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)alamín

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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(II)alamin [Cbl(II)] 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 nitroxycovalamin [nitroxylobalamin, NO–Cbl(III), NOCbl].

**Introduction**

The importance of the reactive nitrogen species nitrosyl hydride [HNO, nitroxyl, $pK_a(1HNO/3NO–) = 11.4$] in biological systems is increasingly being realized. Although it has not yet been unequivocally demonstrated that HNO exists in biological systems,[5] 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$^*$. In addition, HNO generation has recently been demonstrated in cells pretreated with S-nitroso-glutathione exposed to H$_2$S using the HNO sensor Cu-BOT1[a] [a nitrosothiol (HSNO) intermediate is proposed, which reacts further with H$_2$S to give HNO + H$_2$S$_2$.] Fe$^{3+}$ heme-catalyzed reduction of nitrite by H$_2$S 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$^*$. [12a,21] However, direct observation and use of HNO is limited by its rapid dimerization and decomposition to N$_2$O and H$_2$O [$k = (8 \pm 3) \times 10^6$ M$^{-1}$ s$^{-1}$, 22 °C][13] [Equation (1)].[14] HNO donor molecules are therefore required to generate HNO in situ in HNO studies. Sodium α-oxynitrite or Angeli’s salt (Na$_2$N$_2$O$_3$, AS) is the most commonly used HNO donor for chemical and biological studies of HNO’s reactivity.[14] The monoprotonated form of AS, HN$_2$O$_3$$^-$ [$pK_a(HN_2O_3^-) = 9.70$][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 $\rightarrow$ [cis-N$_2$O$_2$H$^+$] $\rightarrow$ N$_2$O + H$_2$O**

**N$_2$O$_2$$^{2-}$ + H$^+$ $\rightarrow$ HN$O_3$$^-$ $\rightarrow$ HNO + NO$_2^-$

HNO donors show considerable promise in treating alcoholism,[14] cardiovascular disease, ischemia/reperfusion injury and heart failure.[12a,17] The main biological targets of HNO are believed to be thiols, DNA and oxidized metals and oxidized metalloprotein centers.[16] 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$_2$OH.[5a,16c,21]

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Vitamin B₁₂ derivatives (also known as cobalams, Cbls; Figure 1) are essential cobalt-containing micronutrients synthesized by microorganisms.[22] Methylcobalamin (MeCbl; X = CH₃; Figure 1) and adenosylcobalamin (AdoCbl; X = Ado; Figure 1) serve as cofactors for two mammalian enzymatic reactions.[23] Cob(III)alamins (Co³⁺) are reduced to cob(II)alamin [Cbl(II)⁺⁻] upon cellular uptake,[24] and cob(I)alamin [Cbl(I)⁻] is a short-lived precursor of the two B₁₂ 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 B₁₂: X = CN⁻, CH₃, Ado, H₂O, 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 (H₂OCbl+/HOCbl) reacts with AS to form nitrosylcobalamin [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 (H₂N₂O₃⁻) decomposition to give HNO and NO₂⁻ is pH-independent in the pH 4–8 range (t₁/₂ = 17 min at 25 °C)[28] and becomes increasingly slower for pH > 8.[28] Upon the addition of excess AS (4.00 × 10⁻⁵ m) to Cbl(II) (4.00 × 10⁻⁵ m) buffer (pH 8.00) under strictly anaerobic conditions, Cbl(II) is cleanly converted into nitroxylcobalamin [nitrosylcobalamin, NOCbl, NO––Cbl(III); λₘₐₓ = 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 NO₂⁻), followed by a rapid reaction between HNO and Cbl(II).

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 (kobs) of (3.58 ± 0.01) × 10⁻² min⁻¹ (Figure S1, Supporting Information). This rate constant is similar to the rate of spontaneous decomposition of AS at pH 8.00 [kL = (3.06 ± 0.01) × 10⁻² 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 (kobs) and the spontaneous decomposition of AS (kL) as a function of pH [25.0 °C, I = 1.0 m (NaCF₃SO₃)] under anaerobic conditions.

