MECHANISTIC STUDIES ON THE REACTION OF COB(I)ALAMIN AND NITRITE

A thesis submitted to the
Kent State University Honors College
in partial fulfillment of the requirements
for University Honors

by
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May, 2011
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ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Nicola Brasch for all of her support and
guidance along the way. She was instrumental in making this experience a good one. I
would also like to thank my graduate mentor, Dr. Hanaa Hassanin for helping me get
started. I would also like to thank my thesis committee, Dr. Doug Kline, Dr. Brett
Ellman, and Dr. Edwin Gould. I am extremely grateful to the Brasch Lab, Dr. Riya
Mukherjee, Harishchandra Subedi, Rohan Dassanayake, Jeffery Brown, David Walker,
and Brian Smith. Finally, I would like to acknowledge and thank the NSF for funding this
work and making this experience possible.
I. INTRODUCTION

Reactive molecules can pose many problems in the delicate balance of biological systems. Of particular importance are reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can cause cellular oxidative and nitrosative stress. The most abundant ROS include superoxide ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$), and hydroxyl radical (HO$^-$) and RNS include NO$_2^-$, ONOO($\text{H}$), and NO$_2^-$.$^{1-3}$

'NO is produced from L-arginine, which is enzymatically converted to L-citrulline by nitric oxide synthase.$^{4-6}$ 'NO is an important signaling molecule in biology that is implicated in vasodilation and neurotransmission at nanomolar (nM) concentrations.$^7$ Additionally, higher concentrations of 'NO are known to occur as part of the immune response to inflammation caused by infection.$^8,9$ 'NO is oxidized to NO$_2^-$, which can be further oxidized to NO$_3^-$. The production of NO$_2^-$ and NO$_3^-$ is used as an indicator of the production of 'NO.$^{10-14}$ NO$_2^-$ concentrations are high in plasma and tissue, which, under normal conditions, contain up to 0.5 and 10 μM NO$_2^-$ respectively.$^8,15$ During sepsis, serum levels of NO$_3^-$ and NO$_2^-$ are significantly elevated.$^{16}$ NO$_2^-$ may also be produced by the reduction of NO$_3^-$, which is present in significantly higher quantities than NO$_2^-$ or 'NO.$^{17}$ NO$_3^-$ is reduced by mammalian enzymes possessing nitrate reductase activity or by bacteria in the mouth and intestines possessing nitrate reductase.$^8$ Indeed, mounting evidence suggests that NO$_2^-$ may function as a biological signaling molecule and play an important role in vasodilation and intravascular endocrine 'NO transport.$^{18}$
Vitamin B\textsubscript{12} derivatives, known as cobalams (Cbl), are essential nutrients synthesized only by microorganisms and obtained by higher organisms through the diet.\textsuperscript{19,20} The three biologically relevant oxidation states of cobalams are Cbl(I), Cbl(II), and Cbl(III), in which the cobalt metal center is in the +1, +2, and +3 oxidation state, respectively. Oxidation-reduction processes play an important role in the biochemistry of cobalamin.\textsuperscript{21} Insufficient Cbl in the metabolism is associated with neurological disorders and pernicious anemia.\textsuperscript{19} The general structure of Cbl(III) (Figure 1) consists of an octahedral cobalt center coordinated to a corrin ring, a nucleotide base, and any of a

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cobalamin_structure.png}
\caption{Structure of cob(III)alamins. The $\beta$-axial site (indicated by the X) can be occupied by a variety of ligands. X = CN: cyanocobalamin; CH\textsubscript{3}: methylcobalamin (CH\textsubscript{3}Cbl); 5’-deoxyadenosyl: adenosylcobalamin (AdoCbl); H\textsubscript{2}O: aquacobalamin; HO\textsuperscript{-}: hydroxocobalamin (HOCbl); NO: nitrosylcobalamin; NO\textsubscript{2}: nitrocobalamin; glutathione: glutathionylcobalamin.\textsuperscript{20,21}}
\end{figure}
variety of ligands in the upper β-axial position. The major form of cobalamin found in cells is the oxygen-sensitive, pentacoordinate Cbl(II) complex, which lacks the ligand in the upper β-axial site.\textsuperscript{21,22} Cbl(I), which is far more sensitive to oxidation than Cbl(II), lacks both axial ligands. Hence, changes in oxidation states of cobalamin is accompanied by changes in the number of axial ligands associated with the complex.\textsuperscript{21}

There are two well-characterized B\textsubscript{12}-dependent enzyme reactions in mammals, which involve cycling of the Cbl oxidation state. The first utilizes adenosylcobalamin (AdoCbl) as an enzyme cofactor in the methylmalonyl-CoA mutase (MMCM) reaction (Scheme 1). The Cbl is cycled between AdoCbl with the 5’-deoxyadenosyl bound to the cobalt at the β-axial site and Cbl(II) with the 5’-deoxyadenosyl unbound.\textsuperscript{20,21} The methionine synthase reaction is the other B\textsubscript{12}-dependent reaction in mammals (Scheme 2). The Cbl cycles between methylcobalamin (CH\textsubscript{3}Cbl) and Cbl(I), catalyzing the transfer of a methyl group from methylenetetrahydrofolate to homocysteine to form the amino acid methionine.\textsuperscript{20} Cbl(I) very easily oxidized to Cbl(II), and under microaerophilic conditions it is known that for ~2000 turnovers of the methionine synthase catalytic cycle, Cbl(I) is oxidized to Cbl(II).\textsuperscript{20,21}

\begin{align*}
\text{MMCM} & \quad \text{AdoCbl} \\
\begin{array}{c}
\text{L-Methylmalonyl-CoA} \\
\text{Succinyl-CoA}
\end{array}
\end{align*}

**Scheme 1**: L-methylmalonyl-CoA is isomerized to succinyl-CoA by the AdoCbl-dependent enzyme methylmalonyl-CoA mutase (MMCM). The Cbl cycles between AdoCbl and Cbl(II).\textsuperscript{20,21}
undergoes oxidation to Cbl(II).\textsuperscript{20,21} In order to complete the cycle, Cbl(II) must then be reduced by flavodoxin prior to methyl donation from S-adenosylmethionine to reform CH\textsubscript{3}Cbl (Scheme 2).

**Scheme 2:** The B\textsubscript{12}-dependent methionine synthase reaction, in which methyltetrahydrofolate methylates homocysteine to give methionine. The process involves the cycling of Cbl between CH\textsubscript{3}Cbl and Cbl(I). Under microaerophilic conditions, Cbl(I) is oxidized to Cbl(II) every ~2000 enzyme turnovers. Reactivation involves flavodoxin, S-adenosylmethionine, and methionine synthase reductase.\textsuperscript{20}

Cbl is taken up and transported by three B\textsubscript{12} binding proteins – intrinsic factor (IF), transcobalamin (TC), and haptocorrin (HC).\textsuperscript{21} These proteins are associated with membrane receptor proteins that allow intracellular uptake of the B\textsubscript{12} to ultimately deliver it to the methionine synthase and MMCM enzymes. Chronic inflammation is associated with elevated levels of TC\textsuperscript{23} and its membrane receptor.\textsuperscript{24} Thus, B\textsubscript{12} is thought to
participate in the cellular response to inflammation. It has also been suggested that B12 has antioxidant properties in part because of its ability to regulate cytokines.$^{25}$

ROS and RNS are implicated in numerous diseases and health conditions, including septic shock, ischemic / reperfusion injury, atherosclerosis, and asthma.$^{26-29}$ These conditions are generally associated with inflammation. Oxidative stress is known to produce a cellular redox imbalance, a common occurrence in several types of cancer.$^{30}$ Additionally, megaloblastic anemia is associated with elevated levels of $^\text{\textbullet} \text{NO}$,$^{31}$ and plasma levels of NO$_2^-$ and NO$_3^-$ are reportedly significantly elevated in patients with vitiligo,$^{32}$ a skin pigmentation disorder. It has been reported that vitamin B$_{12}$ can restore normal levels of $^\text{\textbullet} \text{NO}$ in patients with megaloblastic anemia.$^{31}$

In addition to playing a key role in the methionine synthase reaction, Cbl(I) is a transient intermediate in the biosynthesis of both CH$_3$Cbl and AdoCbl.$^{20}$ Importantly both mammalian B$_{12}$-dependent enzyme reactions are inactivated under oxidative / nitrosative stress conditions.$^{33}$ The purpose of this study is to determine whether oxidation of Cbl(I) by the RNS NO$_2^-$ could occur under oxidative / nitrosative stress conditions. The mechanism and rate constants of the reaction of Cbl(I) with NO$_2^-$ / HNO$_2$ have been determined by studying the kinetics of the reaction under varying conditions, determining the reaction stoichiometry, and elucidating the Cbl and non-Cbl products of the reaction.
II. EXPERIMENTAL SECTION

2.1 Chemicals

Hydroxycobalamin hydrochloride, HOCbl·HCl(nH2O) (≥ 96%, 10-15% water, batch dependent) was purchased from Fluka. BIS-TRIS and TAPS buffers were purchased from Sigma. Sodium borohydride (NaBH₄, ≥ 98%), anaerobic acetone (99.8%), CAPS, CHES, and MES buffer, sodium nitrite (NaNO₂, 99.6%), sodium hydroxide (NaOH), and hydroxylamine hydrochloride (NH₂OH·HCl, ≥ 97%) were purchased from Acros. Disodium hydrogen phosphate (Na₂HPO₄), sodium dihydrogen phosphate (NaH₂PO₄), sodium carbonate (Na₂CO₃), potassium cyanide (KCN, ≥ 99.1%), and 8-hydroxyquinoline (≥ 99%) were purchased from Fisher Scientific. Nessler reagent was purchased from RICCA Chemical. Water was purified using a Barnstead Nanopure Diamond water purification system.

