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Mechanistic studies of the reactions of the reduced vitamin B_{12} derivatives with the HNO donor Piloty's acid: further evidence for oxidation of cob(I)alamin by (H)NO†

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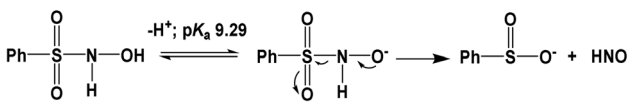
There is accumulating evidence for the existence of HNO in biological systems. Compared with NO (•NO), much less is known about the chemical and biochemical reactivity of HNO. Kinetic and mechanistic studies have been carried out on the reaction between the vitamin B_{12}-derived radical complex cob(II)alamin (Cbl(II)•, Cbl(II)) with the widely used HNO donor Piloty's acid (PA). A stoichiometry of 1 : 2 Cbl(II) : PA was obtained and PA decomposition to HNO and benzenesulfinate ($C_6H_5SO_2^-$) is the rate-determining step. No evidence was found for nitrite (Griess assay), ammonia (Nessler's test) or NH_2OH (indooxine test) in the product solution, and it is likely that HNO is instead reduced to N_2. A mechanism is proposed in which reduction of Cbl(II) by (H)NO results in formation of cob(I)alamin (Cbl(I)$^-$) and •NO. The Cbl(I)$^-$ intermediate is subsequently oxidized back to Cbl(II) by a second (H)NO molecule, and Cbl(II) reacts rapidly with •NO to form nitroxylcobalamin (NOCbl). Separate studies on the reaction between Cbl(I)$^-$ and PA shows that this system involves an additional step in which Cbl(I)$^-$ is first oxidized by (H)NO to Cbl(II), which reacts further with (H)NO to form NOCbl, with an overall stoichiometry of 1 : 3 Cbl(I)$^-$: PA. Experiments in the presence of nitrite for both systems support the involvement of a Cbl(I)$^-$ intermediate in the Cbl(II)/PA reaction. These systems provide the second example of oxidation of cob(I)alamin by (H)NO.

Introduction

There is increasing evidence that the protonated form of nitroxyl, nitrosyl hydride, HNO ($pK_a(^1HNO/^3NO^-) \sim 11.4$ (ref. 1 and 2)) is a biologically important species. Generated *in vitro* by a variety of biochemical routes,[3–10] HNO shows distinctly different biochemical properties from its one-electron redox sibling •NO.[11–15] HNO is short-lived in aqueous solution due to spontaneous and rapid dimerization to ultimately form N_2O and H_2O ($k = 8 \times 10^6$ M^{-1} s^{-1}, 22 °C (ref. 1)); eqn (1).[16]

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

HNO donor molecules are therefore required to generate HNO *in situ* in HNO studies. The rate constant for the reaction between ^1HNO and $^3NO^-$ (to give N_2O and OH^-) has also been indirectly determined ($k = 6.6 \times 10^9$ M^{-1} s^{-1} (ref. 17)). Sodium α-oxyhyponitrite (Angeli's salt, $Na_2N_2O_3$, AS) and *N*-hydroxybenzenesulfonamide (Piloty's acid, $C_6H_5SO_2NHOH$, PA) are the most common HNO donors currently used in chemical and biological studies.[18] The monoprotonated form of AS ($HN_2O_3^-$, $pK_a(HN_2O_3^-) = 9.70$ (ref. 19)) decomposes to give HNO and NO_2^- at pH 4–8, with much slower decomposition rates at higher pH conditions. PA, however, decomposes faster in alkaline solutions to produce HNO and benzenesulfinate ($C_6H_5SO_2^-$), eqn (2).[20] At pH > 9.5 HNO undergoes slow spin-forbidden deprotonation to form $^3NO^-$, eqn (3) ($k_f = (4.9 \pm 0.5) \times 10^4$ M^{-1} s^{-1}; $k_r \sim 1.2 \times 10^2$ s^{-1} (ref. 1)).



(2)

$$^1HNO + OH^- \underset{k_r}{\overset{k_f}{\rightleftharpoons}} {}^3NO^- + H_2O \quad (3)$$

Numerous studies report the reactivity of HNO and HNO donors with transition metal complexes including porphyrins.[21–29] The redox potential of the metal center of the complex is an important factor in determining whether HNO

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and/or the HNO donor itself reacts with the complex.[30] Transition metal centers are typically reduced by HNO and HNO is oxidized to $^{\bullet}NO$.[24–29] We recently studied the reactions of AS with the structurally related cobalt complexes of vitamin B_{12}, cob(II)alamin and cob(I)alamin, Fig. 1.[31] Vitamin B_{12} complexes are essential mammalian coenzymes[32,33] synthesized by microorganisms.[34] Cob(III)alamins (Co^{3+}) are reduced to cob(II)alamin (Cbl(II)$^{\bullet}$, Cbl(II), B_{12r}) upon uptake into cells,[35] and protein-bound cob(I)alamin (Cbl(I)$^{-}$, B_{12s}) is a short-lived precursor of the two coenzyme forms of vitamin B_{12}, methylcobalamin and adenosylcobalamin.[35]

Recent studies on the reactions of cob(II)alamin and cob(I)alamin with AS showed that the mechanisms of these reactions are complex, with multiple reactions occurring.[31] Kinetic and especially product studies ultimately led us to propose that HNO oxidizes the Co(I) center of cob(I)alamin.[31] To our knowledge this is the first report of the oxidation of a metal center by HNO. In this paper we present detailed mechanistic studies on the reactions of cob(II)alamin and cob(I)alamin with PA. Importantly, PA has the distinct advantage compared with AS since it decomposes to give $C_6H_5SO_2^-$ in addition to HNO in alkaline solution,[20] whereas the byproduct of Angeli's salt decomposition to HNO is the redox-active species nitrite which also oxidizes cob(I)alamin.[31,36] The studies presented herein provide further support for oxidation of cob(I)alamin by (H)NO.

