equiv. AS at pD 8.00 under anaerobic conditions was recorded (Figure S2, Supporting Information). The observed chemical shift values match well with the literature values for NOCbl.[30] The indooxine test and Nessler's test were independently performed to check the presence of significant amounts of NH_2OH or NH_3, respectively, in the product solution of the reaction between Cbl(II) and 2.0 mol-equiv. AS (pH 8.00). Both tests were negative (see Exp. Sect.). Importantly, a control experiment showed that NH_2OH is stable in pH 8.00 buffer solution (at lower pH conditions, NH_2OH can undergo disproportionation[31]).

It was also of interest to see if N_2O [the product of HNO dimerization; Equation (1)] reacts with Cbl(II). The UV/Vis spectrum of a solution of Cbl(II) (200 μM) exposed to excess N_2O (20.0 mM) was identical to that for Cbl(II) (λ_{max} = 405 and 475 nm[31a,32]). There is, therefore, no reaction between Cbl(II) and N_2O, as expected based on work of others who propose that Cbl(II) and N_2O are the products of oxidation of Cbl(I)$^-$ by NO [Cbl(I)$^-$ + NO + H^+ → Cbl(II) + ½ N_2O + ½ H_2O].[33] The reaction of Cbl(II) with NO_2^- is negligible under the experimental conditions.[29a]

Cbl(I)$^-$ is the Intermediate of the Reaction between Cbl(II) and AS

Given that NOCbl has a single NO^- ligand, a Cbl(II)/AS = 1:1 stoichiometry was expected. However, the reaction between Cbl(II) and AS is complete only after the addition of 2.0 mol-equiv. AS. Since N_2O and nitrite do not react with Cbl(II), this suggests that Cbl(II) is either oxidized by HNO to cob(III)alamin [Cbl(III)], which reacts with a second HNO molecule, or Cbl(II) is reduced by HNO to cob(I)alamin [Cbl(I)$^-$], which reacts further with HNO. Note that a Cbl(II)/HNO = 1:2 stoichiometry was also found for the nitrite-free HNO donor Piloty's acid (unpublished results). Given that a substantial fraction of cob(III)alamin [Cbl(III)] exists as aquacobalamin [pK_a(H_2OCbl^+) = 7.8[34]] and that independent experiments have shown that H_2OCbl^+ reacts rapidly with AS to give NOCbl,[27] this suggests that the Cbl(II) + AS reaction proceeds via a Cbl(III) intermediate. In order to probe this further, experiments were carried out in the presence of an efficient H_2OCbl^+ trap cyanide, which reacts rapidly and essentially irreversibly with H_2OCbl^+ to form cyanocobalamin [CNCbl; k_f = 250 M^{-1} s^{-1} and $K_{(CNCbl)} \geq 10^{12}$ M^{-1}].[35] If H_2OCbl^+ is produced as an intermediate of the reaction between Cbl(II) and AS in the presence of excess cyanide, CNCbl, not NOCbl, would be the anticipated cobalamin product.

Figure S3 in the Supporting Information shows spectral changes that occur upon treating Cbl(II) (5.00×10^{-5} M) with 2.0 mol-equiv. AS in the presence of 20.0 mol-equiv. cyanide (pH 8.50; 0.10 M phosphate buffer) under anaerobic conditions. The spectral changes and isosbestic points (330, 385 and 484 nm) are identical to those observed in the absence of cyanide, and NOCbl is formed. Importantly, control experiments showed that the presence of 20.0 mol-equiv. cyanide does not affect the rate of decomposition of AS [pH 8.00; k_L = $(4.76 \pm 0.01) \times 10^{-4}$ s^{-1} and $(5.10 \pm 0.01) \times 10^{-4}$ s^{-1}, in the presence and absence of cyanide, respectively], and that NOCbl does not react to any significant extent with cyanide (Figure S4, Supporting Information). Furthermore, the reaction of Cbl(III) (5.00×10^{-5} M) with 20.0 mol-equiv. cyanide in the presence or absence of 1.0 mol-equiv. AS results in instantaneous formation of CNCbl (Figure S5, Supporting Information). Finally, note that Cbl(II) does not react to any significant extent with cyanide (Figure S6, Supporting Information). The results of these multiple experiments do not support a Cbl(III) intermediate for the reaction of Cbl(II) with AS,

despite the fact that Cbl(III) reacts rapidly with AS with a 1:1 stoichiometry to form NOCbl.[27] In addition, data simulation using Pro-Kineticist software also suggest that Cbl(III) is not the intermediate in the reaction between Cbl(II) and AS (data not shown).

Since experiments in the presence of the efficient H_2OCbl^+ trap cyanide suggest that Cbl(I)$^-$ is formed and consumed during the reaction between Cbl(II) and HNO, this suggests that Cbl(II) is reduced to Cbl(I)$^-$ by HNO, with concurrent oxidation of HNO to NO$^\cdot$. Cbl(I)$^-$ could subsequently react with a second HNO molecule to regenerate Cbl(II), which rapidly reacts with NO$^\cdot$ to form NOCbl (Scheme 1). This scheme assumes that N_2 is the ultimate product of HNO oxidation of Cbl(I)$^-$, which is shown later to likely be the case. The reaction between Cbl(II) and NO$^\cdot$ is very rapid and thermodynamically favorable [k = 7.4×10^8 M^{-1} s^{-1}, $K_{(NOCbl)} \approx 1 \times 10^8$ M^{-1}, 25 °C[29]].