2.2 Instrumental and Procedures

For all Cbl(I) experiments, anaerobic solutions were degassed by four freeze-pump-thaw cycles under argon gas using standard Schlenk techniques. Solutions prepared for Cbl(II) work were degassed by bubbling through argon for at least 2 hr. Preparation of all anaerobic solutions was carried out in an MBRAUN Labmaster 130 (1250/78) Glove box filled with argon, equipped with O₂ and H₂O sensors and a freezer at -24 °C. The pH measurements were done at room temperature using an Orion model
710A pH meter equipped with Mettler-Toledo Inlab 423 or 421 pH electrodes. The electrode was filled with a 3 M KCl / saturated AgCl solution (pH 7.0) and standardized with standard buffer solutions at pH 4.00, 7.00, 10.00, and 12.45. Solution pH was adjusted using H₃PO₄ or NaOH solutions as necessary.

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

2.3 Synthesis of Cbl(I)

Cbl(I) was synthesized under anaerobic conditions according to a modified published procedure. HOCbl·HCl (~25 mg, 1.6 x 10⁻⁵ mol (10-15 % H₂O)) was dissolved in anaerobic water (0.75 ml) in a vial. An aqueous, anaerobic stock solution of NaBH₄ (~10 mg, 2.6 x 10⁻⁴ mol, 1.00 mL) was prepared and NaBH₄ (6.0 mole equiv.) was added to the HOCbl·HCl. The vial was shaken vigorously for ~1 min and the reaction was allowed to occur for an additional 15 min. After the reaction was complete, the excess NaBH₄ was quenched by addition of acetone (0.200 mL) except when noted
otherwise. Note that for all experiments performed with the stopped-flow instrument, the excess NaBH₄ was not quenched in order to provide added stability to the Cbl(I) reagent solution. The product was characterized by UV-Vis spectrophotometry ($\lambda_{\text{max}}$ 280, 288, 386, 455, 545, 680 nm) (Figure 2) and stored under anaerobic conditions at -24 °C.

**Figure 2:** UV-Vis spectrum of Cbl(I) (50 µM) in anaerobic water (250 – 800 nm).

### 2.4 Synthesis of Cbl(II)

Cbl(II) was synthesized under anaerobic conditions according to a modified published procedure.³⁴ HOCbl·HCl (~25 mg, 1.6 x 10⁻⁵ mol (10-15 % H₂O)) was dissolved in anaerobic water (0.75 ml) in a vial under anaerobic conditions. An aqueous, anaerobic stock solution of NaBH₄ (~5 mg, 2.6 x 10⁻⁴ mol, 1.00 mL) was prepared and NaBH₄ (1.1 mole equiv.) was added to the HOCbl·HCl solution. The vial was shaken vigorously for ~1 min and the reaction was allowed to occur for an additional ~20 min.
Cbl(II) was characterized by UV-Vis spectrophotometry ($\lambda_{\text{max}}$ 267, 285, 315, 467 nm$^{35}$ (Figure 3), and solutions were stored under anaerobic conditions at -24 °C.

![UV-Vis spectrum of Cbl(II) (50 µM) in anaerobic water (250 – 800 nm).](image)

**Figure 3:** UV-Vis spectrum of Cbl(II) (50 µM) in anaerobic water (250 – 800 nm).

2.5 Determining Cobalamin Concentrations

The concentration of cobalamin in stock solutions was determined by converting the cobalamins to dicyanocobalamin, (CN)$_2$Cbl$^-$, with cyanide. Unless otherwise stated, the concentration determination was performed in triplicate under aerobic conditions, in order to convert all cobalamin species to (CN)$_2$Cbl$^-$. The reaction was allowed to occur for at least 2 hr and the stock solution was diluted for analysis by UV-Vis spectrophotometry. It was found that performing six replicate dilutions and allowing the reaction to occur over ~3 hr improved precision of the results. Additionally, maintaining dilution ratios less than 1:10 improved precision as well. UV-Vis spectra (250 – 800 nm)
were collected, and the absorbance at 368 nm was used to determine the concentration of the stock solution ($\varepsilon_{368} = 3.04 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$ for $(\text{CN})_2\text{Cbl}^{36}$.)

2.6 Attempts to Stabilize Cbl(I) by Precipitation

Cbl(I) was synthesized according to the procedure in Section 2.3. The product Cbl(I) stock solution was added drop wise to chilled acetone (~20 mL, -24 °C). The resulting precipitate was filtered using vacuum filtration on a microporous frit in the glove box. Attempts were also made to synthesize solid Cbl(I) by evaporating the solvent to dryness under vacuum on a Schlenk line. The solid Cbl(I) products were characterized by re-dissolving in water and obtaining a UV-Vis spectrum.

2.7 Determination of the Cbl Product of the Reaction of Cbl(I) with Nitrite

A phosphate buffer (0.050 M, pH 12.00) and a phosphate buffer containing NaNO$_2$ (30.03 mM) were prepared. Cbl(I) was synthesized according to the procedure described in Section 2.3 and the concentration (0.0122 M) was estimated by assuming the HOCbl·HCl contained 12 % H$_2$O. Cbl(I) (50 µM) was reacted with NO$_2^-$ (9.1 mM) in an anaerobic Schlenk cuvette, and UV-Vis spectra were collected every 2.5 min for a total of 40 min. The cuvettes used were equipped with a cavity where the Cbl(I) reagent was stored so the reactants could be combined immediately before beginning data collection.
2.8 Determining the Stoichiometry of the Reaction between Cbl(I) and Nitrite

The stoichiometry of the Cbl(I) + NO$_2^-$ reaction was determined in anaerobic CHES buffer ($5.0 \times 10^{-3}$ M, pH 9.51). A solution containing NaNO$_2$ ($2.96 \times 10^{-4}$ M) in CHES buffer ($5.0 \times 10^{-3}$ M, pH 9.51) was prepared. Cbl(I) was synthesized by dissolving HOCbl·HCl (50.26 mg) in anaerobic water (1.50 mL) and adding aqueous, anaerobic NaBH$_4$ (0.318 M, 0.608 mL, 6.0 mole equiv.) with shaking for ~1 min. The reaction was allowed to occur for ~15 min and excess NaBH$_4$ was quenched with anaerobic acetone (0.200 mL). The Cbl(I) concentration was determined by conversion to (CN)$_2$Cbl$^-$ (Section 2.5). This was performed in triplicate, diluting 50.0 µL, 100 µL, and 150 µL of cobalamin stock solution to 1.00 mL, 1.00 mL, and 2.00 mL respectively with aqueous 0.10 M KCN. The reaction was allowed ~2 hr to take place. The Cbl(I) concentration was determined to be $(1.29 \pm 0.06) \times 10^{-2}$ M.

The Cbl(I) solution was combined with NO$_2^-$ solution in CHES buffer under anaerobic conditions. Cbl(I) ($200 \pm 9$ µM) was added to solutions containing varying mole equiv. of NO$_2^-$ (0.050, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, and 0.50). The minimal time required for each reaction to proceed to completion was determined to be ~30 min by observing the time required for the spectrum of the solution containing Cbl(I) with 0.050 mole equivalents of NO$_2^-$ to come to equilibrium. UV-Vis spectra for each solution were collected from 250 – 800 nm. Absorbance versus equiv. of NO$_2^-$ plots were generated at 500 nm and 700 nm.

A second trial of the experiment was performed in a similar manner with some minor modifications. Cbl(I) was synthesized following the same general procedure by
dissolving HOCbl·HCl (100.31 mg) in 3.0 mL of anaerobic water and adding aqueous, anaerobic NaBH₄ (1.469 mL, 0.261M, 6.0 mole equiv.). The reaction was allowed to proceed as before and acetone (0.200 mL) was added. The Cbl(I) concentration was determined according to the protocol described in Section 2.5. Six replicate dilutions of 0.500 mL Cbl(I) stock solution to 5.00 mL with aqueous, aerobic KCN (0.100 M) were performed followed by two 1:10 dilutions (0.200 mL to 2.00 mL and 0.500 mL to 5.00 mL, sequentially). After completion of the stoichiometry experiment, 3 additional replicates for the determination of the Cbl(I) concentration were completed to confirm the Cbl(I) concentration by following a slightly altered dilution protocol. First, 1.00 mL Cbl(I) solution was diluted with 1.00 mL of anaerobic water and 3 samples of 0.500 mL of this solution were subsequently diluted to 5.00 mL with aerobic KCN (0.100 M). Subsequent dilutions (0.200 mL to 2.00 mL and 1.00 mL to 5.00 mL, sequentially) were performed using KCN (0.100 M). Each dilute solution was allowed to react for ~2 hr and UV-Vis spectra were recorded from 250 – 800 nm. The absorbance at 368 nm was used as before to determine the concentration of the original stock Cbl(I) solution, giving $(1.25 \pm 0.05) \times 10^{-2}$ M. The Cbl(I) solution was diluted with equal parts of anaerobic water to give a final Cbl(I) concentration of $(6.2 \pm 0.3) \times 10^{-3}$ M. The stoichiometry experiment was carried out as described above.

The stoichiometry of the reaction of Cbl(I) and NO₂⁻ was also determined at pH 7.40 ($5.0 \times 10^{-3}$ M phosphate buffer). An aqueous, anaerobic NaNO₂ ($6.39 \times 10^{-4}$ M) solution was prepared in the phosphate buffer. The same general procedure was followed as for the stoichiometry experiment at pH 9.53. The Cbl(I) concentration was determined
by performing six replicate dilutions of 0.500 mL Cbl(I) stock solution to 5.00 mL with aqueous, aerobic KCN (0.100 M) (Section 2.5). Each replicate solution was further diluted 1:10 (0.500 mL diluted to 5.00 mL) and then 1:5 (1.00 mL diluted to 5.00 mL). Additionally, this reaction was carried out for ~3 hr to assure reaction completion. The Cbl(I) concentration was determined as described previously to give (1.22 ± 0.02) x 10^{-2} M. The stoichiometry experiment was performed as described above.