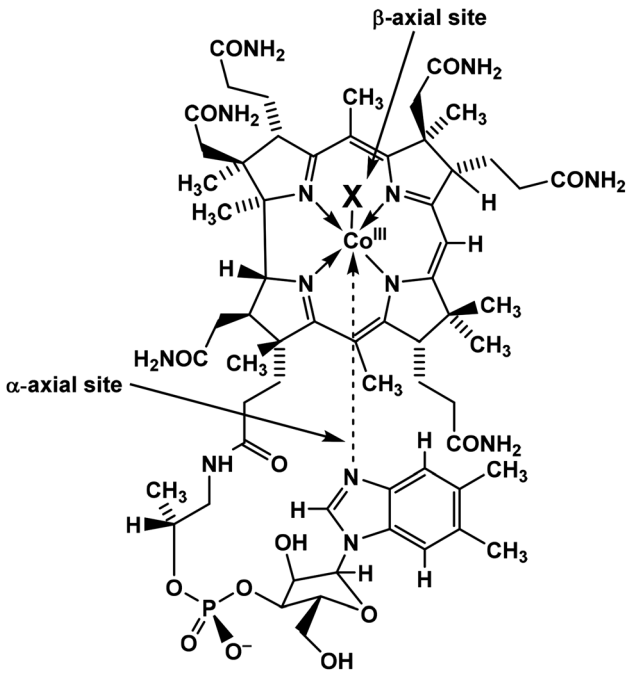


Fig. 1 The structure of vitamin B_{12} complexes (cob(III)alamins, Cbl(III); X = H_2O/OH^- (aquacobalamin/hydroxycobalamin), NO (nitroxylcobalamin/nitrosylcobalamin), CN^-, CH_3, Ado, *etc*. The β-axial ligand X is cleaved upon reduction of Cbl(III) to pentacoordinate cob(II)alamin (Cbl(II)). The bond to the 5,6-dimethylbenzimidazole at the α-axial site is broken upon reduction of Cbl(II) to tetracoordinate cob(I)alamin (Cbl(I)$^-$).

Experimental

Reagents

Hydroxycobalamin hydrochloride (HOCbl·HCl, 98% stated purity by the manufacturer) was purchased from Fluka. The percentage of water in HOCbl·HCl ($\cdot nH_2O$) (10–15% water, batch dependent) was determined by converting HOCbl·HCl to dicyanocobalamin, $(CN)_2Cbl^-$ (0.10 M KCN, pH 11.0, $\varepsilon_{368\ nm}$ = 30.4 $mM^{-1}\ cm^{-1}$).[37] Piloty's acid (PA, 98%) was purchased from Cayman Chemical Company and used without further purification. ^{15}N-labeled PA (^{15}N-PA) was synthesized using ^{15}N-labeled hydroxylamine precursor following the literature procedure with slight modifications.[38] $NaBH_4$ (≥98%), $NaNO_2$ (99.6%), $NH_2OH{\cdot}HCl$ (≥97%), KCN (99%), 8-hydroxyquinoline (≥99%), diethylenetriaminepentaacetic acid (DTPA; ≥98%), D_2O (99.8 atom% D), methanol-d_4 (99.8 atom% D), acetone, triflic acid (99%), NaOH, biological buffers (MES, TES, TAPS, CHES and CAPS) and inorganic buffers (KH_2PO_4, K_2HPO_4, $NaHCO_3$ and Na_2CO_3) were obtained from either Fisher Scientific or Acros Organics. The Griess reagent, $^{15}NH_2OH{\cdot}HCl$ (98%), benzenesulfonyl chloride (99%), TSP (3-(trimethylsilyl)-propionic 2,2,3,3-d_4 acid, sodium salt), benzenesulfinic acid, sodium salt (98%), and methanol were obtained from Sigma Aldrich. Nessler's reagent was purchased 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 inorganic buffers (5.0 mM–0.10 M) and a constant ionic strength was maintained using sodium triflate ($NaCF_3SO_3$; I = 1.0 M). Details on the instrumentation and the procedures used for UV–vis and NMR spectroscopy experiments, pH measurements, the preparation and manipulation of air-free solutions, data fitting, the syntheses of cob(II)alamin and cob(I)alamin, and the determination of cobalamin concentrations are given elsewhere.[31]

Determination of the rate constant for the spontaneous decomposition of Piloty's acid (PA)

Stock solutions were prepared by dissolving PA (solid) in CH_3OH and further dilutions were made with water. The amount of CH_3OH in final reaction solution was <3% v/v. The reaction was initiated by adding an aliquot (0.200 mL, 1.50 mM) of a solution of PA in water/CH_3OH to a cuvette containing buffer solution (2.80 mL) which had been thermostated in the cell holder of the Cary 5000 spectrophotometer. The decomposition of PA (1.00×10^{-4} M) was monitored at 250 nm and the absorbance *versus* time data were fitted to a first-order rate equation. Biological buffers were found to alter the rate of the decomposition of PA; hence phosphate and carbonate buffers (I = 1.0 M, $NaCF_3SO_3$) were used in all kinetic experiments.