$$4\ HN_2O_3^- \xrightarrow[R.D.S.]{k_L} 4\ HNO + 4\ NO_2^-$$

$$2\ Cbl(II)^\cdot + 2\ HNO \longrightarrow 2\ Cbl(I)^- + 2\ NO^\cdot + 2\ H^+$$

$$2\ Cbl(I)^- + 2\ HNO + 2\ H^+ \longrightarrow 2\ Cbl(II)^\cdot + N_2 + 2\ H_2O$$

$$2\ Cbl(II)^\cdot + 2\ NO^\cdot \longrightarrow 2\ NOCbl$$

$$2\ Cbl(II)^\cdot + 4\ HN_2O_3^- \longrightarrow 2\ NOCbl + 4\ NO_2^- + N_2 + 2\ H_2O$$

$$\text{or: } Cbl(II)^\cdot + 2\ HN_2O_3^- \longrightarrow NOCbl + 2\ NO_2^- + 0.5\ N_2 + H_2O$$

Scheme 1. Proposed reaction pathway for the reaction between Cbl(II) and AS.

To probe whether Cbl(I)$^-$ is indeed a reaction intermediate, the products of the reaction between Cbl(II) and 1.0 mol-equiv. AS in the presence of excess nitrite were determined. Previous experiments from our laboratory have established that Cbl(I)$^-$ is rapidly oxidized to Cbl(II) by nitrite;[31a] hence, in the presence of excess nitrite it was anticipated that nitrite could replace the second HNO molecule as the Cbl(I)$^-$ oxidant (Scheme 1). This would mean that only 1.0 mol-equiv. AS would be required for full conversion of Cbl(II) to NOCbl by HNO in the presence of excess nitrite. This was observed experimentally (Figure 4), providing further support for a Cbl(I)$^-$ intermediate.

The reaction pathways shown in Scheme 1 assume that the nitrite released from AS decomposition is not of a sufficiently high concentration to oxidize Cbl(I)$^-$ to Cbl(II); hence, nitrite is not consumed in the reaction. The Griess assay is a well-established method to quantify nitrite.[36] A calibration curve of absorbance at 586 nm vs. nitrite concentration (0–100 μM) was generated (Figure S7, Supporting Information). The Griess assay for quantification of nitrite was used to determine the amount of nitrite released upon decomposition of AS in the absence of Cbl(II). Upon decomposition of 50 μM AS, 51 ± 1 μM NO_2^- is recovered (two experiments). If 2.0 mol-equiv. AS is allowed to react with Cbl(II) [25 μM Cbl(II), 50 μM AS], 49 ± 1 μM nitrite is recovered (two experiments). Hence nitrite is not consumed in the reaction between Cbl(II) and AS, consistent with the reaction pathways shown in Scheme 1.

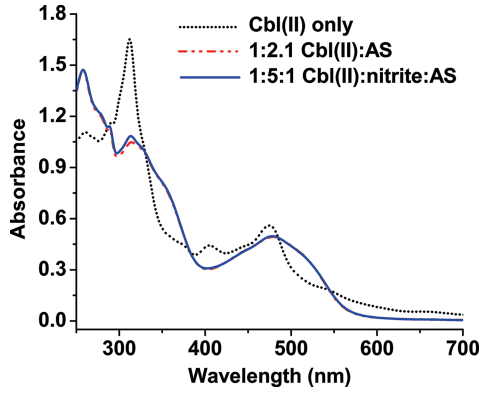


Figure 4. UV/Vis spectra of the product of reaction between Cbl(II) (50.0 μM) and AS in the presence and absence of excess NO_2^- at pH 8.00 (25.0 °C, 0.30 M TAPS buffer) under anaerobic conditions. Cbl(II) (black dotted trace) is converted to NOCbl with 2.1 mol-equiv. AS (red dashed trace) or with 1.0 mol-equiv. AS in the presence of 5.0 mol-equiv. NO_2^- (blue solid trace). Note that NO_2^- absorbs in the 300–400 nm region.

Studies on the Reaction of Cbl(I)$^-$ with AS

Given that Cbl(I)$^-$ is likely to be a reaction intermediate, kinetic studies on the reaction between Cbl(I)$^-$ and AS were independently carried out. High cobalamin concentrations were used to ensure that Cbl(I)$^-$ was stable in solution. UV/Vis spectra for the reaction between Cbl(I)$^-$ (2.00×10^{-4} M) and excess AS (50.0 mol-equiv.) at pH 7.40 [25.0 °C, 0.01 M TES buffer, I = 1.0 M ($NaCF_3SO_3$)] under strictly anaerobic conditions show that the reaction is rapid, and interestingly, two reactions are observed, with NOCbl once again being formed. Upon addition of AS to the Cbl(I)$^-$ solution (λ_{max} = 388, 464 and 547 nm), Cbl(II) (λ_{max} = 405 and 475 nm) is formed (Figure 5a) which is subsequently converted into NOCbl (λ_{max} = 478 nm; Figure 5b). The complete spectra for this reaction are given in Figure S8 (Supporting Information).

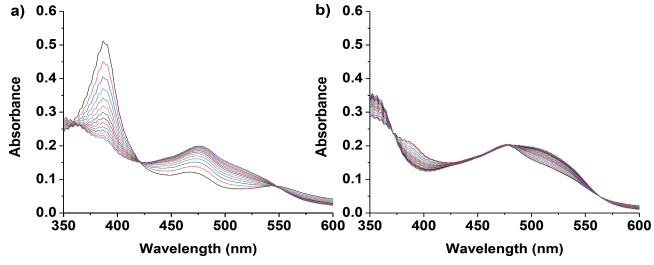


Figure 5. UV/Vis spectra obtained as a function of time for the reaction of Cbl(I)$^-$ (2.00×10^{-4} M) with 50.0 mol-equiv. AS at pH 7.40 [25.0 °C, 0.1 M TES buffer, I = 1.0 M ($NaCF_3SO_3$)] under strictly anaerobic conditions. (a) The first 14 spectral traces (spectra recorded every 0.5 s using a stopped-flow instrument, 2 mm path length) showing the formation of Cbl(II) intermediate. (b) Selected spectra at longer reaction times (7–120 s) showing the formation of final product, NOCbl.