<table>
<thead>
<tr>
<th>pH (± 0.03)</th>
<th>kobs × 10² [min⁻¹][a]</th>
<th>kL × 10² [min⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.00</td>
<td>2.43 ± 0.01</td>
<td>2.40 ± 0.01</td>
</tr>
<tr>
<td>8.00</td>
<td>3.58 ± 0.01</td>
<td>3.06 ± 0.01</td>
</tr>
<tr>
<td>7.00</td>
<td>3.58 ± 0.02</td>
<td>3.25 ± 0.01</td>
</tr>
</tbody>
</table>

[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. 
showed that NH₂OH is stable in pH 8.00 buffer solution negatively (see Exp. Sect.). Importantly, a control experiment respectively, in the product solution of the reaction between 0.10 absence of DTPA, respectively [1.0 mol-equiv. AS, pH 7.40, presence of significant amounts of NH₂OH or NH₃, respectively\[|30|\]. The indooxine test and 
etions 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 NH₂OH or NH₃\[\text{respectively, in the product solution of the reaction between Cbl}(II)\text{ and 2.0 mol-equiv. AS} (pH 8.00)\text{. Both tests were negative (see Exp. Sect.). Importantly, a control experiment showed that NH₂OH is stable in pH 8.00 buffer solution (at lower pH conditions, NH₂OH can undergo disproportionation\[|31|\]).

It was also of interest to see if N₂O [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 N₂O (20.0 nm) was identical to that for Cbl(II) (\(\lambda_{\max } = 405\text{ and 475 nm}\[|31a,32|\]). There is, therefore, no reaction between Cbl(II) and N₂O, as expected based on work of others who propose that Cbl(II) and N₂O are the products of oxidation of Cbl(I) by NO [Cbl(I) + NO + H⁺ → Cbl(II) + ½ N₂O + ½ H₂O].\[|33|\] The reaction of Cbl(II) with NO\₂ is negligible under the experimental conditions.\[|29a|\]

**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.

**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 N₂O 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(II)alamin [Cbl(II)\text{⁻}], 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(II)alamin [Cbl(II)] exists as aquacobalamin \[pKₐ(H₂OCbl⁺) = 7.8\[|34|\] and that independent experiments have shown that H₂OCl⁺ 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 H₂OCl⁺ trap cyanide, which reacts rapidly and essentially irreversibly with H₂OCl⁺ to form cyanocobalamin [CNCbl; \(kₕ = 250 \text{m}⁻¹\text{s}⁻¹ \text{and } K_{CNCbl} \approx 10^{12} \text{M}⁻¹\)],\[|35|\] If H₂OCl⁺ 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_1 = (4.76 ± 0.01) \times 10⁻⁴ \text{s}⁻¹ \text{and (5.10 ± 0.01) } \times 10⁻⁴ \text{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.\textsuperscript{[23]} 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$_2$OCl$^+$ 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 \times 10^8 \text{ M}^{-1} \text{s}^{-1}, K_{(\text{NOCbl})} = 1 \times 10^9 \text{ M}^{-1}, 25 \text{ ºC}^{[29]})\].

\[
4 \text{HN}_2\text{O}_3^– \rightarrow \frac{k_1}{R.D.S.} \rightarrow 4 \text{HNO} + 4 \text{NO}_2^–
\]

\[
2 \text{Cbl(II)}^+ + 2 \text{HNO} \rightarrow 2 \text{Cbl(I)}^- + 2 \text{NO}^- + 2 \text{H}^+
\]

\[
2 \text{Cbl(I)}^- + 2 \text{HNO} + 2 \text{H}^+ \rightarrow 2 \text{Cbl(II)}^+ + 2 \text{N}_2 + 2 \text{H}_2\text{O}
\]

\[
2 \text{Cbl(II)}^+ + 2 \text{NO}^- \rightarrow 2 \text{NOCbl}
\]

\[
2 \text{Cbl(II)}^+ + 4 \text{HN}_2\text{O}_3^– \rightarrow 2 \text{NOCbl} + 4 \text{NO}_2^– + 2 \text{N}_2 + 2 \text{H}_2\text{O}
\]

or: \[
2 \text{Cbl(I)}^- + 2 \text{HN}_2\text{O}_3^– \rightarrow \text{NOCbl} + 2 \text{NO}_2^– + 0.5 \text{N}_2 + 2 \text{H}_2\text{O}
\]