A replicate of the stoichiometry experiment at pH 7.40 was performed using separately prepared phosphate buffer (5.0 x 10^{-3} M, pH 7.40) and NaNO₂ (5.07 x 10^{-4} M in phosphate buffer) solutions. The Cbl(I) concentration was determined in the same way as for the previous replicate, giving (1.24 ± 0.03) x 10^{-2} M. The stoichiometry experiment was performed according to the same procedure described above.

2.9 Determining the Amount of Hydroxylamine Product Formed: Indooxine Test

A modified literature procedure³⁷ (indooxine test) was used to determine the amount of NH₂OH produced by the reaction of Cbl(I) and NO₂⁻. Aqueous NH₂OH·HCl standard solutions (25.00 mL) of varying concentrations (0, 20.0, 40.0, 50.0, 60.0, 80.0, and 100 μM) were prepared by diluting a stock NH₂OH·HCl solution (10.0 mL, 3.42 x 10^{-3} M). An 8-hydroxyquinoline solution (25.00 mL, 4.0 % w/v) was prepared in pure ethanol with stirring (~2 min). This concentration was found to be optimum for the assay. An aqueous Na₂CO₃ solution (100 mL, 1.00 M) was prepared. Calibration standards were prepared by the addition of 8-hydroxyquinoline (1.00 mL, 4.0 % w/v) to 1.00 mL of an NH₂OH solution of a specific concentration, followed by the addition of Na₂CO₃ (1.00
mL, 1.00 M). The reaction was allowed to proceed for 15 min and absorbance spectra for the standards were subsequently measured from 450 – 800 nm. The absorbance at 710 nm was plotted against the concentration of NH$_2$OH. The calibration curve is shown in Figure 4.

**Figure 4:** Calibration curve for the indooxine test showing a line of best fit for the plot of absorbance at 710 nm versus NH$_2$OH concentration. A linear fit with intercept forced through the origin gives a slope of 0.0107 ± 0.0001 μM$^{-1}$.

Aqueous, anaerobic solutions of CHES buffer (5.0 x 10$^{-3}$ M, pH 9.51) and CHES buffer (5.0 x 10$^{-3}$ M, pH 9.51) containing NaNO$_2$ (2.96 x 10$^{-4}$ M) were prepared. Cbl(I) was prepared (Section 2.3) and the concentration ((1.28 ± 0.05) x 10$^{-2}$ M) was determined by conversion to (CN)$_2$Cbl$^-$ (Section 2.5). The reaction of Cbl(I) (200 ± 7 μM) and NaNO$_2$ was carried out under anaerobic conditions for Cbl(I):NO$_2^-$ ratios of 1:0.27 and 1:0.50, each in triplicate. After 1 hr reaction time, the indooxine test was performed on
each sample under aerobic conditions by combining the reaction product (1.00 mL) with
8-hydroxyquinoline solution (1.00 mL) and immediately adjusting the pH with Na₂CO₃
solution (1.00 mL). After 30 min at room temperature, the absorbance spectrum was
collected (450 – 800 nm).

2.10 Indooxine Control Experiments

2.10.1 Testing for the Stability of Hydroxylamine in Anaerobic CHES Buffer

The stability of NH₂OH in neutral and alkaline solution was determined. An
anaerobic solution of NH₂OH (10.00 mL, 2.93 x 10⁻³ M) was prepared and diluted (10.00
mL, 2.93 x 10⁻⁴ M) in the glove box. From this solution, an anaerobic NH₂OH solution
(3.0 mL, 50 μM) was prepared in CHES buffer (5.0 x 10⁻³ M, pH 9.51) and an aerobic
NH₂OH solution (3.0 mL, 50 μM) was prepared in water. After ~1 hr, the indooxine test
was carried out on each sample, as described in Section 2.9, giving 48.1 μM NH₂OH for
the sample in aerobic water and 52.2 μM for the sample in anaerobic pH 9.51 buffer.

2.10.2 Determining whether Cbl(II) Reacts with Hydroxylamine

A stock Cbl(II) solution was prepared according to the procedure described in
Section 2.4 and the concentration (0.0172 M) was estimated by assuming the HOCbl·HCl
reagent contained 12 % H₂O. An anaerobic NH₂OH stock solution was prepared in
anaerobic water and diluted by a factor of 10 giving a concentration of 2.93 x 10⁻⁴ M. An
aqueous, anaerobic Cbl(II) solution (3.0 mL, 50 μM) was prepared in water and an
aqueous, anaerobic Cbl(II) (3.0 mL, 50 μM) solution containing NH₂OH (50 μM) was
prepared in CHES buffer (5.0 x 10^{-3} M, pH 9.51). UV-Vis spectra were collected for both solutions (250 – 800 nm) after 5, 15, and 25 min.

2.10.3 Determining whether Cbl(I) Reacts with Hydroxylamine

A stock Cbl(I) solution was prepared according to the procedure in Section 2.3 and the concentration (0.0130 M) was estimated by assuming the HOCbl·HCl reagent contained 12 % H_{2}O. An anaerobic NH_{2}OH stock solution was prepared in anaerobic water and diluted by a factor of 25 to give a concentration of 1.47 x 10^{-4} M. The spectrum of an aqueous, anaerobic Cbl(I) solution (3.0 mL, 50 μM) was compared with the spectrum of a solution of Cbl(I) (3.0 mL, 50 μM) and NH_{2}OH (50 μM) after 5, 15, and 25 min in CHES buffer (5.0 x 10^{-3} M, pH 9.51).

2.10.4 Determining whether Hydroxylamine Reacts with Nitrite or Acetone

NH_{2}OH (50 μM) was reacted separately with NaNO_{2} (54 μM), acetone (6.3 μM, ~70x higher than the amount used in the indoxine test for NH_{2}OH, Section 2.9) or a solution containing NaNO_{2} (54 μM) and acetone (6.3 μM) for 1 hr under anaerobic conditions in CHES buffer (5.0 x 10^{-3} M, pH 9.51, 3 mL reaction volume). An indoxine test was carried out on each sample in addition to a control NH_{2}OH solution (50 μM) of known concentration (Section 2.9). The absorbance of each solution at 710 nm was recorded and the NH_{2}OH concentration was determined using the calibration curve (Figure 4).
2.10.5 Determining the Hydroxylamine Content of the Cbl(I)/Nitrite Product Solution

Spiked with Additional Hydroxylamine

In the glove box, Cbl(I) (200 μM, 0.600 mmol) was reacted with NaNO₂ (54 μM, 0.163 mmol, 1.0 mL reaction volume) in a 1:0.27 Cbl(I):NO₂⁻ ratio for 1 hr, in duplicate. The indooxine test (Section 2.9) was performed directly on one sample. An additional amount of NH₂OH (79.9 μM, 50.0 nmol, 2.0 mL reaction volume) was added to the second sample immediately prior to performing the indooxine test on that sample. An absorbance spectrum was collected (450 – 800 nm) and the absorbance at 710 nm was used to determine the NH₂OH concentration.

2.10.6 Determining if Nitrite Interferes with a Positive Indooxine Test

A solution of NaNO₂ was prepared in anaerobic CHES buffer (5.0 x 10⁻³ M, pH 9.51) An indooxine test was performed (Section 2.9) on the NO₂⁻ solution by combining 1.00 mL of the NO₂⁻ solution with the reagents for the indooxine test. An absorbance spectrum was collected (450 – 800 nm) and the absorbance at 710 nm was used to determine the NH₂OH concentration.

2.10.7 Determining whether the Presence of Cbl(II) has an Effect on Hydroxylamine Concentration Determined by the Indooxine Test Over Time

Cbl(II) was synthesized as described in Section 2.4 and the concentration (0.0154 M) was approximated by assuming the HOCbl·HCl contained 12 % H₂O. A stock NH₂OH solution (5.97 x 10⁻³ M) was prepared under anaerobic conditions and diluted by a factor of 10 with CHES buffer (pH 9.53, 5.0 x 10⁻³ M) to give 5.97 x 10⁻⁴ M NH₂OH. Anaerobic samples were prepared containing Cbl(II) (200 μM) and NH₂OH (50 μM) in
CHES buffer, and control samples were prepared containing NH$_2$OH (50 µM) only. The
NH$_2$OH concentration was determined for the Cbl(II) + NH$_2$OH samples and control
samples by the indooxine test (Section 2.9) after 0, 30, and 60 min under anaerobic
conditions. All samples were prepared in duplicate.

2.10.8 Determining Whether Bubbling Air through Anaerobic Cbl(II) plus
Hydroxylamimine Solutions Recovers NH$_2$OH

Cbl(II) was synthesized as described in Section 2.4 and the concentration (0.0154
M) was approximated by assuming the HOCbl·HCl contained 12 % H$_2$O. A stock
NH$_2$OH solution (5.97 x 10$^{-3}$ M) was prepared under anaerobic conditions and diluted by
a factor of 10 with CHES buffer (pH 9.53, 5.0 x 10$^{-3}$ M) to give 5.97 x 10$^{-4}$ M NH$_2$OH.
Anaerobic samples were prepared containing Cbl(II) (200 µM) and NH$_2$OH (50 µM) in
CHES buffer, and control samples were prepared containing NH$_2$OH (50 µM) only. The
NH$_2$OH concentration of the Cbl(II) + NO$_2^-$ samples and control samples was determined
by the indooxine test (Section 2.9) after 0, 3, and 10 min of bubbling air through the
samples.

2.11 Determining whether Ammonia is a Product of the Reaction between Cbl(I)
and Nitrite

A Nessler test for the presence of NH$_3$ was performed on a solution containing the
reaction products of 200 µM Cbl(I) with 50 µM NO$_2^-$ (0.050 M CHES buffer, pH 9.51)
under aerobic conditions, in which the Cbl species was oxidized to HOCbl.
Approximately 8 drops of the Nessler reagent were added to the product solution. A
positive result for NH₃ is indicated by a yellow or, at high concentrations, brown coloring in the reaction solution.³⁸ In this case, no brown or yellow coloring was observed above the pink color of the HOCbl species.