Sample preparation for kinetic measurements on the reaction of Cbl(II) with PA

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 PA solutions were prepared by dissolving PA in anaerobic CH_3OH and further dilutions were made in water. Stock solutions were stored in the freezer (−24 °C) inside the glove box and used within 24 h. Strictly air-free conditions were required since the major reaction product nitroxylcobalamin is extremely air-sensitive.[39]

Sample preparation for kinetic measurements on the reaction of $Cbl(I)^-$ with PA

Similar procedures were used as for the studies on the Cbl(II) + AS system.[31] High $Cbl(I)^-$ concentrations (100–200 μM) and low buffer concentrations (5–10 mM) were used. Stock PA solutions were prepared and handled as described above.

Sample preparation for ^1H NMR experiments for the reaction of Cbl(II) with PA

For ^1H NMR experiments, stock PA solutions were prepared in anaerobic deuterated methanol and added to the Cbl(II) solution in anaerobic buffer (pD 10.00, 0.10 M carbonate buffer). The reaction was allowed to proceed to completion inside the glove box and subsequently transferred to an air-free NMR tube before recording the ^1H NMR spectra. TSP was added as the internal reference.

Determination of the stoichiometry of the reaction of Cbl(II) and $Cbl(I)^-$ with PA at pH 10.00

Inside the glove box a series of vials were prepared containing Cbl(II) (50.0 μM) and varying aliquots of a stock PA solution (0, 0.25–3.5 mol equiv. PA added). The total volume of final solution in each vial was 3.00 mL. After the addition of PA, the vials were quickly capped and wrapped with Parafilm. The reaction was allowed to proceed to completion for at least 5 half-lives for the slowest reaction (Cbl(II) + 0.25 mol equiv. PA). The product solutions were transferred to an air-free cuvette and equilibrated at 25.0 °C for at least for 15 min prior to UV–vis measurements.

For the stoichiometry of the reaction between $Cbl(I)^-$ and PA, 200 μM $Cbl(I)^-$ was reacted with varying mol equiv. of PA (0, 1.0–5.0 mol equiv.). All other procedures were similar to that for the Cbl(II)/PA system.

Indooxine test to determine if hydroxylamine is formed in the reaction of Cbl(II) with PA

A modified literature procedure was used.[31,40,41] The reaction of Cbl(II) (1.00×10^{-3} M) and 2.2 mol equiv. PA at pH 10.00 (0.10 M carbonate buffer) was allowed to proceed to completion under anaerobic conditions inside the glove box for ~3.5 h. Aerobic 8-hydroxyquinoline and Na_2CO_3 were added and the UV–vis spectrum recorded as for the Cbl(II) + AS system.[31] The product solution contained ~6% NH_2OH which was found to originate from the commercially available PA itself (see below).

Our lab and others have shown that at acidic pH conditions, NH_2OH can undergo disproportionation.[36,42,43] The stability of authentic NH_2OH in pH 10.00 buffer was therefore checked using the same procedure. NH_2OH (100 μM) was added to the carbonate buffer (0.10 M) and the solution was kept inside the glove box overnight. The indooxine test was carried out after 12 h. About 8% of the NH_2OH had decomposed.

Indooxine test with commercially available PA

The indooxine test was carried out on PA itself at pH 10.00, using the procedure described above. Commercially available PA was found to contain ~6% NH_2OH as an impurity.

Indooxine test of the product solution of the reaction between PA (HNO) and NH_2OH

An indooxine test of an equilibrated solution (2.5 h) of NH_2OH (50.0 μM) with 1.1 mol equiv. PA at pH 10.00 under anaerobic conditions shows that NH_2OH is stable in the presence of HNO under these conditions, although under other experimental conditions HNO reacts with NH_2OH to form $N_2 + H_2O$.[44]

Nessler's test to determine whether ammonia is formed in the reaction of Cbl(II) and $Cbl(I)^-$ with PA

The reaction between Cbl(II) (1.00×10^{-3} M) and 2.2 mol equiv. PA at pH 10.00 (0.10 M carbonate buffer) was allowed to proceed to completion in a vial for ~3.5 h inside the glove box. The product solution (1.00 mL) was taken outside the glove box and 8–10 drops of Nessler's reagent added. A positive result for NH_3 is indicated by a yellow or brown (at high concentrations) coloring in the reaction solution.[45] In this case, no brown or yellow coloring was observed above the pink color of the HOCbl complex. A similar procedure showed that there was no detectable formation of ammonia from the reaction between $Cbl(I)^-$ and 3.0 mol equiv. PA at pH 10.00.

Griess test to determine whether nitrite is formed in the reaction of Cbl(II) and $Cbl(I)^-$ with PA

Standard procedures for the Griess assay[46] were used to determine if nitrite is formed in the reaction of Cbl(II) with 2.0 mol equiv. PA and that of $Cbl(I)^-$ with 3.0 mol equiv. PA at pH 10.00 under anaerobic conditions. Negative results were obtained for both systems indicating the absence of nitrite as a reaction product.

Reaction of $Cbl(I)^-$, Cbl(II) and NOCbl with benzenesulfinate

Cbl(II) (50 μM) and $Cbl(I)^-$ (200 μM) were independently reacted with excess sodium benzenesulfinate (20.0 mol equiv.) overnight or for 2 h, respectively, under anaerobic conditions. No spectral changes were observed by UV–vis spectroscopy. Similarly, no reaction was observed between NOCbl and sodium benzenesulfinate.