To confirm that Cbl(I)$^-$ is first oxidized to Cbl(II), which can react further with HNO generated by AS decomposition, spectral scans were obtained for the reaction of Cbl(I)$^-$ (2.00×10^{-4} M) with 0.25 mol-equiv. AS (pH 7.40,

25.0 °C, 5.00×10^{-3} M phosphate buffer) (Figure 6a). Since Cbl(I)$^{-}$ is in excess, one would expect that Cbl(I)$^{-}$ is oxidized cleanly to Cbl(II) and that the amount of AS is insufficient for NOCbl formation. Figure 6a shows the clean conversion of Cbl(I)$^{-}$ to Cbl(II) with isosbestic points occurring at 417 and 542 nm, which are in good agreement with literature values for Cbl(I)$^{-}$/Cbl(II) conversion.[31a,32] The absorbance at 475 nm vs. time data for the reaction of Cbl(I)$^{-}$ (2.00×10^{-4} M) with 0.25 mol-equiv. AS at pH 8.00 [25.0 °C, 5.00×10^{-3} M TAPS buffer, I = 1.0 M ($NaCF_3SO_3$)] fit well to a first-order rate equation giving k_{obs} = $(4.33 \pm 0.04) \times 10^{-2}$ min^{-1} (Figure 6b). This k_{obs} value is slightly higher than that for AS decomposition at pH 8.00 [k_{AS} = $(3.25 \pm 0.01) \times 10^{-2}$ min^{-1}; Table 1]. Upon reducing the amount of AS to 0.04 mol-equiv. (7 times less than stoichiometric amount), the observed rate constant is closer to that observed for AS decomposition [k_{obs} = $(3.71 \pm 0.01) \times 10^{-2}$ min^{-1}] (Figure S9, Supporting Information). The rate-determining step of the reaction between Cbl(I)$^{-}$ and AS is therefore decomposition of AS to give HNO and nitrite.

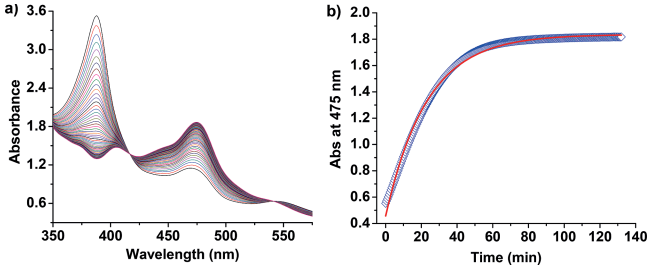


Figure 6. (a) UV/Vis spectra obtained as a function of time for the reaction of Cbl(I)$^{-}$ (2.00×10^{-4} M) with 0.25 mol-equiv. AS at pH 7.40 [25.0 °C, 5.00×10^{-3} M phosphate buffer, I = 1.0 ($NaCF_3SO_3$)] under strictly anaerobic conditions. (b) Plot of absorbance at 475 nm vs. time for the same reaction at pH 8.00 [25.0 °C, 5.00×10^{-3} M TAPS buffer, I = 1.0 ($NaCF_3SO_3$)]. The data were fitted to a first-order rate equation giving the observed rate constant: k_{obs} = $(4.33 \times 0.04) \times 10^{-2}$ min^{-1}.

The stoichiometry of the reaction between Cbl(I)$^{-}$ and AS was investigated (pH 7.00). Anaerobic solutions of Cbl(I)$^{-}$ (200 μM) with varying mol-equiv. AS (0, 0.05–3.0 mol-equiv.) were equilibrated (ca. 4 h; at least 5 half-lives for the slowest reaction), and UV/Vis spectra recorded. From the spectra of the equilibrated product solutions (Figure S10, Supporting Information), a plot of absorbance at 387 nm vs. mol-equiv. AS was generated (Figure 7a). This wavelength was chosen since 387 nm is an isosbestic point for the Cbl(II)/NOCbl conversion. The absorbance at 387 nm linearly decreases up to 0.25 mol-equiv. AS and becomes constant upon further addition of AS, consistent with a Cbl(I)$^{-}$/AS = 1:0.25 stoichiometry for the oxidation of Cbl(I)$^{-}$ to Cbl(II)$^{-}$ by one of the decomposition products of AS (HNO or nitrite). A similar plot of absorbance at 360 nm [which is not an isosbestic point for Cbl(II)/NOCbl conversion] vs. mol-equiv. AS shows that the absorbance decreases linearly up to 0.25 mol-equiv. AS, then increases linearly up to 2.25 mol-equiv. AS, and is unchanged upon further addition of AS (Figure 7b). This is consistent with an overall reaction stoichiometry of Cbl(I)$^{-}$/AS = 1:2.25.

