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Mechanistic Studies on the Reaction of Nitrocobalamin with Glutathione: Kinetic evidence for formation of an aquacobalamin intermediate

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Abstract

The essential but also toxic gaseous signaling molecule nitric oxide is scavenged by the reduced vitamin B_{12} complex cob(II)alamin. The resulting complex, nitroxylcobalamin (NO^--Cbl(III)), is rapidly oxidized to nitrocobalamin (NO_2Cbl) in the presence of oxygen; however it is unlikely that nitrocobalamin is itself stable in biological systems. Kinetic studies on the reaction between NO_2Cbl and the important intracellular antioxidant, glutathione (GSH), are reported. In this study, a reaction pathway is proposed in which the β-axial ligand of NO_2Cbl is first substituted by water to give aquacobalamin (H_2OCbl^+), which then reacts further with GSH to form glutathionylcobalamin (GSCbl). Independent measurements of the four associated rate constants k_1, k_{-1}, k_2, and k_{-2} support the proposed mechanism. These findings provide insight into the fundamental mechanism of ligand substitution reactions of cob(III)alamins with inorganic ligands at the β-axial site.

Keywords

Vitamin B_{12}; Cob(III)alamin; Bioinorganic chemistry; Kinetics; Reaction mechanisms

Introduction

Two essential enzymes in mammals, L-methylmalonyl-CoA mutase, and methionine synthase, and numerous bacterial enzymes require the vitamin B_{12} derivatives (= cobalamins, Cbls) adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) as cofactors, Figure 1.[1] MeCbl-dependent methionine synthase transfers a methyl group from methyltetrahydrofolate to homocysteine to generate tetrahydrofolate and methionine, whereas AdoCbl-dependent L-methylmalonyl-CoA mutase catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA.[1] Cobalamins may also have additional roles in biological systems, including regulating the immune response and protecting against intracellular oxidative stress.[2]

The signaling molecule nitric oxide (•NO) plays a key role in the immune response, vasodilation, and neurotransmission.[3] However, high levels of NO can be deleterious and can result in sepsis and septic shock,[4] leading to organ failure and even death. Importantly,

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administering cobalamins suppresses •NO-induced relaxation of smooth muscle,[5] •NO-induced vasodilation[6] and •NO-mediated inhibition of cell proliferation.[7] Cobalamins also reverse •NO-induced neural tube defects.[8] Both mammalian B_{12}-dependent enzymes are inhibited by •NO.[9] With the exception of glutathionylcobalamin (X = glutathione, Figure 1), •NO does not directly react with cob(III)alamins,[10] whereas the rate of the reaction between cob(II)alamin and nitric oxide to form nitrosylcobalamin (NOCbl) is almost diffusion controlled and essentially irreversible.[11] Given that all cob(III)alamins are readily reduced by intracellular reductases,[12] it is likely that cob(II)alamin reacts with •NO in biological systems, to form NOCbl.

In discussions of the biological relevance of NOCbl formation, we have observed that authors neglect to mention that NOCbl itself is not a stable entity.[5a, 6–8, 9c–e] However, in the presence of even minute amounts of air, orange NOCbl rapidly oxidizes to form red nitrocobalamin, NO_2Cbl.[13] Indeed, we have used this property of NOCbl in our laboratory numerous times to check the condition of valves and taps used in strictly anaerobic experimental setups. The intracellular fate of NO_2Cbl is unclear. Possibilities include NO_2Cbl being reduced by intracellular reductases, and/or NO_2Cbl reacting with the strong nucleophile and reductant glutathione (GSH), which is present in mM concentrations in cells.[14] In this work we report kinetic studies on the reaction of NO_2Cbl with glutathione. Interestingly, our kinetic data show that GSH does not directly react with NO_2Cbl, but instead reacts with aquacobalamin, which is always present in equilibrium, albeit typically in small amounts, with cobalamins incorporating inorganic ligands at the β-axial cobalamin site (Figure 1).

Results and Discussion

Kinetic data were collected for the reaction of NO_2Cbl with glutathione (GSH). Experiments were initiated by adding a small aliquot of concentrated aqueous NO_2Cbl solution (final concentration 5.0×10^{-5} M) to a buffered GSH solution (3.00 mL) at a specific pH condition (I = 1.0 M ($NaCF_3SO_3$)). Control experiments established that rate constants were identical within experimental error in the absence and presence of air; hence all experiments were carried out under aerobic conditions. Importantly, dissolving NO_2Cbl in water rather than in buffer minimized the decomposition of NO_2Cbl to aquacobalamin (H_2OCbl^+) prior to the addition of an aliquot of this solution into a buffered GSH solution.