2.12 Kinetic Studies on the Reaction between Cbl(I) and Nitrite

All kinetic experiments for the reaction of Cbl(I) and NO₂⁻ were performed using at least ten times excess NO₂⁻ to ensure pseudo-first-order kinetics. A total ionic strength of 0.50 M was maintained for all experiments using Na₂HPO₄/NaH₂PO₄. Data at pH 10.80 were carried out in 0.050 M CAPS buffer using the Cary UV-Vis spectrophotometer and Schlenk cuvettes equipped with a cavity for the storage of the Cbl(I) reagent, allowing the Cbl(I) and NO₂⁻ reagents to be combined immediately before beginning data collection. The solutions were thermostated at 25.0 °C for at least 10 min before initiating the reaction. Data at pH < 10.80 were collected using the stopped-flow spectrophotometer in buffer (CHES, 0.050 M, pH 9.51; TAPS, 0.050 M, pH 8.51; phosphate, pH 7.40). Experiments performed at pH < 7.40 required that the buffer be mixed with a Cbl(I) solution in water as part of the stopped-flow procedure, since Cbl(I) is less stable in solution at lower pH. The Cbl(I) concentration was also elevated to 300 µM before mixing to provide additional stability. Buffers at pH 6.92 and 6.50 were prepared at twice the desired concentration and mixed with Cbl(I) within the stopped-flow itself. Data at pH 6.92 and 6.50 were carried out using 0.050 M buffers (BIS-TRIS and MES, respectively). Data at pH > 6.92 were collected and analyzed at 388 nm for
which the absorbance change for the reaction is largest. At pH 6.92 and 6.50, data was collected and analyzed at 500 nm to accommodate higher Cbl concentrations.

The source of the intercepts in the $k_{\text{obs}}$ versus $\text{NO}_2^-$ concentration plots at pH 6.92 and 6.50 (Figure 13(a,b), Section 3.5) was determined. Cbl(I) was diluted in anaerobic H$_2$O and was reacted with BIS-TRIS and MES buffers. Stopped-flow data at 500 nm was recorded for 60 sec.
III. RESULTS AND DISCUSSION

3.1 Attempts to Prepare Solid Cbl(I)

Cbl(I) is an unstable radical complex that requires a highly anaerobic environment for synthesis and storage. Others have termed it a supernucleophile that is known to react \(\sim 10^5\) times faster than standard nucleophiles, such as thiols.\(^{39,40}\) Moreover, slow spontaneous oxidation of Cbl(I) to Cbl(II) was observed in aqueous, anaerobic solution at room temperature. It was hypothesized that Cbl(I) may be more stable as a solid.

Three separate precipitations of Cbl(I) solution in chilled acetone were performed (Section 2.6). A fraction of the precipitated Cbl(I) was immediately re-dissolved in anaerobic water and the UV-Vis spectrum recorded. The results from the most successful trial are shown in Figure 5. The results of this experiment were not reproduced well by the other two trials, which yielded more significant oxidation of Cbl(I) to Cbl(II) immediately after precipitation. The most successful precipitation procedure resulted in \(\sim 10\%\) oxidation of Cbl(I) to Cbl(II) immediately after precipitation. The precipitated Cbl(I) sample was re-dissolved in water after 11 days of storage in the freezer of the glove box (-24 °C), and analyzed by UV-Vis spectrophotometry. The spectrum indicated complete conversion to Cbl(II) after 11 days of storage. It was therefore determined that precipitation by addition to chilled acetone does not achieve pure solid Cbl(I).

Alternatively, attempts were made to synthesize solid Cbl(I) by evaporation of the solvent of a Cbl(I) solution under vacuum. This method, however, requires a lengthy
period in which Cbl(I) is in solution at room temperature. The obtained solid was re-
dissolved in H$_2$O, a UV-Vis spectrum obtained, and found to be $\sim$70% oxidized to
Cbl(II). Thus, both methods failed to produce pure Cbl(I) in the solid state.
Consequentially, all of the subsequent experiments were carried out using freshly
prepared Cbl(I) in solution as the Cbl(I) reagent.

![UV-Vis spectra of Cbl(I) (λ$_{\text{max}}$ 280, 288, 386, 455, 545, 680 nm$^{35}$) before
precipitation (solid trace) and after precipitation and resuspension in solution.
Immediately following precipitation, Cbl(I) was partially (\sim10%) oxidized to Cbl(II)
(λ$_{\text{max}}$ 267, 285, 315, 467 nm$^{35}$) (dashed trace). 11 days after precipitation, near complete
oxidation to Cbl(II) (70 %) was observed (dotted trace). Large differences in absorbance
are primarily due to differences in concentration of the samples.]

**Figure 5:** UV-Vis spectra of Cbl(I) (λ$_{\text{max}}$ 280, 288, 386, 455, 545, 680 nm$^{35}$) before
precipitation (solid trace) and after precipitation and resuspension in solution.
Immediately following precipitation, Cbl(I) was partially (~10%) oxidized to Cbl(II)
(λ$_{\text{max}}$ 267, 285, 315, 467 nm$^{35}$) (dashed trace). 11 days after precipitation, near complete
oxidation to Cbl(II) (70 %) was observed (dotted trace). Large differences in absorbance
are primarily due to differences in concentration of the samples.

**3.2 Identification of Cbl(II) as the Cbl Product of the Reaction of Cbl(I) with Nitrite**

The Cbl product of the reaction of Cbl(I) with NO$_2^\cdot$ was identified by UV-Vis
spectrophotometry (Section 2.7). UV-Vis spectral data as a function of time were
collected for the reaction of Cbl(I) (50 μM) with NO$_2^\cdot$ (9.1 mM), in phosphate buffer
(0.050 M, pH 12.00), Figure 6(a). The initial and final spectra correspond to that of Cbl(I), and Cbl(II) respectively, Figure 6(b). From Figure 6(a) it can be seen that the reaction exhibits clean isosbestic points at 307, 346, 417 and 543 nm in agreement with literature values for the conversion of Cbl(I) to Cbl(II), indicating a direct and clean conversion of Cbl(I) to Cbl(II) for the rate-determining step of the reaction.

Figure 6: (a) UV-Vis spectra as a function of time for the reaction of Cbl(I) ($\lambda_{\text{max}}$ 280, 288, 386, 455, 545, and 680 nm, 50 µM) with NO$_2^-$ (9.1 mM) in 0.050 M phosphate buffer at pH 12.00, 25.0 °C. Spectra were recorded every 2.5 min for 40 min. (b) Initial and final spectra for the reaction shown in (a). The final spectrum corresponds to that of Cbl(II) ($\lambda_{\text{max}}$ 267, 285, 315, 467 nm).

3.3 Determination of the Stoichiometry of the Reaction of Cbl(I) with Nitrite

UV-Vis spectra of the product solution obtained upon equilibrating Cbl(I) (200 µM) with 0 – 0.50 mole equiv. NO$_2^-$ under anaerobic conditions were recorded for pH 9.51 (5.0 x 10$^{-3}$ M CHES, 25.0 °C). The Cbl(I) concentration was elevated for this experiment to enhance its stability in buffered solution. The minimum time for the reaction to proceed to completion was found to be ~30 min, so each solution was allowed
to react under anaerobic conditions for at least this amount of time. The absorbance at 700 nm (Figure 7(a)) and 500 nm (Figure 7(b)) for each reaction was plotted against the mole equiv. of NO$_2^-$ added. These wavelengths were found to be convenient for analysis because the absorbance of the Cbl species is within the detectable range for the instrument at these wavelengths (Abs < 2). From these plots, it is clear that the reaction is complete after the addition of ~0.25 mole equiv. of NO$_2^-$. Tables 1 and 2 summarize the experimental data obtained at 700 and 500 nm, respectively. The mean value of the equiv. of NO$_2^-$ required is 0.24 ± 0.02. This suggests a 4:1 Cbl(I):NO$_2^-$ mole ratio.

Figure 7: Plot of absorbance at (a) 700 nm or (b) 500 nm versus mole equiv. of NaNO$_2$ for equilibrated solutions of Cbl(I) (200 ± 9 μM) with NO$_2^-$ (5.0 x 10$^{-3}$ M, CHES buffer, pH 9.51, 25.0 °C).

The stoichiometry experiment was repeated at pH 9.53 using a slightly modified procedure for determination of the Cbl(I) concentration (Section 2.8). Once again, the number of mole equiv. of NO$_2^-$ added was varied and the absorbance at 700 nm (Figure 8(a)) and 500 nm (Figure 8(b)) for each reaction was plotted against the number of NO$_2^-$ equiv. added. Tables 3 and 4 summarize the data, which gives a mean value of
Table 1: Determination of the stoichiometry of the reaction between Cbl(I) and NO$_2^-$ at pH 9.51 (5.0 x 10$^{-3}$ M CHES buffer). Absorbances were measured at 700 nm.

\[ \text{Fraction Cbl(I) Reacted} = \frac{\text{Abs}_{\text{Cbl(I)}} - \text{Abs}_{\text{obs}}}{\text{Abs}_{\text{Cbl(I)}} - \text{Abs}_{\text{Cbl(II)}}} \]

\[ \text{Mole Equiv. NO}_2^- \text{ Required} = \left( \frac{[\text{NO}_2^-]/[\text{Cbl(I)}]}{[\text{Cbl(I)}]} \right) / \text{Fraction Cbl(I) Reacted} \]

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Table 2: Determination of the stoichiometry of the reaction between Cbl(I) and NO$_2^-$ at pH 9.51 (5.0 x 10$^{-3}$ M CHES buffer). Absorbances were measured at 500 nm.

\[ \text{Fraction Cbl(I) Reacted} = \frac{\text{Abs}_{\text{Cbl(I)}} - \text{Abs}_{\text{obs}}}{\text{Abs}_{\text{Cbl(I)}} - \text{Abs}_{\text{Cbl(II)}}} \]

\[ \text{Mole Equiv. NO}_2^- \text{ Required} = \left( \frac{[\text{NO}_2^-]/[\text{Cbl(I)}]}{[\text{Cbl(I)}]} \right) / \text{Fraction Cbl(I) Reacted} \]

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0.26 ± 0.02 mole equiv. NO$_2^-$: This value is in excellent agreement with that obtained from the earlier experiment. Combining the results of both experiments gives a NO$_2^-$:Cbl(I) stoichiometry of (0.25 ± 0.02):1.