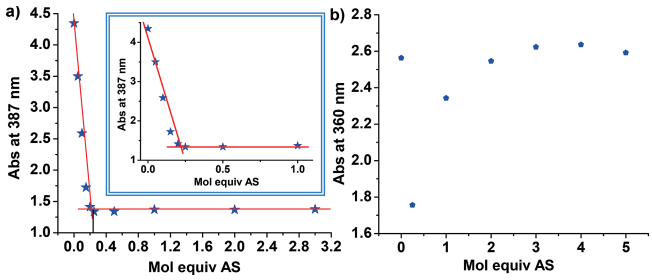
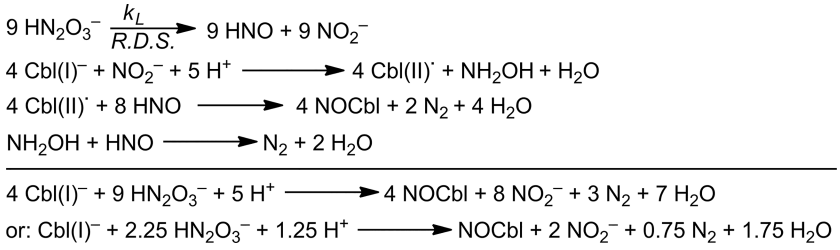


Figure 7. (a) Plot of absorbance at 387 nm vs. mol-equiv. AS obtained for anaerobic equilibrated solutions of Cbl(I)$^{-}$ (200 μM) with AS (0, 0.05, 0.10–3.0 mol-equiv.) at pH 7.00 (25.0 °C, 5.00×10^{-3} M phosphate buffer). Inset: expanded version of the plot for 0–1.0 mol-equiv. AS. (b) Plot of absorbance at 360 nm vs. mol-equiv. AS for the reaction of Cbl(I)$^{-}$ (2.00×10^{-4} M) with AS (0–5.0 mol-equiv.) at pH 8.00 (25.0 °C, 5.00×10^{-3} M phosphate buffer) under strictly anaerobic conditions.

Since Cbl(I)$^{-}$ is first oxidized to Cbl(II), control experiments with excess nitrite were carried out to confirm that the reaction of Cbl(I)$^{-}$ with AS proceeds similarly to the Cbl(II) + AS reaction after the initial oxidation of Cbl(I)$^{-}$ to Cbl(II) has occurred. In the presence of excess nitrite (20.0 mol-equiv.), 1.0 mol-equiv. AS is once again sufficient to convert Cbl(I)$^{-}$ to NOCbl (Figure S11, Supporting Information). In other words, the stoichiometry of Cbl(I)$^{-}$/AS = 1:2.25 changes to Cbl(I)$^{-}$/AS = 1:1 in the presence of excess nitrite (pH 8.00).

The amount of nitrite consumed was investigated using the Griess assay. Upon the reaction of Cbl(I)$^{-}$ (25 μM) with 2.25 mol-equiv. AS (56.25 μM), 50 ± 1 μM nitrite is produced (two experiments). In the absence of Cbl(I)$^{-}$, AS decomposes to produce 58 ± 1 μM nitrite (two experiments). Hence ca. 0.25 mol-equiv. nitrite is consumed during the reaction, consistent with initial oxidation of Cbl(I)$^{-}$ to Cbl(II) by nitrite[31a] (Scheme 2). The Nessler's test for the presence



Scheme 2. Proposed reaction pathway for the reaction between Cbl(I)$^{-}$ and AS.

of significant amounts of ammonia in the reaction product solution was negative (see Exp. Sect.). Finally, indooxine tests were carried out to check whether significant amounts of hydroxylamine are present in the product solution of the reaction of Cbl(I)$^-$ (200 μM) with 3.0 mol-equiv. AS at pH 8.00. A small amount of NH_2OH (ca. 9 μM) was detected.

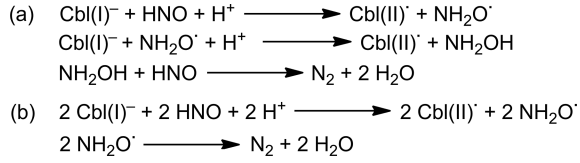
Discussion

Kinetic studies of the reaction between Cbl(II) and AS were carried out using UV/Vis spectroscopy in the pH range 7.00–9.00 under anaerobic conditions. NOCbl is formed under all conditions. The rate of the reaction between Cbl(II) and AS is independent of the Cbl(II) concentration (Figure 2); that is, the rate of the reaction between Cbl(II) and AS depends only on the rate of AS decomposition. This is also evident from a comparison of the rate constants for AS decomposition with rate constants for the reaction between Cbl(II) and 1.0 mol-equiv. AS under the same reaction conditions (Table 1).

The stoichiometry of the reaction between Cbl(II) and AS at pH 8.00 was found to be Cbl(II)/AS = 1:2 by UV/Vis spectroscopy. This unusual stoichiometry suggests that multiple reactions occur involving the cobalamin intermediates Cbl(III) or Cbl(I)$^-$. The redox potentials ($E°$) for the NO, H^+/HNO, HNO, 2 H^+/NH_2OH, Cbl(III)/Cbl(II) and Cbl(II)/Cbl(I)$^-$ redox couples are –0.55 V (pH 7, NHE),[1a] +0.30 V (pH 7, NHE),[1a] +0.20 V (pH 7.4, NHE)[37] and –0.61 V (pH 7.4, NHE),[37] respectively. (Note that the redox potential values for NO/HNO and HNO, 2 H^+/NH_2OH are estimated values.) The oxidation of Cbl(II) by HNO to Cbl(III) [= H_2OCbl^+/HOCbl; pK_a(H_2OCbl^+) = 7.8[34]] and the reduction of Cbl(II) by HNO to Cbl(I)$^-$ therefore are both thermodynamically feasible processes. However, experiments in the presence of the efficient H_2OCbl^+ trapping agent cyanide (which reacts rapidly with H_2OCbl^+ to form cyanocobalamin, CNCbl) once again resulted in formation of NOCbl upon treating Cbl(II) with 2.0 mol-equiv. AS, consistent with Cbl(I)$^-$ being the cobalamin intermediate. Further support for a Cbl(I)$^-$ intermediate was also obtained from experiments in the presence of nitrite. A change in the reaction stoichiometry from Cbl(II)/AS = 1:2 in the absence of nitrite to Cbl(II)/AS = 1:1 in the presence of excess nitrite (5.0 mol-equiv.) was observed, with nitrite replacing AS (HNO) as the oxidant of Cbl(I)$^-$. Cbl(I)$^-$ is also the intermediate of the reaction between Cbl(II) and the nitrite-free HNO donor, Piloty's acid (unpublished data).