Figure 2(a) shows UV-vis spectral changes observed upon the addition of NO_2Cbl to a buffered GSH solution (5.00×10^{-2} M) at pH 4.00. NO_2Cbl (λ_{max} 354, 413 and 532) is converted to GSCbl (λ_{max} 333, 372, 428 and 534[15]) with isosbestic points at 336, 367, 452 and 543, indicating that a single reaction occurs. The corresponding plot at 354 nm versus time is given in Figure 2(b). The data fit well to the first-order rate equation, giving an observed rate constant, $k_{obs} = (1.15 \pm 0.07) \times 10^{-2}$ s^{-1}.

Kinetic data were collected at pH 4.00 and 7.00, in order to determine whether the rate of the reaction is pH dependent. Plots of k_{obs} versus total GSH concentration are shown in Figures 3(a) and 3(b). These plots indicate that the observed rate constant reach a limiting value at high GSH concentrations and that there is no pH dependence in this pH region. There are two plausible mechanisms by which NO_2Cbl reacts with GSH to give a plot exhibiting curvature to reach a limiting value of k_{obs} at high GSH concentrations (saturation kinetics). The first involves rapid equilibration to form a NO_2Cbl•GSH association complex prior to rate-determining substitution of the β-axial NO_2^- ligand of NO_2Cbl by GSH to give GSCbl. The other alternative is a two step process, in which H_2OCbl^+, which is in equilibrium with NO_2Cbl, reacts with GSH, Scheme 1. Given that all Cbls with β-axial inorganic ligands exist in equilibrium with H_2OCbl^+ and that Cbls undergo β-axial ligand

exchange via a dissociative interchange mechanism,[16] the latter possibility is more likely to occur. Kinetic studies on the reaction between H_2OCbl^+/HOCbl and GSH have been reported,[17] and show that H_2OCbl^+ reacts rapidly with GSH to form GSCbl under the conditions of our study.

The rate expression corresponding to the reaction pathway shown in Scheme 1 is [18]

$$k_{obs}=(k_1k_2[GSH]/(k_{-1}[NO_2^-]))+k_{-2})/(1+k_2[GSH]/(k_{-1}[NO_2^-])) \quad (1)$$

$$=(k_1K[GSH])+k_{-2})/(1+K[GSH]) \quad (2)$$

where $K = k_2/(k_{-1}[NO_2^-])$. The rate constant, k_{-2}, for decomposition of GSCbl to H_2OCbl^+ and GSH was independently determined at pH 4.00 and found to be $(7.4 \pm 0.5) \times 10^{-4}$ s^{-1}, Figure S1, Supporting Information. Previous studies have shown that the observed equilibrium constant for formation of GSCbl, K_{obs}(GSCbl), = k_2/k_{-2}, increases by approximately one order of magnitude for each unit change in pH.[17] Since k_2 is pH independent,[17] k_{-2} is therefore negligible at pH 7.00. Data in Figure 3(a) were fitted to eq (2) fixing $k_{-2} = 7.4 \times 10^{-4}$ s^{-1}, giving $k_1 = (1.75 \pm 0.02) \times 10^{-2}$ s^{-1} and $K = 94.5 \pm 3.7$ M^{-1}. Data in (b) were fitted to the same equation fixing $k_{-2} = 0$ s^{-1}, giving $k_1 = (1.73 \pm 0.05) \times 10^{-2}$ s^{-1} and $K = 102.1 \pm 9.7$ M^{-1}. K is therefore also pH independent in the pH 4–7 region, within experimental error.

Importantly, if our model is correct, then $K = k_2/(k_{-1}[NO_2^-])$. Note, however, that the free nitrite concentration is not strictly constant during the reaction, but will vary from 0 to a maximum value of 5.0×10^{-5} M as the reaction proceeds, as NO_2Cbl (5.0×10^{-5} M) reacts with GSH to give GSCbl plus NO_2^-. Hence K is not strictly constant during the reaction, as reflected in the error associated with K. In order to provide support for our model, new data was therefore collected at pH 7.00 under the same conditions as the experiments summarized in Figure 3(b), except that 5.00×10^{-4} M $NaNO_2$ was added to each solution, so the nitrite concentration is constant (pseudo-first order conditions) during the reaction. These data are shown in Figure 4. Fitting this data to eq (2) fixing $k_{-2} = 0$ s^{-1} gave $k_1 = (1.60 \pm 0.05) \times 10^{-2}$ s^{-1} and $K = 16.2 \pm 0.2$ M^{-1}. The data now fit considerably better to eq (2) as expected (the error in K is now one order of magnitude smaller), since the nitrite concentration remains constant during the reaction.