Results and discussion

The reaction of cob(II)alamin (Cbl(II)) with Piloty's acid (PA)

Kinetic studies on the reaction between Cbl(II) and PA at different pH conditions were carried out using UV–vis spectroscopy. Decomposition of PA requires deprotonation ($pK_a(C_6H_5SO_2NHOH)$ = 9.29 (ref. 20)); hence the decomposition is slow at neutral pH, increases from pH 8–10.5 and becomes essentially pH independent at pH > 10.5.[20] Upon the addition of excess PA (1.00×10^{-3} M) to Cbl(II) (5.00×10^{-5} M) in buffer (pH 10.00) under strictly anaerobic conditions, Cbl(II) is cleanly converted to nitroxylcobalamin (nitrosylcobalamin, NOCbl, NO^-–Cbl(III); λ_{max} = 289, 315 and 478 nm (ref. 39 and 47–49)) with sharp isosbestic points occurring at 330, 377, 479 and 541 nm, Fig. 2. These values are in agreement with literature values for the Cbl(II)/NOCbl conversion.[47,48] The corresponding plot of absorbance at 510 nm *versus* time given in the inset to Fig. 2 is essentially linear (0–5 min), which indicates that the rate determining step is independent of the Cbl(II) concentration. This suggests that the rate-determining step is the decomposition of PA to give HNO (and $C_6H_5SO_2^-$), followed by a rapid reaction between HNO and Cbl(II). This was confirmed by obtaining kinetic data for the reaction of Cbl(II) (1.00×10^{-4} M) with 1.0 mol equiv. PA at pH 10.00, which fitted very well to a first-order reaction, Fig. S1, ESI.† The observed rate constant (k_{obs}) is similar to the rate constant for PA decomposition to HNO and $C_6H_5SO_2^-$ (k_L) under the same pH conditions (k_{obs} = $(3.20 \pm 0.01) \times 10^{-4}$ s^{-1} and k_L = $(3.65 \pm 0.01) \times 10^{-4}$ s^{-1} respectively; see Table 1). Similar experiments at other pH values (pH 8.00–12.00) showed that NOCbl is also formed upon reacting Cbl(II) with PA at these pH conditions and PA decomposition remains the rate-determining step for the reaction (Table 1 and Fig. S2–S8, ESI†). At pH ≤ 7 decomposition of PA to HNO is very slow ($t_{1/2}$ ~ 4 days at pH 7 (ref. 20)).

Table 1 Observed rate constants for the spontaneous decomposition of PA (k_L) and the reaction of Cbl(II) with 1.0 mol equiv. PA (k_{obs}) as a function of pH (25.0 °C, 0.10 M phosphate buffer, I = 1.0 M ($NaCF_3SO_3$)) under anaerobic conditions. The values of k_L agree well with values reported in the literature[20]

pH (±0.02)	Literature value for $10^4 \times k_L$ (s^{-1})	Experimental value for $10^4 \times k_L$ (s^{-1})	$10^4 \times k_{obs}$ (s^{-1})
12.00	4.22	3.98 ± 0.01	3.93 ± 0.01
10.00	3.47	3.65 ± 0.01	3.20 ± 0.01
9.00	1.28	1.57 ± 0.01	1.50 ± 0.01
8.60	0.68	0.50 ± 0.01	0.58 ± 0.01
8.00	0.20	0.13 ± 0.01	0.15 ± 0.01

In order to probe the mechanism of the reaction, the stoichiometry of the reaction between Cbl(II) and PA at pH 10.00 was determined. From UV–vis spectra of equilibrated solutions of Cbl(II) (50.0 μM) with PA (0–3.5 mol equiv.; Fig. S9, ESI†), a plot of absorbance at 355 nm *versus* mol equiv. PA was generated, Fig. 3. The absorbance at 355 nm increases linearly up to 2.0 mol equiv. PA and is unchanged upon the further addition of PA, consistent with a stoichiometry of 1 : 2 Cbl(II) : PA. A similar conclusion was reached by plotting absorbance data at 312 or 510 nm *versus* mol equiv. PA, Fig. S10, ESI.† NOCbl and benzenesulfinate were confirmed by ^{1}H NMR spectroscopy as the products of the reaction of anaerobic Cbl(II) with 2.2 mol equiv. PA at pD 10.00, Fig. 4.[49,50]

Given that NOCbl has a single NO^- ligand, a 1 : 1 Cbl(II) : PA stoichiometry was expected. However Fig. 3 clearly shows a stoichiometry of 1 : 2 Cbl(II) : PA. This stoichiometry suggests that the reaction occurs *via* multiple steps. A stoichiometry of 1 : 2 Cbl(II) : HNO donor was also found for the reaction between Cbl(II) and AS.[31] Control experiments showed that the $C_6H_5SO_2^-$ byproduct from PA decomposition, eqn (2), does not

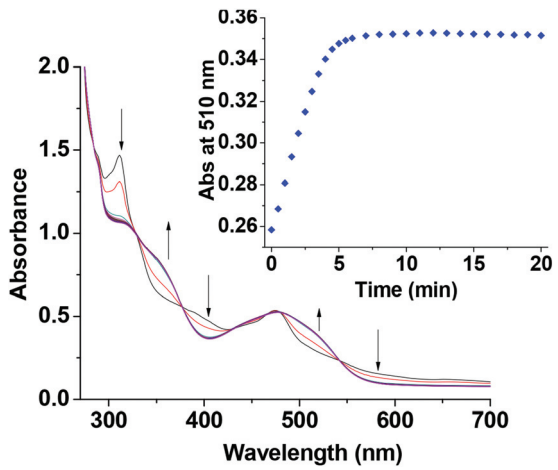


Fig. 2 UV–vis spectra for the reaction between Cbl(II) (5.00×10^{-5} M) and excess PA (1.00×10^{-3} M) at pH 10.00 ± 0.02 (25.0 °C, 0.30 M CAPS buffer, I = 1.0 M ($NaCF_3SO_3$)) under strictly anaerobic conditions. Inset: plot of absorbance at 510 nm *versus* time for the same reaction.