The proposed reaction scheme for the reaction between Cbl(II) and AS is given in Scheme 1. HNO reduces Cbl(II) to Cbl(I)$^-$, being itself oxidized to NO$^·$. Cbl(II) does not react with NO_2^- or the product of HNO dimerization, N_2O, under the conditions of our experiments. Others have observed one-electron reduction of transition-metal centers by HNO in which HNO is oxidized to NO$^·$.[20] Cbl(I)$^-$ is then oxidized back to Cbl(II) by a second molecule of HNO. The Cbl(II) produced from the oxidation of Cbl(I)$^-$ by HNO subsequently reacts rapidly with NO$^·$ to form NOCbl.[29] NO$^·$ could potentially oxidize Cbl(I)$^-$ back to Cbl(II) [Cbl(I)$^-$ + NO$^·$ + H^+ → Cbl(II)$^·$+ ½ N_2O + ½ H_2O];[33] however, in this case NO would be consumed and NOCbl would therefore not be formed; hence the oxidation of Cbl(I)$^-$ by HNO must be faster than this reaction. Although nitrite produced from AS decomposition can oxidize Cbl(I)$^-$ to Cbl(II),[31a] the observation of a Cbl(II)/AS = 1:2 stoichiometry and the results of the Griess assay, which show that nitrite is not consumed in the reaction, do not support this occurring for this system. Finally, a Cbl(II)/HNO = 1:2 stoichiometry was also observed for the nitrite-free Cbl(II)/Piloty's acid system (unpublished results); providing further evidence that nitrite is not involved.

HNO is reduced by Cbl(I)$^-$. Possible HNO reduction products from the reaction between Cbl(I)$^-$ and AS are the iminoxyl radical ($H_2NO^·$[38]), NH_2OH, NH_4^+ and/or N_2. The Nessler's test suggests that appreciable amounts of NH_4^+ are not present in the product solution. Others have shown that HNO is reduced to NH_2OH by reductants for other systems.[5a,18c,21] However, the indooxine test for NH_2OH showed only trace amounts of NH_2OH present in the product solution, consistent with insignificant amounts of NH_2OH being formed during the reaction and/or NH_2OH reacting further with HNO to give N_2 and H_2O. Two possible mechanisms are shown in Scheme 3. In Scheme 3a Cbl(I)$^-$ is oxidized by HNO to give Cbl(II) and $H_2NO^·$, which oxidizes a second molecule of Cbl(I)$^-$ to give Cbl(II) + NH_2OH. $H_2NO^·$ is a strong oxidant.[38] The oxidation of NH_2OH by HNO to give N_2 and H_2O is well known (k = 4×10^3 M^{-1} s^{-1}[18c]). A control experiment showed that NH_2OH does react with HNO under the conditions of our experiments (see Exp. Sect.), although the reaction is incomplete. A further possibility (Scheme 3b) is that $NH_2O^·$ instead rapidly dimerizes to give N_2 and H_2O (k = 1.4×10^8 M^{-1} s^{-1}[39]). Transition-metal-catalyzed decomposition of NH_2OH is also well known,[31b,31c] and it has previously been shown that Cbl(II) catalyzes the decomposition of NH_2OH.[31a] Further experiments to probe the mechanism of oxidation of Cbl(I)$^-$ by HNO will require the development of HNO donor molecules that release HNO on the sub-second time scale. Experiments to detect nitrogen using headspace-gas analysis of the product mixture generated from treating Cbl(II) with 1.8 mol-equiv. ^{15}N-AS were unfortunately unsuccessful. It is likely that the small amounts of N_2 in the headspace of the product solution were insufficient for detection by the available instrumentation.



Scheme 3. Possible reaction pathways for the reaction between Cbl(I)$^-$ and HNO.

Separate studies on the reaction of Cbl(I)$^{-}$ with AS were carried out. Once again, the rate-determining step was the decomposition of AS. The stoichiometry of the reaction was Cbl(I)$^{-}$/AS = 1:2.25. This intriguing stoichiometry was initially difficult to rationalize and led us to carry out a detailed study on the nitrite-free Cbl(I)$^{-}$ + Piloty's acid system (unpublished data). In this latter system a Cbl(I)$^{-}$/PA = 1:3 stoichiometry was obtained, consistent with HNO being the sole oxidant of Cbl(I)$^{-}$. Previous studies in our laboratory have shown that Cbl(I)$^{-}$ is oxidized to Cbl(II) with a stoichiometry of Cbl(I)$^{-}$/NO_2^{-} = 1:0.25.[31a] A Cbl(I)$^{-}$/AS = 1:2.25 stoichiometry is therefore consistent with the reaction scheme shown in Scheme 2 for the AS system, in which nitrite carries out the initial oxidation of Cbl(I)$^{-}$. If this is indeed the case, 0.25 mol-equiv. NO_2^{-} will be consumed during the reaction, which was observed experimentally using the Griess assay. Why nitrite oxidizes only one molecule of Cbl(I)$^{-}$ is not entirely clear to us. One possible explanation is that the first Cbl(I)$^{-}$ oxidation consumes the nitrite impurity in AS (both commercial AS and AS synthesized in our laboratory contain small amounts of nitrite; see Exp. Sect.). The stoichiometry of the reaction changed to Cbl(I)$^{-}$/AS = 1:1 in the presence of excess nitrite, as expected, since nitrite out-competes HNO in the oxidation of Cbl(I)$^{-}$.