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,\textsuperscript{[11a]} 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.\textsuperscript{[30]} A calibration curve of absorbance at 586 nm vs. nitrite concentration (0–100 \(\text{μM}\)) 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 \(\text{μM}\) AS, \(51 \pm 1 \text{ μM} \text{NO}_2^\cdot\) is recovered (two experiments). If 2.0 mol-equiv. AS is allowed to react with Cbl(II) (25 \(\text{μM}\) Cbl(II), 50 \(\text{μM}\) AS), \(49 \pm 1 \text{ μM}\) 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.

**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 \(\text{×}10^{-4} \text{ M}\)) and excess AS (50.0 mol-equiv.) at pH 7.40 [25.0 ºC, 0.01 M TES buffer, \(I = 1.0 \text{ M} (\text{NaCF}_3\text{SO}_3)\)] under 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 (\(\text{λ}_\text{max} = 388, 464 \text{ and } 547 \text{ nm}\)), Cbl(II) (\(\text{λ}_\text{max} = 405 \text{ and } 475 \text{ nm}\)) is formed (Figure 5a) which is subsequently converted into NOCbl (\(\text{λ}_\text{max} = 478 \text{ nm};\) Figure 5b). The complete spectra for this reaction are given in Figure S8 (Supporting Information).

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 \(\text{×}10^{-4} \text{ M}\)) with 0.25 mol-equiv. AS at pH 7.40, 25.0 ºC, 0.01 M TES buffer, \(I = 1.0 \text{ M} (\text{NaCF}_3\text{SO}_3)\) 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.
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⁻) (200 μm) with 0.25 mol-equiv. AS at pH 8.00 [25.0 °C, 5.00 × 10⁻³ m TAPS buffer, I = 1.0 m (NaCF₃SO₃)] fit well to a first-order rate equation giving the observed rate constant: k_{obs} = (4.33 ± 0.04) × 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 ± 0.01) × 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 ± 0.01) × 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.

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.

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). The Nitrite’s test for the presence

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 m (NaCF₃SO₃)] 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 m (NaCF₃SO₃)] fit well to a first-order rate equation giving the observed rate constant: k_{obs} = (4.33 ± 0.04) × 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 ± 0.01) × 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 ± 0.01) × 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 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.

9 HN₃O₅⁻ $\xrightarrow{R.D.S.}$ 9 HNO + 9 NO₂⁻

4 Cbl(II⁻) + NO₂⁻ + 5 H⁺ $\rightarrow$ 4 Cbl(III⁻) + NH₂OH + H₂O

4 Cbl(II⁻) + 8 HNO $\rightarrow$ 4 NOCbl + 2 N₂ + 4 H₂O

NH₂OH + HNO $\rightarrow$ N₂ + 2 H₂O

or: Cbl(I⁻) + 2.25 HN₃O₅⁻ + 1.25 H⁺ $\rightarrow$ NOCbl + 2 NO₂⁻ + 0.75 N₂ + 1.75 H₂O

Scheme 2. Proposed reaction pathway for the reaction between Cbl(I⁻) and AS.