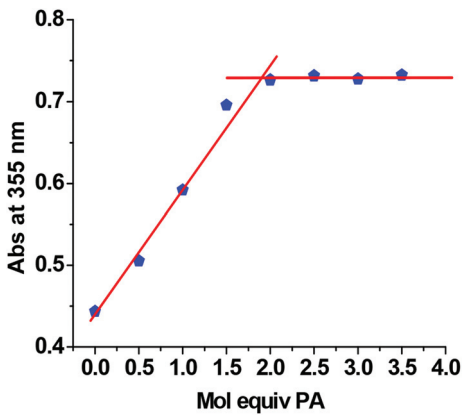


Fig. 3 Plot of absorbance at 355 nm *versus* mol equiv. PA for the reaction of Cbl(II) (50.0 μM) with PA (0–3.5 mol equiv.) at pH 10.00 (25.0 °C, 0.10 M phosphate buffer) under strictly anaerobic conditions. Spectra are given in Fig. S9 in the ESI.†

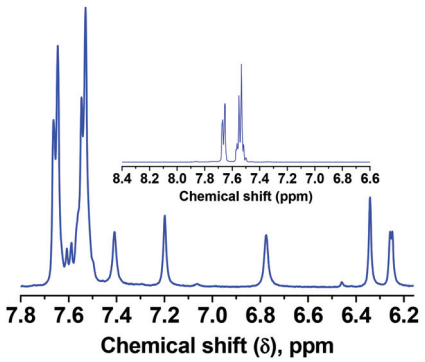
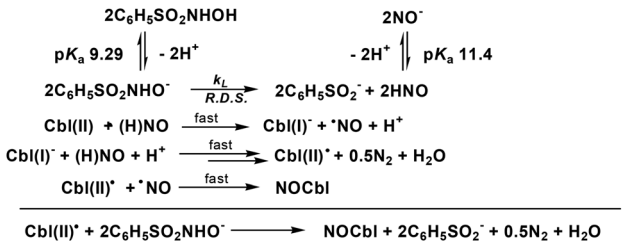


Fig. 4 Aromatic region of the ^{1}H NMR spectrum of the products of the reaction between Cbl(II) (6.04×10^{-3} M) and 2.2 mol equiv. PA at pD 10.00 (0.10 M carbonate buffer) under anaerobic conditions. The peaks at 7.42, 7.20, 6.79, 6.35 and 6.25 ppm correspond to NOCbl and those at 7.66, 7.65, 7.55 and 7.53 ppm correspond to benzenesulfinate. Inset: ^{1}H NMR spectrum (aromatic region) for authentic sodium benzenesulfinate (δ = 7.67, 7.65, 7.55 and 7.53 ppm).



Scheme 1 Proposed reaction pathway for the reaction of Cbl(II) with PA.

react with Cbl(I)$^-$, Cbl(II) or NOCbl (see Experimental section). It is well established that HNO and HNO donors coordinate to metal centers[21–23] and/or reduce M^{n+} to $M^{(n-1)+}$ (HNO is oxidized to $^{\bullet}$NO).[24–29] The redox potentials (E°, NHE) for the $^{\bullet}$NO, H^+/HNO (pH 10) and Cbl(II)/Cbl(I)$^-$ redox couples are −0.77 V (ref. 2) and −0.61 V (ref. 51) respectively; hence reduction of Cbl(II) by (H)NO to Cbl(I)$^-$ and $^{\bullet}$NO is thermodynamically feasible. (Importantly, although HNO is the predominant species in solution at pH 10 (~95%), since ^{3}NO$^-$ is a stronger reducing agent, it is also possible that NO$^-$, not HNO, reduces Cbl(II) to Cbl(I)$^-$. This will be discussed in more detail below). Note that Cbl(II) does not react with the product of HNO dimerization or the product of the reaction of HNO and ^{3}NO$^-$,[17] N_2O.[31] Although $^{\bullet}$NO cannot react with Cbl(I)$^-$ to give the observed NOCbl product (Cbl(I)$^-$ + $^{\bullet}$NO + H^+ → Cbl(II)$^{\bullet}$ + $^1/_2N_2O$ + $^1/_2H_2O$[52]), as for the Cbl(II) + AS system,[31] Cbl(I)$^-$ could be oxidized back to Cbl(II) by a second (H)NO molecule, consistent with the observed 1 : 2 Cbl(II) : PA stoichiometry, Scheme 1. Cbl(II), a radical species, may then subsequently rapidly react with $^{\bullet}$NO to form NOCbl ($k = 7.4 \times 10^8$ M^{-1} s^{-1}, $K_{(NOCbl)} \approx 1 \times 10^8$ M^{-1}, 25 °C (ref. 47 and 48)). Scheme 1 assumes N_2 is formed, which will be shown later to be the likely product of (H)NO oxidation by Cbl(I)$^-$. Oxidation of NAD(P)H, ascorbate, nitrosothiols and thiols by HNO has also been observed, in which HNO is reduced to NH_2OH.[6,44,53]

The reaction of Cbl(I)$^-$ with PA

In order to determine whether Cbl(I)$^-$ is a viable intermediate of the reaction, the reaction between Cbl(I)$^-$ and PA was directly investigated. UV–vis spectra for the reaction between Cbl(I)$^-$ and excess PA at pH 10.0 under strictly anaerobic conditions once again showed that NOCbl is ultimately formed. Fig. 5a shows that the reaction proceeds *via* a Cbl(II) intermediate (the isosbestic points at 347, 417 and 542 nm agree well with values for the conversion of Cbl(I)$^-$ to Cbl(II)[36,54]). Cbl(II) subsequently reacts further at longer times to give NOCbl, Fig. 5b (the isosbestic points at 377, 488 and 546 nm are consistent with Cbl(II)/NOCbl conversion[47,48]). The complete spectra for this reaction are given in Fig. S11, ESI.† Further