Conclusions

Kinetic and mechanistic studies have been carried out on the reaction of Cbl(II) with the HNO donor Angeli's salt. NOCbl is formed, a stoichiometry of Cbl(II)/AS = 1:2 is observed, and NO_2^{-} and most likely N_2 are the non-cobalamin products. The rate-determining step is decomposition of AS to give HNO and NO_2^{-}. Studies on the reaction between Cbl(I)$^{-}$ and AS and experiments in the presence of cyanide and nitrite provide support for a Cbl(I)$^{-}$ reaction intermediate. A mechanism is proposed in which Cbl(II) is reduced by HNO, giving Cbl(I)$^{-}$ and NO$^{\cdot}$. A second HNO molecule oxidizes Cbl(I)$^{-}$ back to Cbl(II), which reacts rapidly with NO$^{\cdot}$ to form NOCbl. The oxidation of a transition-metal center by HNO, which may occur for this system, is, to the best of our knowledge, unprecedented. Oxidation of transition metals by HNO including transition-metal centers of metalloproteins could potentially occur in biological systems, although further studies are required to confirm or refute this.

Experimental Section

Reagents: Hydroxycobalamin hydrochloride (HOCbl·HCl, 98% stated purity by the manufacturer) was purchased from Fluka. The percentage of water in HOCbl·HCl(·$n$$H_2O$) (10–15% water, batch-dependent) was determined by converting HOCbl·HCl into dicyanocobalamin, $(CN)_2Cbl^{-}$ (0.10 M KCN, pH 11.0, $\varepsilon_{368\ nm}$ = 30.4 $mM^{-1}cm^{-1}$).[40] Angeli's salt (AS) was either purchased from Cayman Chemical (and used without further purification) or synthesized according to a published procedure.[28] ^{15}N-labeled Angeli's salt (^{15}N-AS) was synthesized using the ^{15}N-labeled hydroxylamine precursor. $NaBH_4$ (≥ 98%), $Na_2S_2O_4$ (≥ 85%), $NaNO_2$ (99.6%), NH_2OH·HCl (≥ 97%), n-butyl nitrate (> 99%), KCN (≥ 99.1%), 8-hydroxyquinoline (≥ 99%), diethylenetriaminepentaacetic acid (DTPA; ≥ 98%), D_2O (99.8 atom-% D), acetone, triflic acid (99%), NaOH, biological buffers (MES, TES, TAPS and CHES) and inorganic buffers (Na_2CO_3, $NaHCO_3$, KH_2PO_4 and K_2HPO_4) were obtained from either Fisher Scientific or Acros Organics. $^{15}NH_2OH$·HCl (98%), TSP [2,2,3,3-[D_4]-3-(trimethylsilyl)-propionic acid, sodium salt] and the Griess reagent were obtained from Sigma Aldrich. Nessler's reagent was obtained from Spectrum 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). All pH measurements were carried out at room temperature using an Orion Model 710A pH meter equipped with a Mettler-Toledo Inlab 423 or 421 electrode. The 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. The pH of the solutions was adjusted using H_3PO_4 or NaOH solutions as necessary.

Air-Free Chemistry: Anaerobic solvent and buffer solutions were prepared by bubbling argon through the solution for ca. 24 h. Stock solutions were stored 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. Temperature-sensitive solutions were stored in the freezer. Air-free UV/Vis spectrometric measurements were carried out in Schlenk cuvettes (cuvettes fitted with a J-Young or an equivalent stopcock) with 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 fast kinetics under anaerobic conditions, experiments were carried out 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 105 Viewer (version 4.1.10) software, using a 2 mm path-length cell. The system was pre-treated with anaerobic sodium dithionite (for at least 1 h) to remove oxygen and subsequently thoroughly flushed with anaerobic water. The instrument was continuously purged with nitrogen gas during data collection. Hamilton gas-tight syringes filled with the anaerobic reactant solutions in the glove box were used to introduce the reactant solutions into the reservoir syringes of the stopped-flow instrument. For ^1H NMR spectroscopy experiments under anaerobic conditions, air-tight J-Young NMR tubes (Wilmad, 535-JY-7) were used. ^1H NMR spectra were recorded with a Bruker 400 MHz spectrometer equipped with a 5 mm probe at 23 ± 1 °C. TSP was used as an internal reference. Kinetic data were fitted using the program Microcal Origin version 8.0.

Synthesis of Cob(II)alamin [Cbl(II)]: Cbl(II) was prepared by reducing HOCbl·HCl with $NaBH_4$ under anaerobic conditions inside the glove box according to a procedure reported in the literature.[41]

Synthesis of Cob(I)alamin [Cbl(I)$^{-}$]: Cbl(I)$^{-}$ was prepared by reducing HOCbl·HCl with excess $NaBH_4$ (6.0 mol-equiv.) under anaerobic conditions inside the glove box according to a literature procedure.[42]

Determination of Cobalamin (Cbl) Concentrations: The Cbl concentrations were determined by converting Cbls 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 ($\varepsilon_{368\,nm}$ = 30 mM^{-1} cm^{-1}).[40]

Determination of Rate Constants for the Spontaneous Decomposition of Angeli's Salt (AS): Rate constants for the spontaneous decomposition of AS at different pH values were measured using UV/Vis spectrophotometry under strictly anaerobic conditions by monitoring 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 NaOH (0.01 M) to a cuvette containing buffer (2.95 mL), which had been thermostatted at 25.0 °C in the cell holder of the Cary 5000 spectrophotometer. TES, TAPS and CHES buffers (0.30 M) were used, and the total ionic strength was maintained at 1.0 M ($NaCF_3SO_3$). The absorbance vs. time data were fitted to a first-order rate equation.