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of significant amounts of ammonia in the reaction product solution was negative (see Exp. Sect.). Finally, indoxine tests were carried out to check whether significant amounts of hydroxylamine are present in the product solution of the reaction of Cbl(I) \((200 \mu \text{m})\) with 3.0 mol-equiv. AS at pH 8.00. A small amount of NH2OH (ca. 9 \(\mu \text{m}\)) 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 \(E^\circ\) for the NO, H\(^+\)/NO, HNO, 2 H\(^+\)/NH\(_2\)OH, Cbl(III)/Cbl(II) and Cbl(II)/Cbl(I) redox couples are \(-0.55\) V (pH 7, NHE), \(+0.30\) V (pH 7, NHE), \(+0.20\) 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\(_2\)OH are estimated values.) The oxidation of Cbl(II) by HNO to Cbl(III) \([= \text{H}_2\text{O}Cbl^+ / \text{HOCbl}^+: \kappa_{\text{H}}(\text{H}_2\text{O}Cbl^+) = 7.8^{[32]}\] 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\(_2\)OCl\(^+\) trapping agent cyanide (which reacts rapidly with H\(_2\)OCl\(^+\) 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 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\(_2\). Cbl(II) does not react with NO\(_2\) or the product of HNO dimerization, N\(_2\)O, 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\(_2\).\(^{[20]}\) Cbl(II) 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\(_2\) to form NOCbl.\(^{[29]}\) NO\(_2\) could potentially oxidize Cbl(I) back to Cbl(II) \([\text{Cbl(I)} + \text{NO}_2 + \text{H}^+ \rightarrow \text{Cbl(II)} + \frac{1}{2} \text{N}_2 + \frac{1}{2} \text{H}_2\text{O}].[33]\) However, in this case NO\(_2\) 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\(_2\)NO\(_2\))\(^{[38]}\), NH\(_2\)OH, 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\(_2\)OH by reductants for other systems.\(^{[5a,18c]}\) However, the indoxine test for NH\(_2\)OH showed only trace amounts of NH\(_2\)OH present in the product solution, consistent with insignificant amounts of NH\(_2\)OH being formed during the reaction and/or NH\(_2\)OH reacting further with HNO to give N\(_2\) and H\(_2\)O. Two possible mechanisms are shown in Scheme 3. In Scheme 3a Cbl(I) is oxidized by HNO to give Cbl(II) and H\(_2\)NO\(_2\), which oxidizes a second molecule of Cbl(I) to give Cbl(II) + NH\(_2\)OH. H\(_2\)NO\(_2\) is a strong oxidant.\(^{[38]}\) The oxidation of NH\(_2\)OH by HNO to give N\(_2\) and H\(_2\)O is well known \((k = 4 \times 10^9 \text{ M}^{-1} \text{s}^{-1})^{[18c]}\). A control experiment showed that NH\(_2\)OH 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\(_2\)O\(_2\) instead rapidly dimerizes to give N\(_2\) and H\(_2\)O \((k = 1.4 \times 10^{10} \text{ M}^{-1} \text{s}^{-1})^{[39]}\). Transition-metal-catalyzed dimerization of NH\(_2\)OH is also well known,\(^{[31b,31c]}\) and it has previously been shown that Cbl(II) catalyzes the decomposition of NH\(_2\)OH.\(^{[31a]}\) 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.

(a) \(\text{Cbl(I)}^– + \text{HNO} + \text{H}^+ \rightarrow \text{Cbl(II)}^– + \text{NH}_2\text{O}^–\)
\(\text{Cbl(I)}^– + \text{NH}_2\text{O}^– + \text{H}^+ \rightarrow \text{Cbl(II)}^– + \text{NH}_2\text{OH}\)
\(\text{NH}_2\text{OH} + \text{HNO} \rightarrow \text{N}_2 + 2 \text{H}_2\text{O}\)
(b) \(2 \text{Cbl(I)}^– + 2 \text{HNO} + 2 \text{H}^+ \rightarrow 2 \text{Cbl(II)}^– + 2 \text{NH}_2\text{O}^–\)
\(2 \text{NH}_2\text{OH} \rightarrow \text{N}_2 + 2 \text{H}_2\text{O}\)