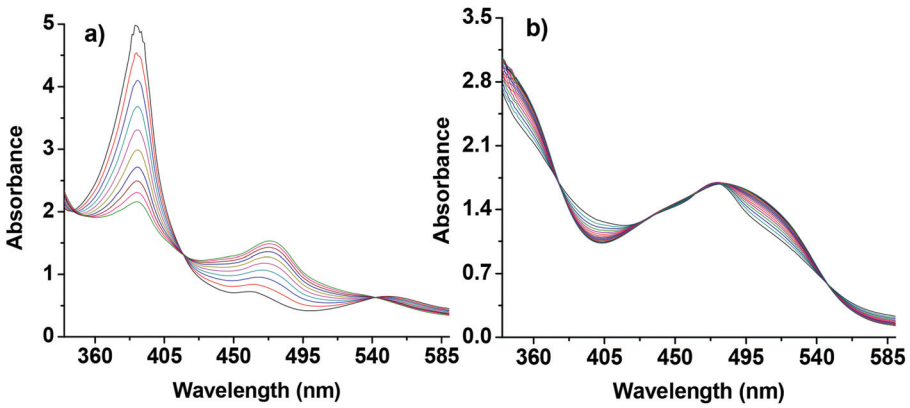


Fig. 5 UV–vis spectra obtained as a function of time for the reaction between Cbl(I)$^-$ (2.00×10^{-4} M) and 5.0 mol equiv. PA at pH 10.00 under strictly anaerobic conditions (25.0 °C, 0.01 M carbonate buffer, I = 1.0 M ($NaCF_3SO_3$)). (a) The first 10 spectra (spectra recorded every 0.5 min) show the formation of the Cbl(II) intermediate. (b) Selected spectra at longer reaction times (10–70 min) showing the conversion of Cbl(II) to the NOCbl product.

confirmation that the reaction proceeds *via* a Cbl(II) intermediate was obtained by recording UV–vis spectra as a function of time for the reaction of Cbl(I)$^-$ with 1.0 mol equiv. PA at pH 10.00. In this case there is insufficient PA for the second reaction to occur and Cbl(I)$^-$ is cleanly converted to Cbl(II) (λ_{max} = 405 and 475 nm (ref. 36 and 54)), Fig. S12, ESI.†

To determine the rate constant for the oxidation of Cbl(I)$^-$ to Cbl(II), a large excess of Cbl(I)$^-$ compared with PA was used, to ensure no contribution to the observed rate constant from the subsequent reaction of Cbl(II) with (H)NO. Fig. S13 and S14 in the ESI† show spectra and a plot of absorbance at 475 nm *versus* time for the reaction between Cbl(I)$^-$ (2.00×10^{-4} M) and PA (2.00×10^{-5} M). The first-order fit of the data gives $k_{obs} = (2.38 \pm 0.01) \times 10^{-4}$ s^{-1}, which is similar to the rate constant for the anaerobic decomposition of PA at pH 10.00 ($(3.27 \pm 0.01) \times 10^{-4}$ s^{-1}, Fig. S15, ESI†); hence the rate determining step for the reaction between Cbl(I)$^-$ and PA at pH 10.00 is once again the decomposition of PA to HNO and $C_6H_5SO_2^-$.

If indeed Cbl(I)$^-$ is first oxidized by 1.0 mol equiv. (H)NO to Cbl(II), and Cbl(II) subsequently reacts further with (H)NO (1 : 2 Cbl(II) : PA) to ultimately form NOCbl, one would expect a 1 : 3 Cbl(I)$^-$: PA stoichiometry for the reaction of Cbl(I)$^-$ with PA. This was confirmed experimentally, Fig. 6. Spectra are given in Fig. S16a, ESI.† A 1 : 3 stoichiometry was also obtained by analyzing the data at 362 nm, Fig. S16b, ESI.† Additional experiments confirmed that Cbl(I)$^-$ is an intermediate of the reaction between Cbl(II) and PA. Specifically, previous studies from our lab have shown that Cbl(I)$^-$ is rapidly oxidized by excess nitrite at pH 10.0 to Cbl(II) (+NH_2OH; HNO_2 is the oxidant;[36] pK_a(HNO_2) ~ 3.2 (ref. 55)), and that Cbl(II) does not react with NO_2^- in alkaline solution.[47] If indeed Cbl(I)$^-$ is the intermediate and Scheme 1 is correct, the complete conversion of Cbl(II) to NOCbl in the presence of excess nitrite should require only 1.0 mol equiv. HNO, since nitrite can replace HNO as the oxidant of the Cbl(I)$^-$ intermediate, Scheme 2. This was confirmed experimentally (50.0 mol equiv. nitrite, Fig. 7). Furthermore, the stoichiometry of the reaction between Cbl(I)$^-$ and PA should also be 1 : 1 in the presence of excess

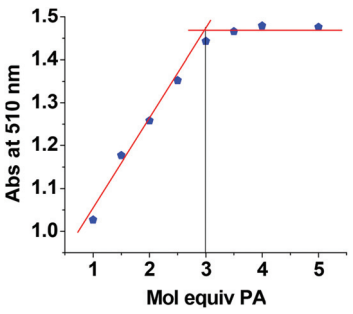
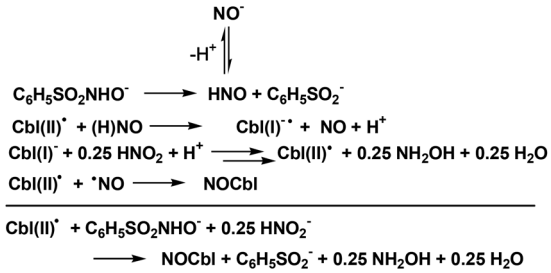