Sample Preparation for Kinetic Measurements on the Reaction of Cbl(II) with AS: All samples were prepared under strictly anaerobic conditions inside the glove box. Stock Cbl(II) solutions were prepared by dissolving solid Cbl(II) in the appropriate buffer. Stock AS solutions were prepared by dissolving solid AS in NaOH (0.01 M). Stock solutions were stored in the freezer (–24 °C) inside the glove box and used within 24 h. The major reaction product in the reaction of Cbl(II) with AS under different pH conditions is the extremely air-sensitive nitroxylcobalamin (NOCbl).[43]

Sample Preparation for Kinetic Measurements on the Reaction of Cbl(I)$^-$ with AS: All samples were prepared under strictly anaerobic conditions inside the glove box. Stock Cbl(I)$^-$ solutions were prepared in H_2O [Cbl(I)$^-$ undergoes slow oxidation to Cbl(II) in the presence of buffers], stored under strictly anaerobic conditions at –24 °C and used within 1 week of preparation. Since Cbl(I)$^-$ is extremely air-sensitive and undergoes self-oxidation to Cbl(II) at lower concentrations even in the absence of oxygen, high concentrations (≥ 200 μM) were used in all kinetic experiments. Low buffer concentrations (5–10 mM) were used to control the pH. Stock AS solutions were prepared and handled as described above.

Determination of the Stoichiometry of the Reaction between Cbl(II) and AS: In the glove box, a series of vials were prepared containing a fixed volume of Cbl(II) solution (50.0 μM), and an aliquot of a stock AS solution (prepared in 0.01 M NaOH) was added to achieve various mol-equiv. AS (0, 0.25–3.5 mol-equiv.; pH 8.00). The total volume of final solution in each vial was 3.00 mL. After the addition of AS, the vials were capped and wrapped with Parafilm to minimize evaporation. The reactions were allowed to proceed to completion for at least 5 half-lives for the slowest reaction [Cbl(II) + 0.25 mol-equiv. AS]. The product solutions were systematically transferred to an air-free cuvette, and the cuvette was equilibrated at 25.0 °C in the cell compartment of the spectrometer for at least for 15 min prior to UV/Vis measurements.

Determination of the Stoichiometry of the Reaction between Cbl(I)$^-$ and AS: High concentrations of Cbl(I)$^-$ (200 μM) were used to avoid the spontaneous self-oxidation of Cbl(I)$^-$ to Cbl(II). Varying aliquots of AS stock solutions (0.0–5.0 mol-equiv.) were added to the vial containing Cbl(II) (200 μM) in buffer, the vials capped and wrapped with Parafilm. The reaction was allowed proceed to completion for ca. 4 h. The product solutions were equilibrated in an air-free cuvette at 25.0 °C for at least for 15 min prior to UV/Vis measurements. A control experiment showed that Cbl(I)$^-$ is stable in solution under the conditions of these experiments for 4 h.

Indooxine Test To Determine if Hydroxylamine is Formed in the Reaction of Cbl(II) or Cbl(I)$^-$ with AS: A modified literature procedure was used.[42,44] The reaction of Cbl(II) (1.00×10^{-3} M) with 2.0 mol-equiv. AS at pH 8.00 (0.30 M CHES buffer) was allowed to proceed to completion under anaerobic conditions inside the glove box for ca. 1 h. Then 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) were added to the product solution (1.00 mL) outside the glove box. After standing at room temperature for 45 min, the UV/Vis spectrum was recorded. The stability of authentic NH_2OH in pH 8.00 buffer was also checked using the same procedure. Our laboratory and others have shown that at lower pH conditions, NH_2OH can undergo disproportionation.[31] In a typical experiment, NH_2OH (50.0 μM) was prepared in TAPS buffer (0.30 M; pH 8.00). After 30 min, 8-hydroxyquinoline (4.0%, w/v in ethanol) and Na_2CO_3 (1.00 M) were added, the reaction allowed to proceed for 40 min, and UV/Vis spectra recorded (carried out in duplicate). From the absorbance at 710 nm the concentration of NH_2OH was calculated to be > 49.0 μM (> 98% recovery); hence, NH_2OH is stable at pH 8.00. Finally, the indooxine test for the reaction product solution upon the addition of NH_2OH (50.0 μM) to 1.0 mol-equiv. AS at pH 8.00 shows that AS reacts incompletely with NH_2OH, with ca. 13 μM NH_2OH unreacted.

Nessler's Test To Determine whether Ammonia Is Formed in the Reaction of Cbl(II) or Cbl(I)$^-$ with AS: The reaction between Cbl(II) (5.50×10^{-3} M) and 2.2 mol-equiv. AS at pH 7.00 (5.00×10^{-3} M phosphate buffer) was allowed to proceed to completion in a vial inside the glove box for ca. 2.5 h. The product solution (1.00 mL) was removed from the glove box, and 8–10 drops of Nessler's reagent were added under aerobic conditions. Formation of a brown precipitate indicates the presence of ammonia in the product solution.[45] However, a red precipitate formed at the bottom of the reaction vial. From similar experiments for Cbl(II) in the absence (red precipitate) or presence of ammonia (brown precipitate), it was clear that significant amounts of ammonia are not formed. Similar procedures were used for the test of ammonia in the products of the reaction between Cbl(I)$^-$ and AS at pH 8.00. Once again, no evidence was found for ammonia production.