**Figure 8**: Plot of absorbance at (a) 700 nm or (b) 500 nm versus mole equiv. NaNO$_2$ for equilibrated solutions of Cbl(I) (200 ± 8 μM) with NO$_2^-$ (5.0 x 10$^{-3}$ M CHES buffer, pH 9.53, 25.0 °C).

The stoichiometry for the reaction of Cbl(I) and NO$_2^-$ was also determined at pH 7.40 (5.0 x 10$^{-3}$ M phosphate buffer). The minimum time for the reaction to equilibrate was found to be < 1.5 min (the time required to remove the sample from the glove box). As before, the absorbance at 700 nm (Figure 9(a)) and 500 nm (Figure 9(b)) for each reaction was plotted against the mole equiv. of NO$_2^-$ added. Again, these plots show that the reaction is complete after the addition of ~0.25 mole equiv. of NO$_2^-$. The experimental data for 700 and 500 nm is summarized in Tables 5 and 6, respectively. The mean value of the equiv. of NO$_2^-$ required is 0.28 ± 0.03 equiv NO$_2^-$. The expected number of equivalents (0.25 equiv.) is contained within the statistical error of the result.
Table 3: Determination of the stoichiometry of the reaction between Cbl(I) and NO$_2^-$ at pH 9.53 (5.0 x 10$^{-3}$ M CHES buffer). Absorbances were measured at 700 nm.

\[ \text{Fraction Cbl(I) Reacted} = \frac{\text{Abs}_{\text{Cbl(I)}} - \text{Abs}_{\text{obs}}}{\text{Abs}_{\text{Cbl(I)}} - \text{Abs}_{\text{Cbl(II)}}} \]

\[ \text{Mole Equiv. NO}_2^- \text{ Required} = \frac{[\text{NO}_2^-]/[\text{Cbl(I)}]_i}{\text{Fraction Cbl(I) Reacted}} \]

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</table>

Table 4: Determination of the stoichiometry of the reaction between Cbl(I) and NO$_2^-$ at pH 9.53 (5.0 x 10$^{-3}$ M CHES buffer). Absorbances were measured at 500 nm.

\[ \text{Fraction Cbl(I) Reacted} = \frac{\text{Abs}_{\text{Cbl(I)}} - \text{Abs}_{\text{obs}}}{\text{Abs}_{\text{Cbl(I)}} - \text{Abs}_{\text{Cbl(II)}}} \]

\[ \text{Mole Equiv. NO}_2^- \text{ Required} = \frac{([\text{NO}_2^-]/[\text{Cbl(I)}])_i}{\text{Fraction Cbl(I) Reacted}} \]

<table>
<thead>
<tr>
<th>10$^4$[Cbl(I)]$_i$ (M)</th>
<th>10$^5$[NO$_2^-$]$_i$ (M)</th>
<th>[NO$_2^-$]$_i$ /[Cbl(I)]$_i$</th>
<th>Abs$_{\text{Cbl(I)}}$</th>
<th>Abs$_{\text{Cbl(II)}}$</th>
<th>Abs$_{\text{obs}}$</th>
<th>Fraction Cbl(I) Reacted$^a$</th>
<th>Mole Equiv. NO$_2^-$ Required$^b$</th>
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</thead>
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</tbody>
</table>
Figure 9: Plot of absorbance at (a) 700 nm or (b) 500 nm versus mole equiv. NaNO₂ for equilibrated solutions of Cbl(I) (200 ± 3 μM) with NO₂⁻ (5.0 x 10⁻³ M phosphate buffer, pH 7.40, 25.0 °C)

Table 5: Determination of the stoichiometry of the reaction between Cbl(I) and NO₂⁻ at pH 7.40 (5.0 x 10⁻³ M phosphate buffer). Absorbances were measured at 700 nm.

<table>
<thead>
<tr>
<th>10⁻⁴[Cbl(I)]₀ (M)</th>
<th>10⁻³[NO₂⁻]₀ (M)</th>
<th>[NO₂⁻]₀ /[Cbl(I)]₀</th>
<th>Absₐₐₐ(Cbl(I))</th>
<th>Absₐₐₐ(Cbl(II))</th>
<th>Absₐₐₐ</th>
<th>Fraction Cbl(I) Reactedᵃ</th>
<th>Mole Equiv. NO₂⁻ Requiredᵇ</th>
</tr>
</thead>
<tbody>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.00</td>
<td>10.00</td>
<td>0.50</td>
<td>0.389</td>
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<td>0.111</td>
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</tbody>
</table>
Table 6: Determination of the stoichiometry of the reaction between Cbl(I) and NO$_2^-$ at pH 7.40 (5.0 x 10$^{-3}$ M phosphate buffer). Absorbances were measured at 500 nm.

\(^a\)Fraction Cbl(I) Reacted = (Abs$_{\text{Cbl(I)}}$ – Abs$_{\text{obs}}$) / (Abs$_{\text{Cbl(I)}}$ – Abs$_{\text{Cbl(II)}}$)

\(^b\)Mole Equiv. NO$_2^-$ Required = ([NO$_2^-$]$_i$ / [Cbl(I)]$_i$) / Fraction Cbl(I) Reacted

<table>
<thead>
<tr>
<th>$10^4$[Cbl(I)]$_i$ (M)</th>
<th>$10^4$[NO$_2^-$]$_i$ (M)</th>
<th>/[Cbl(I)]$_i$</th>
<th>Abs$_{\text{Cbl(I)}}$</th>
<th>Abs$_{\text{Cbl(II)}}$</th>
<th>Abs$_{\text{obs}}$</th>
<th>Fraction Cbl(I) Reacted$^a$</th>
<th>Mole Equiv. NO$_2^-$ Required$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00</td>
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<td>0.322</td>
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<td>1.13</td>
<td>-</td>
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</table>

The stoichiometry experiment at pH 7.40 (5.0 x 10$^{-3}$ M phosphate buffer) was repeated to verify the result. The absorbance at 700 nm (Figure 10(a)) and 500 nm were measured. The absorbance at 700 nm (Figure 10(a)) and 500 nm (Figure 10(b)) are plotted versus mole equiv. NaNO$_2$ for equilibrated solutions of Cbl(I) (200 ± 6 μM) with NO$_2^-$ (5.0 x 10$^{-3}$ M phosphate buffer, pH 7.40, 25.0 °C).

Figure 10: Plot of absorbance at (a) 700 nm or (b) 500 nm versus mole equiv. NaNO$_2$ for equilibrated solutions of Cbl(I) (200 ± 6 μM) with NO$_2^-$ (5.0 x 10$^{-3}$ M phosphate buffer, pH 7.40, 25.0 °C).
(Figure 10(b)) for each reaction was plotted against the mole equiv. of NO$_2^-$ added. The experimental data for 700 and 500 nm is summarized in Tables 7 and 8, respectively. The mean value of the equiv. of NO$_2^-$ required was determined to be $0.22 \pm 0.02$ equiv NO$_2^-$. This result suggests the possibility of a 5e$^-$ process; however this would result in reduction of nitrogen to the -2 oxidation state. This is unlikely, which suggests that the reaction stoichiometry is 4:1 Cbl(I):NO$_2^-$ at pH 7.40. Combining the results of both experiments at pH 7.40 gives a NO$_2^-$:Cbl(I) stoichiometry of $(0.25 \pm 0.04)$:1. The reaction stoichiometry was, therefore, found to be 0.25:1 NO$_2^-$:Cbl(I) for both pH 9.51 and pH 7.40.

**Table 7:** Determination of the stoichiometry of the reaction between Cbl(I) and NO$_2^-$ at pH 7.40 ($5.0 \times 10^{-3}$ M phosphate buffer). Absorbances were measured at 700 nm.

- **Fraction Cbl(I) Reacted =** $(\text{Abs}_{\text{Cbl(I)}} - \text{Abs}_{\text{obs}}) / (\text{Abs}_{\text{Cbl(I)}} - \text{Abs}_{\text{Cbl(II)}})\)$
- **Mole Equiv. NO$_2^-$ Required =** $\left(\frac{[\text{NO}_2^-]/[\text{Cbl(I)}]}{0.29}\right) / \text{Fraction Cbl(I) Reacted}$

<table>
<thead>
<tr>
<th>$10^4[\text{Cbl(I)}]$</th>
<th>$10^3[\text{NO}_2^-]$</th>
<th>$[\text{NO}_2^-]/[\text{Cbl(I)}]$</th>
<th>$\text{Abs}_{\text{Cbl(I)}}$</th>
<th>$\text{Abs}_{\text{Cbl(II)}}$</th>
<th>$\text{Abs}_{\text{obs}}$</th>
<th>Fraction Cbl(I) Reacted$^a$</th>
<th>Mole Equiv. NO$_2^-$ Required$^b$</th>
</tr>
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<tr>
<td>2.00</td>
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Table 8: Determination of the stoichiometry of the reaction between Cbl(I) and NO$_2^-$ at pH 7.40 (5.0 $\times$ 10$^{-3}$ M phosphate buffer). Absorbances were measured at 500 nm.