Fig. 6 Plot of absorbance at 510 nm *versus* mol equiv. PA for equilibrated solutions of Cbl(I)$^-$ (200 μM) with PA (0, 1.0–5.0 mol equiv.) at pH 10.00 (0.01 M carbonate buffer) under anaerobic conditions. Spectra are shown in Fig. S16a, ESI.†



Scheme 2 Proposed reaction pathway for the reaction between Cbl(II) and PA in the presence of excess NO_2^-.

nitrite. Indeed Fig. S17 in the ESI† shows that Cbl(I)$^-$ is completely converted to NOCbl upon the addition of 1.0 mol equiv. PA in the presence of 50.0 mol equiv. NO_2^-. Finally, experiments showed that Cbl(III) does not react with 1.1 mol equiv. PA to form NOCbl in the presence of 50.0 mol equiv. nitrite, Fig. S18, ESI.† This result is anticipated because Cbl(III) exists as the substitution-inert form hydroxycobalamin, HOCbl (pK_a(H_2OCbl^+/HOCbl) = 7.8 (ref. 56)), not as aquacobalamin (H_2OCbl^+), under the alkaline pH conditions. Hence all three experiments in the presence of excess nitrite support Cbl(I)$^-$, not Cbl(III), being the Cbl reaction intermediate.

Experiments were carried out to identify the nitrogen products from (H)NO reduction. If Cbl(I)$^-$ is oxidized by (H)NO to Cbl(II), then possible (H)NO reduction products are NH_2OH, NH_4^+, and/or N_2. Although others have shown that NH_2OH can disproportionate[42,43] or react directly with HNO to form N_2 and H_2O,[44] control experiments using the indooxine test to determine the NH_2OH concentration under our alkaline experimental conditions showed that NH_2OH is stable at pH 10.00 (10 mM carbonate buffer), and that NH_2OH does not react with (H)NO produced from the decomposition of PA,[44] Experimental section. The indooxine test of the products of the reaction between Cbl(II) and 2.2 mol equiv. PA unexpectedly showed that 0.06 mol equiv. NH_2OH was formed, which was subsequently found to be originated from PA itself (see Experimental section; the standard method to synthesize PA is by reacting benzenesulfonyl chloride with NH_2OH[38]). Both the Griess test for NO_2^- and the Nessler's test for NH_3 were negative (see Experimental section). Hence N_2 is the likely product of (H)NO reduction by Cbl(I)$^-$. The Nessler's test for NH_3 and the Griess test for NO_2^- were also both negative for the reaction of Cbl(I)$^-$ with 3.0 mol equiv. PA at pH 10.00, suggesting again that N_2 is the only nitrogen-containing product from the reaction of Cbl(I)$^-$ with PA. Attempts to detect nitrogen in the product mixture of the reaction of Cbl(II) with 1.8 mol equiv. ^{15}N-PA at pH 10.00 by mass spectrometry headspace gas analysis were unfortunately unsuccessful. (Note that slightly less than the stoichiometric amount of ^{15}N-PA was used to ensure that all HNO was converted to $^{15}N_2$ rather than $^{15}N_2O$; the latter species was also not detected.) Attempts to detect N_2 (headspace gas of the Cbl(II) + PA reaction) using Raman spectroscopy were also unfortunately unsuccessful. Subsequent control experiments

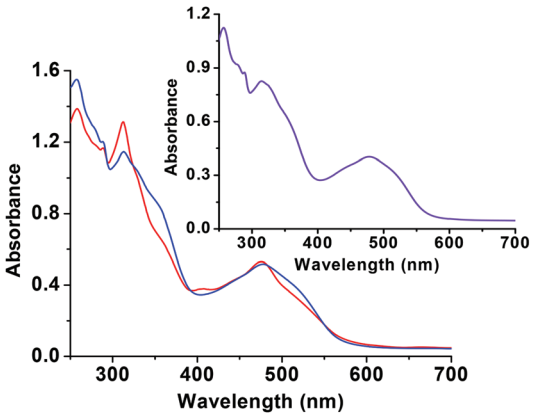


Fig. 7 UV–vis spectrum of the product mixture of the reaction between Cbl(II) (50.0 μM) with 1.0 mol equiv. PA in the absence and presence of 50.0 mol equiv. NO_2^- at pH 10.00 (25.0 °C, 0.10 M carbonate buffer) under anaerobic conditions. The inset shows the spectrum for authentic NOCbl. 1 : 1 Cbl(II) : PA reaction gives incomplete formation of NOCbl (red trace) whereas 1 : 50 : 1 Cbl(II) : NO_2^- : PA results in the complete formation of NOCbl (blue trace). In other words, 1 : 2 Cbl(II) : PA stoichiometry can be changed to 1 : 1 Cbl(II) : PA in the presence of excess NO_2^-. A small bump appears at ~350 nm because NO_2^- strongly absorbs in 300–400 nm region.