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 \( \text{Cbl(I)} / \text{AS} = 1.2:2.25 \). 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(II) is oxidized to Cbl(II) with a stoichiometry of Cbl(I)/NO2− = 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. NO2− 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 NO2− and most likely N2 are the non-cobalamin products. The rate-determining step is decomposition of AS to give HNO and NO2−. 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. A second HNO molecule oxidizes Cbl(I)− back to Cbl(II), which reacts rapidly with NO 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 was determined by converting HOCbl·HCl into dicyanocobalamin, (CN)2Cbl (0.10 M KCN, pH 11.0, \( \varepsilon_{368 \text{ nm}} = 30.4 \text{ M}^{-1} \text{cm}^{-1} \)). Angeli’s salt (AS) was either purchased from Angeli’s salt (15N-AS) was synthesized using the15N-labeled hydroxycobalamin products. The rate-determining step is decompositon of AS to give HNO and NO2−. 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. A second HNO molecule oxidizes Cbl(I)− back to Cbl(II), which reacts rapidly with NO 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.

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product was determined using UV/Vis spectrometry ($\varepsilon_{368\text{ nm}} = 30\ \text{mm}^{-1}\text{cm}^{-1})$.[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 (NaCl, Na$_2$SO$_4$). 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).[41]

**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$_2$O [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 (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 a fixed volume of Cbl(II) + 0.25 mol-equiv. AS. The reaction was allowed to proceed to completion for ca. 4 h. Then aerobic 8-hydroxyquinolinoine solution (1.00 mL, 4.0% w/v in ethanol) and aqueous aerobic Na$_2$CO$_3$ 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$_2$OH in pH 8.00 buffer was also checked using the same procedure. Our laboratory and others have shown that at lower pH conditions, NH$_2$OH can undergo disproportionation.[31] In a typical experiment, NH$_2$OH (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$_2$CO$_3$ (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$_2$OH was calculated to be $>49.0$ μm (98% recovery); hence, NH$_2$OH is stable at pH 8.00. Finally, the indoxine test for the reaction product solution upon the addition of NH$_2$OH (50.0 μm) to 1.0 mol-equiv. AS at pH 8.00 shows that AS reacts incompletely with NH$_2$OH, with ca. 13 μM NH$_2$OH 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) (5.00 × 10$^{-3}$ M) and 2.2 mol-equiv. AS at pH 7.00 was analyzed using Nessler’s reagent inside 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. 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$_2$O:** Since N$_2$O is the product of HNO dimerization, the reactivity of N$_2$O with Cbl(II) was investigated. Excess (20.0 mM) N$_2$O-saturated buffer (pH 8.00; concentration of N$_2$O in N$_2$O-saturated buffer: 28.8 mM[46]) was injected into a septum-capped flask containing an anaerobic solution of Cbl(II) (2.00 × 10$^{-3}$ 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(II) (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^{-3} m) with 2.0 mol-equiv. AS in the presence of 20.0 mol-equiv. NO2 and 1.0 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 H2OCl+/HOCbl (5.00 × 10^{-3} 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. NO2 at pH 8.00 (5.0 mM TAPS buffer) under anaerobic conditions. The product spectrum remains the same as those obtained from the reaction of Cbl(II) (200 μM) and 1.0 mol-equiv. AS in the presence of 20.0 mol-equiv. NO2 at pH 8.00 (5.0 mM TAPS buffer) under strictly anaerobic conditions is also identical with the NOCbl spectrum.

Synthesis of 15N-Labeled AS (15N-AS; Na15N4NO3): 15NH2OH·HCl (0.500 g) was dissolved in warm water (0.50 mL). Solid NaNO3 (0.888 g) was dissolved in CH3OH (6.00 mL) with sonication. The 15NH2OH·HCl solution was added dropwise with stirring to the NaNO3 solution. The reaction mixture was filtered through a sintered funnel and the NaCl precipitate discarded. The filtrate containing pure 15NH2OH was transferred to a Schlenk flask and purged with nitrogen for ca. 15 min. N2-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. 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 15N-AS was checked by UV/Vis spectroscopy (ε296 nm = 8.30 × 10^{-3} m·cm^{-1}·mol^{-1}) and found to be ≥ 99%.

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

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