*a*Fraction Cbl(I) Reacted = (Abs$_{Cbl(I)}$ – Abs$_{obs}$) / (Abs$_{Cbl(I)}$ – Abs$_{Cbl(II)}$)

*b*Mole Equiv. NO$_2^-$ Required = ([NO$_2^-$]/[Cbl(I)]$_i$) / Fraction Cbl(I) Reacted

<table>
<thead>
<tr>
<th>10$^{-5}$[Cbl(I)]$_i$ (M)</th>
<th>10$^{-5}$[NO$_2^-$]$_i$ (M)</th>
<th>[NO$_2^-$]$_i$/[Cbl(I)]$_i$</th>
<th>Abs$_{Cbl(I)}$</th>
<th>Abs$_{Cbl(II)}$</th>
<th>Abs$_{obs}$</th>
<th>Fraction Cbl(I) Reacted$^a$</th>
<th>Mole Equiv. NO$_2^-$ Required$^b$</th>
</tr>
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<td>0.313</td>
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<td>1.08</td>
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<td>-</td>
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<tr>
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<td>0.313</td>
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<td>1.08</td>
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3.4 Determining the Amount of Hydroxylamine Product Formed in the Reaction of Cbl(I) with Nitrite

A 0.25:1 NO$_2^-$:Cbl(I) reaction stoichiometry suggests that the non-Cbl reaction product is NH$_2$OH. Whether the reactive species is NO$_2^-$ or HNO$_2$ will be considered later. The balanced equation for the reaction is shown in equation (1).

(4)5H$^+$ + 4Cbl(I)$^-$ + (H)NO$_2$ $\rightarrow$ 4Cbl(II) + NH$_2$OH + H$_2$O

An indoxine test for the presence of NH$_2$OH was carried out as described in Section 2.9. A calibration curve (absorbance at 710 nm versus NH$_2$OH concentration) was generated and is shown in Figure 4, Section 2.9. Cbl(I) and NO$_2^-$ were combined in 1:0.27 (near
stoichiometric) and 1:0.50 (excess NO$_2^-$) mole ratios and the indooxine test was performed on these solutions. Only the indooxine product absorbs in the 650 – 750 nm region ($\lambda_{\text{max}}$ 710 nm$^{37}$) and the NH$_2$OH concentration was determined by use of the calibration curve in Figure 4, Section 2.9. Table 9 summarizes the results. The average NH$_2$OH concentration in the product solution was found to be 21 ± 2 µM. Given that 200 µM Cbl(I) was reacted with sufficient NO$_2^-$ for the reaction to proceed to completion according to the results of the stoichiometry experiment, 50.0 µM NH$_2$OH should theoretically be produced for both 1:0.27 and 1:0.50 Cbl(I):NO$_2^-$ ratios. However, only ~40 % of the NH$_2$OH product (21 ± 2 µM) was obtained using the indooxine test to quantify the amount of product formed for both reactant ratios.

The low recovery of the NH$_2$OH species by the indooxine test was investigated. A series of control experiments to determine what was responsible for the reduced yield of NO$_2^-$ were undertaken. First, the stability of NH$_2$OH in anaerobic CHES buffer (pH 9.51)

<table>
<thead>
<tr>
<th>Equivalents of NO$_2^-$</th>
<th>Absorbance at 710 nm</th>
<th>Concentration of NH$_2$OH (µM)</th>
</tr>
</thead>
<tbody>
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Table 9: Detection of NH$_2$OH by the indooxine test, confirming NH$_2$OH as a reaction product in the reaction of Cbl(I) (200 ± 7 µM) with NO$_2^-$ (0.27 and 0.50 mole equiv.) in CHES buffer (5.0 x 10$^{-3}$ M, pH 9.51). The NH$_2$OH concentration was calculated based on the calibration curve in Figure 4, Section 2.9. The average concentration was 21 ± 2 µM.
was determined (Section 2.10.1). NH$_2$OH (50 µM) was incubated in anaerobic CHES buffer and aerobic water for ~1 hr and an indooxine test was used to quantify the amount of NH$_2$OH in each solution. The determined NH$_2$OH concentrations were 48.1 µM for the sample in aerobic water and 52.2 µM for the sample in anaerobic pH 9.51 buffer. Hence the alkaline pH, the presence or absence of air, and the presence of the CHES buffer in the NH$_2$OH sample have no effect within experimental error on the NH$_2$OH concentration determined using the indooxine method; that is, NH$_2$OH is stable in solution under these conditions.

Another possibility is that some of the NH$_2$OH product was oxidized to N$_2$. The reduction potentials of N$_2$/NH$_2$OH, Cbl(III)/Cbl(II), and Cbl(II)/Cbl(I) are -3.04 V (pH 14), 0.200 V, and -0.600 V$^{20}$ versus SHE, respectively. The stability of Cbl(II) and Cbl(I) in solution with NH$_2$OH was determined by observing the spectrum of Cbl(II) (50 µM) and Cbl(I) (50 µM) in solution with NH$_2$OH (50 µM) by UV-Vis spectrophotometry (Sections 2.10.2 and 2.10.3). It was found that the spectra of Cbl(II) and Cbl(I) do not change over 25 min when NH$_2$OH is added under anaerobic conditions; hence neither Cbl(II) nor Cbl(I) are reduced by NH$_2$OH under our experimental conditions.

Others have reported that NH$_2$OH reacts with NO$_2^-$, albeit under strongly acidic conditions.$^{43}$ Whether this reaction occurs under our experimental conditions was therefore also investigated (Section 2.10.4). The potential of NH$_2$OH to react with the small amount of acetone originating from the synthesis of Cbl(I) (Section 2.3) in the product mixture was also investigated (Section 2.10.4). The results of the indooxine test for NH$_2$OH concentration showed that the NH$_2$OH does not decompose or react further
under the experimental conditions used in the presence of NaNO$_2$ (54 µM) or acetone (6.3 µM), or both NaNO$_2$ and acetone.

To ensure that the indooxine test was reliable for the experimental conditions used, the indooxine test was performed on a Cbl(I) + NO$_2^-$ product sample which was spiked with an additional 50.0 nmol NH$_2$OH (Section 2.10.5). Using the calibration curve (Figure 4, Section 2.9), it was determined that the unspiked sample contained 10.9 µM (10.9 nmol) NH$_2$OH and the spiked sample contained 29.4 µM (58.8 nmol) NH$_2$OH. The spiked sample gave the expected increase in NH$_2$OH content.

Excess NO$_2^-$ present in solution may cause interference with the indooxine test under certain conditions. The indooxine test for the NH$_2$OH product of the reaction between Cbl(I) and NO$_2^-$ was performed using no more than 50 µM excess NO$_2^-$. Therefore, the indooxine test was performed on a sample containing 50 µM NO$_2^-$ under the conditions used for the quantification of NH$_2$OH produced by the Cbl(I) + NO$_2^-$ reaction (Section 2.10.6). It was found that NO$_2^-$ does not interfere with the indooxine test at this concentration.

A control experiment was also performed to test the effect of the presence of Cbl(II) (200 µM) on NH$_2$OH (50 µM) for 0, 30, and 60 min under anaerobic conditions (Section 2.10.7). The indooxine test showed that ~30 % of the original NH$_2$OH is absent from the samples containing Cbl(II) regardless of how long the solution was allowed to react (0, 30, or 60 min), while at least 95 % of the NH$_2$OH was recovered in the solutions that did not contain Cbl(II) (Table 10). Additionally, since the indooxine reaction requires aerobic conditions, a control experiment was done to determine the effect of bubbling air
through the Cbl(I) + NO$_2^-$ product solution prior to performing the indooxine test (Section 2.10.8). It was found that bubbling for 3 min recovered ~80% of the original NH$_2$OH concentration, but further bubbling for 10 min was unsuccessful at recovering the remaining NH$_2$OH (Table 11). Importantly, transition metal-catalyzed disproportionation of NH$_2$OH has been reported by others.$^{44,45}$ It is likely that aqueous reduced products of the disproportionated NH$_2$OH are re-oxidized to NH$_2$OH in the presence of oxygen, but the gaseous and oxidized products are not retrieved. Under anaerobic conditions in the presence of transition metals, NH$_2$OH disproportionates to give N$_2$, N$_2$O, and NO$^+$.44

Finally, a test was performed to determine whether NH$_3$ is a product of the reaction of Cbl(I) and NO$_2^-$ (Section 2.11). Cbl(I) (200 µM) was reacted with NO$_2^-$ (50 µM) under anaerobic conditions (0.050 M CHES buffer, pH 9.51). Nessler reagent was added (~8 drops) under aerobic conditions to the product solution. A color change to yellow or brown is indicative of the presence of NH$_3$. Since no color change was observed, NH$_3$ is not a product of the reaction of Cbl(I) and NO$_2^-$.

To summarize, the results of the stoichiometry experiment suggest that the stoichiometry of the reaction between Cbl(I) and NO$_2^-$ is 4:1 Cbl(I):NO$_2^-$. Cbl(II) was shown to be the Cbl product by UV-Vis spectrophotometry. The expected non-Cbl product is NH$_2$OH, and the balanced equation is given by equation (1). The indooxine test for the presence of NH$_2$OH was positive, albeit non-quantitative, in agreement with the elucidated reaction stoichiometry. It is likely that some of the NH$_2$OH disproportionates; hence the lower yield of NH$_2$OH.
Table 10: Absorbance obtained at 710 nm for solutions of NH$_2$OH (50 μM) in the absence and presence of Cbl(II) (200 μM). Solutions were left to react under anaerobic conditions for 0, 30, and 60 min prior to determining the NH$_2$OH concentration using the indooxine test. *Determined from the calibration curve shown in Figure 4, Section 2.9.