showed that the low concentrations of N_2 or $^{15}N_2$ (from ^{15}N-PA) formed using the highest concentrations of reagents possible were still insufficient for detection by the available instrumentation. Note that since NH_2OH was shown to be stable in the presence of HNO under our experimental conditions, and that the indooxine test for NH_2OH of the product mixture was negative, it seems unlikely that NH_2OH is a reaction intermediate. One possible route for obtaining N_2 from the reduction of (H)NO by Cbl(I)$^-$ is *via* aminoxyl radical ($NH_2O^\bullet$) formation.[31] The reduction of HNO to $NH_2O^\bullet$ by Cbl(I)$^-$ is a thermodynamically favorable process (E° (HNO, H^+/NH_2O) = +0.6 V *versus* NHE[57] and the Cbl(II)/Cbl(I)$^-$ redox couple is −0.61 V (ref. 51)), and subsequent dimerization of $NH_2O^\bullet$ to generate N_2 and $2H_2O$ is rapid ($k = 1.4 \times 10^8$ M^{-1} s^{-1}).[58]

An important point mentioned briefly above is that since the rate-determining step of the reaction is decomposition of PA to form HNO (and $C_6H_5SO_2^-$), it is not possible to obtain kinetic information on the rapid reactions of either Cbl(II) or Cbl(I) with (H)NO, and therefore elucidate whether HNO and/or NO^- are the reactive species. At pH 10 HNO is the predominant species in solution (~95%; $pK_a(^1HNO/^3NO^-) \sim 11.4$) and spin-forbidden deprotonation of HNO to form $^3NO^-$ is comparatively slow ($t_{1/2} \sim 0.1$ s at pH 10 (ref. 1)); however at pH 12 $^3NO^-$ predominates in solution and the deprotonation of HNO to NO^- is much faster ($t_{1/2} \sim 1$ ms). The $^3NO^-$, $H^+/^\bullet NO$ redox potential is also much lower than that for $HNO/^\bullet NO$, H^+; hence NO^- is the stronger reducing agent as expected. However one would expect the oxidation of Cbl(I)$^-$ by HNO to be more thermodynamically favorable compared to NO^-. Future studies aimed at probing these reactions further using HNO donor molecules which rapidly release HNO are certainly worthwhile, particularly given the novelty of the cob(I)alamin plus (H)NO reaction.

Finally, in Scheme 1 we have assumed that Cbl(I)$^-$ is oxidized by (H)NO. However others have reported that the product of the rapid dimerization of HNO, N_2O, also rapidly oxidizes Cbl(I)$^-$ to Cbl(II) ($2HNO \rightarrow N_2O + H_2O$; $k = 1.6 \times 10^2$ M^{-1} s^{-1}; pH 8).[59] As discussed earlier, given that N_2O does not react with Cbl(II), (H)NO, not N_2O, must reduce Cbl(II) to Cbl(I)$^-$. Therefore the observed rate of HNO dimerization is ≥5 times slower than the reduction of Cbl(II) by (H)NO, since both of these (H)NO-consuming reactions compete with each other. Complete conversion of Cbl(II) to NOCbl requires the addition of 2.0 mol equiv. PA (= 2HNO), whereas upon the addition of 1.0 mol equiv. PA, the product mixture consists of a 1 : 1 mixture of unreacted Cbl(II) and NOCbl. Importantly, experiments in the presence of nitrite provide strong support for Cbl(I))$^-$, not cob(III)alamin (H_2OCbl^+/HOCbl) being the reaction intermediate, since in the presence of excess nitrite 1 : 1 Cbl(II) : PA results in complete formation of NOCbl (nitrite replaces (H)NO as the oxidant of Cbl(I)$^-$), whereas NOCbl is not formed upon reacting 1 : 1 Cbl(III) : PA. However given that the Cbl(I)$^-$ is never observed experimentally, all reactions subsequent to the reduction of Cbl(II) by (H)NO must also therefore be significantly faster than the reduction of Cbl(II) by (H)NO. Hence oxidation of Cbl(I)$^-$ by N_2O is unlikely, since formation of N_2O first requires (slow) HNO dimerization (or the reaction of HNO with NO^-), and would result in observation of the Cbl(I)$^-$ intermediate upon the addition of 1.0 mol equiv. PA, which is clearly not experimentally observed. Furthermore, upon the direct addition of 1.0 mol equiv. PA (= (H)NO) to Cbl(I)$^-$, complete conversion to a Cbl(II) intermediate is observed, which subsequently reacts to ultimately yield NOCbl upon the addition of more PA. This means that the oxidation of Cbl(I)$^-$ by the nitrogen oxide species (N_2O or (H)NO) is at least 5 times faster than the reduction of Cbl(II) by HNO, which is again consistent with (H)NO, not N_2O, oxidizing Cbl(I)$^-$.

Conclusions

To summarize, kinetic and mechanistic studies have been carried out on the reaction of Cbl(II) with the HNO donor Piloty's acid, to form NOCbl, $C_6H_5SO_2^-$, and most likely N_2. A stoichiometry of 1 : 2 Cbl(II) : PA was observed. The rate-determining step involves PA decomposition to give HNO and benzenesulfinate. (H)NO then reduces Cbl(II) to Cbl(I)$^-$ and is itself oxidized to $^\bullet NO$. The Cbl(I)$^-$ intermediate is, in turn, oxidized back to Cbl(II) by a second molecule of (H)NO and the Cbl(II) and $^\bullet NO$ radicals subsequently rapidly combine to form NOCbl. The reaction between Cbl(I)$^-$ and PA involves an additional step in which Cbl(I)$^-$ is first oxidized by (H)NO to Cbl(II), which reacts further with (H)NO. Experiments in the presence of nitrite and kinetic and stoichiometric data for the reaction of Cbl(I)$^-$ with PA confirm the involvement of a Cbl(I)$^-$ intermediate. This system serves as the second example of oxidation of cob(I)alamin by (H)NO. Given the abundance of

metals in biological systems in addition to about one-third of proteins being metalloproteins, our results may have important implications in regards to elucidating the potential roles and toxicity of HNO in biological systems.

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