Reaction of Cbl(II) with N_2O: Since N_2O is the product of HNO dimerization, the reactivity of N_2O with Cbl(II) was investigated. Excess (20.0 mM) N_2O-saturated buffer (pH 8.00; concentration of N_2O in N_2O-saturated buffer: 28.8 mM[46]) was injected into a septum-capped flask containing an anaerobic solution of Cbl(II) (2.00×10^{-4} M) and the reaction allowed to proceed inside the glove box overnight. After 12 h, the UV/Vis spectrum of the final solution was recorded. The spectrum of the product solution was indistinguishable from that of pure Cbl(II).

Griess Assay To Identify and Quantify the Amount of Nitrite Produced in the Reaction of Cbl(II) or Cbl(I)$^-$ with AS: Experiments were carried out under strictly anaerobic conditions. A calibration curve of absorbance at 586 nm vs. nitrite concentration was generated (Figure S7, Supporting Information). An aliquot of Griess reagent (1.50 mL) was added to an equal volume of buffer (1.50 mL, 0.30 M TAPS buffer, pH 8.00) containing varying concentrations of nitrite (0, 20.0, 40.0, 60.0, 80.0 and 100.0 μM) and NOCbl (40.0 μM), and the absorbance at 586 nm was determined. An aliquot of the product of the reaction between Cbl(II) (25.0 μM) and 2.0 mol-equiv. AS at pH 8.00 was subjected to the same procedure. The resulting absorbance of 0.653 ± 0.003 at 586 nm corresponded to 48.5 μM (1.94 mol-equiv.) NO_2^- produced. Similarly, an aliquot of the product of the reaction between Cbl(I)$^-$ (25.0 μM) and 2.25 mol-equiv. AS at pH 8.00 was subjected to the Griess assay procedure, and in this case 49.5 μM (ca. 2.0 mol-equiv.) NO_2^- was recovered. Griess tests were carried out to test the presence of nitrite in commercially available AS and the AS synthesized in our

laboratory. The Griess assay results show that both AS samples contain ca. 6% nitrite impurities.

Control Experiments in the Presence of Excess Cyanide To Probe the Possible Intermediate in the Reaction of Cbl(II) with AS: Control experiments were carried out to trap the potential cob(III)alamin intermediate. All the experiments were carried out under strictly anaerobic conditions. Kinetic spectra were recorded for the reaction of Cbl(II) (5.00×10^{-5} M) with 2.0 mol-equiv. AS in the presence of 20.0 mol-equiv. cyanide at pH 8.50 (25.0 °C, 0.10 M phosphate buffer). Spectral changes and the isosbestic points in the spectra remain the same as those obtained from the reaction of Cbl(II) with 2.0 mol-equiv. AS in the absence of cyanide. In addition, both Cbl(II) and NOCbl do not react with 20.0 mol-equiv. cyanide. However, the direct reaction of H_2OCbl^+/HOCbl (5.00×10^{-5} M) with cyanide (20.0 mol-equiv.) in the presence or absence of 1.0 mol-equiv. AS at pH 8.50 under anaerobic conditions gives CNCbl as product.

Stoichiometry Experiments in the Presence of Excess Nitrite: To confirm the presence of $Cbl(I)^-$ as an intermediate in the reaction of Cbl(II) with AS, control experiments were carried out in the presence of excess nitrite. UV/Vis spectra were recorded for the product solution of the reaction between Cbl(II) (50 μM) and 1.0 mol-equiv. AS in the presence of 5.0 mol-equiv. NO_2^- at pH 8.00 (5.0 mM TAPS buffer) under anaerobic conditions. The product spectrum resembles the spectrum of NOCbl. Similarly, the spectrum of the product of the reaction between $Cbl(I)^-$ (200 μM) and 1.0 mol-equiv. AS in the presence of 20.0 mol-equiv. NO_2^- at pH 8.00 (5.0 mM TAPS buffer) under strictly anaerobic conditions is also identical with the NOCbl spectrum.

Synthesis of ^{15}N-Labeled AS (^{15}N-AS; $Na_2{}^{15}N^{14}NO_3$): $^{15}NH_2OH{\cdot}HCl$ (0.500 g) was dissolved in warm water (0.50 mL). Solid NaOH (0.888 g) was dissolved in CH_3OH (6.00 mL) with sonication. The $^{15}NH_2OH{\cdot}HCl$ solution was added dropwise with stirring to the NaOH solution. The reaction mixture was filtered through a sintered funnel and the NaCl precipitate discarded. The filtrate containing pure $^{15}NH_2OH$ was transferred to a Schlenk flask and purged with nitrogen for ca. 15 min. N_2-purged *n*-butyl nitrate (0.80 mL) was then added dropwise with stirring using a syringe. The product precipitated upon leaving the solution in a refrigerator overnight. ***Caution:*** *Others have reported that an explosion can potentially occur!*[28] After 12 h, the product was filtered, redissolved in a minimal amount of 0.10 M NaOH (0.50 mL) and reprecipitated in excess ethanol (ca. 20 mL). The product was finally dried under vacuum (2×10^{-2} mbar) at 95 °C overnight. The yield was 0.288 g (57.6%). The purity of ^{15}N-AS was checked by UV/Vis spectroscopy ($\varepsilon_{248\ nm} = 8.30 \times 10^{-3}\ M^{-1}cm^{-1}$[28]) and found to be ≥ 99%.

Supporting Information (see footnote on the first page of this article): Figures S1–S11.

Acknowledgments

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