<table>
<thead>
<tr>
<th>Contents</th>
<th>Replicate</th>
<th>Time under anaerobic conditions (min)</th>
<th>Absorbance at 710 nm</th>
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<td>0.499</td>
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Table 11: Absorbance obtained at 710 nm for solutions of NH$_2$OH (50 μM) in the absence and presence of Cbl(II) (200 μM). Air was bubbled through the solutions for 0, 3, or 10 min prior to determining the NH$_2$OH concentration using the indooxine test. *Determined from the calibration curve shown in Figure 4, Section 2.9.

<table>
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3.5 Kinetic Studies on the reaction of Cbl(I) with Nitrite

The kinetics of the reaction of Cbl(I) with NO$_2^-$ were studied at pH 6.50 (0.050 M MES), 6.92 (0.050 M BIS-TRIS), 7.40 (phosphate), 8.51 (0.050 M TAPS), 9.51 (0.050 M CHES), and 10.80 (0.050 M CAPS) at 25 °C. Data were collected under pseudo-first-order conditions in which the NO$_2^-$ concentration was at least ten times greater than the Cbl(I) concentration. This method allows for direct determination of the reaction order for each reactant. Figure 11(a) gives a typical plot of absorbance versus time for the reaction of Cbl(I) (50 μM) with NO$_2^-$ (5.0 mM). The first-order integrated rate equation

$$A_{obs} = A_f + (A_0 - A_f)e^{-k_{obs}t}$$ (2)

was used to fit the data, where $A_{obs}$ is observed absorbance, $A_f$ is final absorbance, $A_0$ is initial absorbance, $k_{obs}$ is the observed rate constant, and $t$ is time. The data fit well to equation (2), suggesting that the reaction is first-order with respect to the Cbl(I) concentration. Figure 11(b) gives a plot of absorbance versus time for the Cbl(I) solution in the absence of NO$_2^-$ at pH 10.80. Cbl(I) is slowly oxidized to Cbl(II); however, this reaction is significantly slower than the reaction of interest at pH 10.80. The change in absorbance ($\Delta A$) for the complete reaction between Cbl(I) and NO$_2^-$ (Figure 11(a)) was 0.45 at 388 nm. Oxidation of Cbl(I) in buffer over this same period of time (Figure 11(b)) gave a $\Delta A$ of 0.02 at 388 nm, which amounts to 5 % of the total absorbance change. Thus, at pH 10.80, the oxidation of Cbl(I) in buffer is negligible with respect to the rate of the Cbl(I) + NO$_2^-$ reaction. The rate of oxidation of Cbl(I) to Cbl(II) in buffer alone can in fact be determined from the intercept of the $k_{obs}$ versus NO$_2^-$ plots.
Figure 11: (a) Kinetic data showing the change in absorbance at 388 nm versus time for the reaction of Cbl(I) (50 μM) with NO$_2^-$ (5.0 mM) (0.050 M CAPS buffer, pH 10.80, $I = 0.50$ M (Na$_2$HPO$_4$)). Fitting the data to equation (2) gives $k_{obs} = 0.209 \pm 0.001$ s$^{-1}$. (b) Plot of absorbance versus time for Cbl(I) (50 μM, same conditions) at pH 10.80 showing the oxidation to Cbl(II) in buffer alone over the same period of time.

The dependence of the rate of the reaction on NO$_2^-$ concentration can be determined by measuring the observed rate constant as a function of NO$_2^-$ concentration for a given pH condition. The reaction order with respect to NO$_2^-$ is determined by plotting $k_{obs}$ versus NO$_2^-$ concentration, as shown in Figure 12. The slope of each plot represents the apparent rate constant ($k_{app}$) at a given pH value. The linear relationship between $k_{obs}$ and NO$_2^-$ concentration is indicative of the first-order dependence of the reaction rate on NO$_2^-$ concentration.

Under acidic pH conditions (pH 6.50 and 6.92), Cbl(I) was not stable enough to proceed with kinetic measurements by mixing a solution of Cbl(I) in buffer with NO$_2^-$ in buffer. Collection of the Cbl(I) UV-Vis spectrum after dilution in acidic biological buffer (BIS-TRIS and MES) showed partial oxidation to Cbl(II). Therefore, the Cbl(I) solution
was instead prepared in water and only combined with the buffer within the stopped-flow spectrophotometer. The plots of $k_{obs}$ versus NO$_2^-$ concentration had significant intercepts for acidic pH conditions.

The acid dependence for the reaction between Cbl(I) and NO$_2^-$ can be determined from the relationship between $k_{app}$ and the pH. Figure 13 and Table 12 summarize the dependence of $k_{app}$ on pH. The data fit an exponential decay function

$$k_{app} = k10^{-pH}$$  \hspace{1cm} (3)

At each pH condition

$$\text{rate} = k_{app}[\text{Cbl(I)}][\text{NO}_2^-]$$  \hspace{1cm} (4)

The pH-dependent rate law is

$$\text{rate} = k[\text{Cbl(I)}][\text{NO}_2^-][\text{H}^+]$$  \hspace{1cm} (5)

Therefore, $k_{app} = k[\text{H}^+] = k10^{-pH}$. Fitting the $k_{app}$ versus pH data to equation (3) (Figure 13) gives $k = (1.7 \pm 0.1) \times 10^{10}$ M$^{-2}$s$^{-1}$ for the third-order rate equation (5).

Physiological pH is 7.4. Substituting this into equation (5) yields a second-order rate law at pH 7.4

$$\text{rate} = k_1[\text{Cbl(I)}][\text{NO}_2^-]$$  \hspace{1cm} (7)

where $k_1 = (6.6 \pm 0.5) \times 10^2$ M$^{-1}$s$^{-1}$. As the pH decreases, the rate constant is expected to increase according to the model used. The rate of the reaction was not investigated near the pK$_a$ of HNO$_2$ (pK$_a$ 3.16$^{46}$) because the reaction at pH values less than 6.50 was too fast to be measured using stopped-flow spectrophotometry. As the pH increases, the rate approaches 0, as seen in Figure 13. This suggests that the reactive species is HNO$_2$
Figure 12: Plot of $k_{\text{obs}}$ versus NaNO$_2$ concentration (25.0 °C, $I = 0.50$ M NaH$_2$PO$_4$/Na$_2$HPO$_4$) for (a) pH 6.50 (0.050 M MES), (b) pH 6.92 (0.050 M BIS-TRIS), (c) pH 7.40 (Na$_2$HPO$_4$), (d) pH 8.51 (0.050 M TAPS), (e) pH 9.51 (0.050 M CHES), and (f) pH 10.80 (0.050 M CAPS). Linear regressions were performed on the data at each pH. The slopes ($k_{\text{app}}$ in M$^{-1}$s$^{-1}$) were found to be (a) $(4.94 \pm 0.04) \times 10^3$, (b) $(2.8 \pm 0.1) \times 10^3$, (c) $(9.9 \pm 0.3) \times 10^2$, (d) $(1.21 \pm 0.07) \times 10^2$, (e) $12.4 \pm 0.5$, and (f) $0.49 \pm 0.02$, respectively. The intercepts (in s$^{-1}$) were found to be (a) $5.7 \pm 0.3$, (b) $2.5 \pm 0.8$, (c) $0.2 \pm 0.1$, (d) $0.06 \pm 0.02$, (e) no intercept, (f) $(1.3 \pm 0.4) \times 10^{-3}$, respectively.
instead of NO₂⁻. HNO₂ is a stronger oxidant \( E(\text{HNO}_2/\text{N}_2) = 1.45 \text{ V, pH 0; } E(\text{NO}_2^-/\text{N}_2) = 0.41 \text{ V, pH 14 versus SHE}^{42} \) due to electron-withdrawing effects of the acidic proton.

Substituting the equilibrium expression and the \( K_a \) for HNO₂ in the rate law given in equation (7) gives a rate law expressed in terms of HNO₂:

\[
\text{rate} = k_2 [\text{Cbl(I)}][\text{HNO}_2] \tag{8}
\]

where \( k_2 = (1.15 \pm 0.08) \times 10^7 \text{ M}^{-1}\text{s}^{-1} \).

**Table 12:** Apparent rate constants (\( k_{\text{app}} \)) and intercepts of the plots for the reaction of Cbl(I) with NO₂⁻ as a function of pH (25.0 °C, \( I = 0.50 \text{ M NaH}_2\text{PO}_4 / \text{Na}_2\text{HPO}_4 \)).

<table>
<thead>
<tr>
<th>pH</th>
<th>( k_{\text{app}} ) (M⁻¹s⁻¹)</th>
<th>Intercept (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.50</td>
<td>4940 ± 40</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>6.92</td>
<td>2800 ± 100</td>
<td>2.5 ± 0.8</td>
</tr>
<tr>
<td>7.40</td>
<td>990 ± 30</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>8.51</td>
<td>121 ± 7</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>9.51</td>
<td>12.4 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>10.80</td>
<td>0.49 ± 0.02</td>
<td>0.0013 ± 0.0004</td>
</tr>
</tbody>
</table>

**Figure 13:** Plot of \( k_{\text{app}} \) versus pH. The data was fitted to equation (3) in the text, giving \( k = (1.7 \pm 0.1) \times 10^{10} \text{ M}^{-2}\text{s}^{-1} \).
Figure 14 gives a plot of the intercept of the $k_{\text{obs}}$ versus NO$_2^-$ plots (Figure 12) for the reaction between Cbl(I) and NO$_2^-$ at each pH condition. The intercepts are indicative of a second reaction competing with the Cbl(I) + NO$_2^-$ reaction. Figure 14 shows that the rate of the second reaction increases with decreasing pH. For pH 6.92 and 6.50, it was found that decomposition of Cbl(I) to Cbl(II) is significant during the time periods of the reaction. It was assumed that the process would occur within five half-lives derived from the intercepts of the $k_{\text{obs}}$ versus NO$_2^-$ concentration plots; that is, within 1.4 s at pH 6.92 and within 0.61 s at pH 6.50. Although the fit of the Cbl(I) decomposition to a first-order equation was poor, as a first approximation, the data was fitted to the first-order kinetics equation (2). At pH 6.92, the average rate constant was $2.4 \pm 0.2$ s$^{-1}$ in agreement with the intercept value of $2.5 \pm 0.8$ s$^{-1}$. At pH 6.50, the average rate constant was $3.9 \pm 0.7$ s$^{-1}$ compared with the intercept value of $5.7 \pm 0.3$ s$^{-1}$. The results for pH 6.50 were more

![Figure 14](image.png)

**Figure 14:** Plot of the intercepts from the $k_{\text{obs}}$ versus NO$_2^-$ plots (Figure 12) versus pH. The intercepts at pH 7.40 and higher are insignificant, but the intercepts in acidic pH are significant.
variable and appeared to be experiment-dependent. The observation of this process supports the hypothesis that Cbl(I) oxidation to Cbl(II) competes with the reaction of Cbl(I) with NO$_2^-$ under acidic pH conditions. This reaction is most likely attributable to the reduction of H$^+$ by Cbl(I) to give H$_2$ and Cbl(II). The reduction potential of H$^+$ to give H$_2$ is 0 V by definition of the SHE, and the reduction potential of the Cbl(II)/Cbl(I) redox couple is -0.600 V versus SHE.$^{20}$ Therefore, the cell potential ($E_{cell}$) is 0.600 V and, the formula

$$\Delta G = -nFE_{cell}$$

(9)

where $n$ is the number of electrons involved in the redox process and $F$ is the Faraday constant, ($F = 96,485$ Cmol$^{-1}$) allows computation of the Gibbs free energy ($\Delta G$). The $\Delta G$ is negative ($\Delta G = -232$ kJ/mol), indicative of a spontaneous process. Acid dependent decomposition of Cbl(I) has also been reported by others.$^{47}$

A mechanism can be proposed for the reaction between Cbl(I) and NO$_2^-$ based on the complete balanced equation (1) and the kinetic data. Cbl(I) can be oxidized by HNO$_2$ to Cbl(II) by a 1e$^-$ process or to hydroxocobalamin HOCbl by a 2e$^-$ process. Scheme 3(a) outlines the 1e$^-$ process, and Scheme 3(b) outlines the 2e$^-$ process.

The rate-determining step of the 1e$^-$ process (Scheme 3(a)) results in reduction of HNO$_2$ to 'NO, and the subsequent reaction (equation (11)) of Cbl(I) with 'NO has been studied by others.$^{48}$ The subsequent reaction of the mechanism is also known,$^{49}$ and gives N$_2$ as the final product. This reaction pathway does not produce the experimentally observed NH$_2$OH and gives a stoichiometry of 3:1 Cbl(I):HNO$_2$ instead of the experimentally determined 4:1 stoichiometry. Thus, Scheme 3(a) can be ruled out.
\[ \text{Scheme 3: Postulated reaction pathways for the reaction between Cbl(I) and HNO}_2. \text{ (a) gives the pathway for a 1e}^\text{-rate-determining step and (b) gives the pathway for a 2e}^\text{-rate-determining step.} \]

The rate-determining step of the 2e\text{-} process (Scheme 3(b)) results in reduction of HNO\textsubscript{2} to HNO. HNO is an oxidizing agent and is reduced to NH\textsubscript{2}OH.\textsuperscript{50} The reduction potential of HNO, 2H\textsuperscript{+} / NH\textsubscript{2}OH is 0.3 V versus SHE at pH 7,\textsuperscript{51} making it a moderate oxidizing agent capable of oxidizing Cbl(I) (\( E(Cbl(II)/Cbl(I)) = -0.600 \text{ V versus SHE}^\text{20} \)). Thus equation (15) is thermodynamically feasible. Alternatively, N\textsubscript{2}O rather than HNO could be produced in the first step of this pathway, but others have shown that N\textsubscript{2}O reacts with Cbl(I) to give N\textsubscript{2},\textsuperscript{49} which was not the experimentally observed product. Finally, the first step in Scheme 3(b) (equation (14)) must be the slow step of the process because there is no observed accumulation of the HOCl\textsuperscript{+} intermediate. Figure 6(a) shows clean and complete conversion of Cbl(I) to Cbl(II), so equations (15) and (16) must be rapid compared to equation (14). Importantly, the rate constant for the reaction of Cbl(I) with
Cbl(III) is $3.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, which is significantly larger than the second-order rate constant for the reaction of Cbl(I) and NO$_2^-$ at pH 7.4 ($6.6 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$).
IV. CONCLUSIONS

The stoichiometry, reaction products, and the kinetics of the reaction of Cbl(I) with NO$_2^-$ have been determined. The Cbl product of the reaction was identified as Cbl(II) by UV-Vis spectrophotometry. Stoichiometry experiments showed that 0.25 mole equiv. of NO$_2^-$ is required for the reaction to proceed to completion. Thus, Cbl(I) and NO$_2^-$ react in a 4:1 mole ratio. The non-Cbl reaction product was determined to be NH$_2$OH as suggested by the reaction stoichiometry and the indooxine test for NH$_2$OH. The amount of indooxine product formed did not quantitatively account for the expected amount of NH$_2$OH produced according to the reaction stoichiometry, but control experiments showed that the Cbl(II) reaction product catalyzes the disproportionation of NH$_2$OH in solution. Bubbling oxygen through the solution before doing the indooxine test recovers some of the NH$_2$OH, although full recovery could not be achieved. The complete balanced equation for the reaction is

$$4\text{H}^+ + 4\text{Cbl(I)}^- + \text{HNO}_2 \rightarrow 4\text{Cbl(II)} + \text{NH}_2\text{OH} + \text{H}_2\text{O}$$

(18)

The kinetics of the reaction between Cbl(I) and NO$_2^-$ were studied under pseudo-first-order conditions as a function of pH. Each reactant was found to exhibit first-order dependence with respect to its concentration. The rate law of the reaction for Cbl(I) with NO$_2^-$ was found to be

$$\text{rate} = k[\text{Cbl(I)}][\text{NO}_2^-][\text{H}^+]$$

(19)

The rate constant, $k$, is $(1.7 \pm 0.1) \times 10^{10}$ M$^{-2}$s$^{-1}$. Under physiologically relevant conditions
(pH 7.4), the rate expression becomes

$$\text{rate} = k_1 [\text{Cbl(I)}][\text{NO}_2^-]$$  \hspace{1cm} (20)

where $k_1 = (6.6 \pm 0.5) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ for the second-order process. The reactive species is thought to be HNO$_2$, so the rate law can also be expressed as

$$\text{rate} = k_2 [\text{Cbl(I)}][\text{HNO}_2]$$  \hspace{1cm} (21)

where $k_2 = (1.15 \pm 0.08) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.4. It has been determined that the source of the intercepts in the plots of $k_{\text{obs}}$ versus NO$_2^-$ concentration under acidic pH conditions is acid catalyzed decomposition of Cbl(I) to Cbl(II).

The mechanism of the reaction was determined to be as follows:

\[
\begin{align*}
(2)\text{H}^+ + \text{Cbl}(I)^- + \text{HNO}_2 & \quad \xrightarrow{\text{slow}} \quad \text{HOCbl}^+ + (\text{H})\text{NO} + \text{H}_2\text{O} \\
(2)\text{3H}^+ + \text{Cbl}(I)^- + (\text{H})\text{NO} & \quad \xrightarrow{\text{fast}} \quad \text{HOCbl}^+ + \text{NH}_2\text{OH} \\
2\text{Cbl}(I)^- + 2\text{HOCbl}^+ & \quad \xrightarrow{\text{fast}} \quad 4\text{Cbl}(\text{II}) \\
4\text{H}^+ + 4\text{Cbl}(I)^- + \text{HNO}_2 & \quad \xrightarrow{\text{fast}} \quad 4\text{Cbl}(\text{II}) + \text{NH}_2\text{OH} + \text{H}_2\text{O}
\end{align*}
\hspace{1cm} (22, 23, 24, 25)
\]

Tissue NO$_2^-$ concentrations are as high as 10 µM$^{8,15}$ under normal conditions and may be significantly elevated during periods of cellular oxidative and nitrosative stress. The total Cbl concentration in cells is 30-700 nM in humans$^{52}$ which is significantly lower than the concentration of NO$_2^-$. Cbl(I) itself is a transient species present at very low concentrations. To give perspective, the NO$_2^-$ concentration (10 µM) can be substituted into the rate law in equation (20) to give

$$\text{rate} = k_3 [\text{Cbl(I)}]$$  \hspace{1cm} (26)

where $k_3 = (6.6 \pm 0.5) \times 10^{-4} \text{ s}^{-1}$ for the first-order process. The half-life, $t_{1/2}$, for the reaction at pH 7.4 with 10 µM NO$_2^-$ is therefore estimated to be 17 min. Under periods of
oxidative / nitrosative stress, the NO$_2^-$ concentration will be significantly higher, and the half-life therefore shorter.

Both mammalian B$_{12}$-dependent enzymes are oxidized under oxidative and nitrosative stress conditions.$^{53}$ Thus, Cbl(I), while bound to the B$_{12}$ enzymes or B$_{12}$ transport proteins, is still accessible to rapidly react with small ROS and RNS including NO$_2^-$. Cbl(I) is formed as a precursor to the formation of the two B$_{12}$ cofactors CH$_3$Cbl and AdoCbl in addition to its role as a transient intermediate in the methionine synthase reaction.$^{20}$ The B$_{12}$-dependent enzyme reactions are known to be deactivated under periods of oxidative and nitrosative stress.$^{33}$ This study has shown that the reaction of Cbl(I) with HNO$_2$ most likely results in deactivation the Cbl(I) intermediate in the metabolically important methionine synthase and MMCM reactions.
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