The rate constant k_2 for the reaction of H_2OCbl^+ with GSH was also independently determined at pH 4.00 and found to be 12.00 ± 0.25 M^{-1} s^{-1} (25.0 °C, 0.020 M $NaCH_3COO$, I = 1.00 M ($NaCF_3SO_3$)), Figure S2, Supporting Information. This value is in good agreement with a value reported by us under slightly different conditions (k_2 = 18.5 M^{-1} s^{-1} at pH 4.50, 25.0 °C, 0.10 M NaOAc, I = 0.50 M (KNO_3)[17]), and is pH independent in the pH 4–7 range.[17] The rate constant k_{-1} for the reaction between H_2OCbl^+ and NO_2^- was independently determined to be $(1.25 \pm 0.02) \times 10^3$ and $(1.20 \pm 0.02) \times 10^3$ M^{-1} s^{-1} at pH 4.00 and 7.00, respectively, Figures S3 and S4, Supporting Information. Hence k_{-1} is also independent of the pH (pH 4–7), as expected, as the ionization of the reactants is essentially unchanged ($pK_a(HNO_2) \sim 3.2$; $pK_a(H_2OCbl^+) = 7.8$ [17]) in this pH region. The value of k_{-1} agrees well with a value reported by others ($k_{-1} = 99.8 \times 10^2$ M^{-1} s^{-1} at 25 °C, I = 2.2 M ($NaNO_3$)).[19] Using $k_2 = 12.00$ M^{-1} s^{-1}, $k_{-1} = 1.23 \times 10^3$ M^{-1} s^{-1} and $NO_2^- = 5.00 \times 10^{-4}$ M gives K ~20 M^{-1}, which is in very good agreement with the experimental value of K obtained from the best fit of the data (16.2 ± 0.2 M^{-1}), providing strong support for the reaction pathway proposed in Scheme 1.

Finally, the rate constant k_1 for decomposition of NO_2Cbl to H_2OCbl^+ was also independently determined by obtaining kinetic data for the decomposition of NO_2Cbl to H_2OCbl^+ upon dissolving NO_2Cbl in buffer. Figure S5 in the Supporting Information shows the absorbance change (ΔAbs = 0.013 at 350 nm) that occurs at pH 4.00. Only a small fraction of NO_2Cbl is converted to H_2OCbl^+; however the absorbance change is sufficient to allow calculation of k_1. The results at different pH conditions are summarized in Table S1, and give a mean value for k_1 of $(1.48 \pm 0.22) \times 10^{-2}$ M^{-1} s^{-1} (pH 3.5 – 6.0; k_1 is pH independent). The observed rate constant for NO_2Cbl partially decomposing to give H_2OCbl^+ is actually $k_1 + k_{-1}[NO_2^-]$ for the pseudo-first-order reversible process. A separate experiment showed that an absorbance difference of 0.232 is observed upon completely converting H_2OCbl^+ to NO_2Cbl upon the addition of a slight excess of NO_2^- ($Cbl_T = 5.0 \times 10^{-5}$ M). Hence an absorbance change of 0.013 corresponds to formation of 5.6% H_2OCbl^+ (2.8×10^{-6} M H_2OCbl^+ and 2.8×10^{-6} M NO_2^-) upon dissolving NO_2Cbl in H_2OCbl^+; that is, the maximum NO_2^- is 2.8×10^{-6} M. Using $k_1 = 1.48 \times 10^{-2}$ M^{-1} s^{-1}, $k_{-1} = (1.25 \pm 0.02) \times 10^3$ and $[NO_2^-] = 2.8 \times 10^{-6}$ M shows that k_1 is ~ 5 times larger than $k_{-1}[NO_2^-]$, validating the assumption that $k_{obs} \sim k_1$ upon dissolving NO_2Cbl in H_2OCbl^+. Using our values of k_1 and k_{-1}, the equilibrium constant for formation of NO_2Cbl, $K(NO_2Cbl) = k_{-1}/k_1$, is 8.5×10^4 M^{-1}, which is in reasonable agreement with a value reported by others under different ionic strength conditions ($K(NO_2Cbl) = 2.2 \times 10^5$ M^{-1}, 25 °C, I = 2.2 M [20]).

Conclusions

Kinetic studies on the reaction between NO_2Cbl and GSH show that the rate of the reaction is pH independent in the pH 4–7 region. By independently determining values of k_1, k_{-1}, k_2 and k_{-2}, we have shown that the data fits a model involving an H_2OCbl^+ intermediate, which then rapidly reacts with GSH to form GSCbl, Scheme 1. To our knowledge this is the first time that the reaction pathway of β-axial inorganic ligand exchange for cob(III)alamins via an H_2OCbl^+ intermediate has been unequivocally demonstrated. This may have important consequences for free and potentially even protein-bound cob(III)alamins incorporating inorganic ligands (X-ray structures of cobalamins bound to B_{12} transport proteins show that the β-axial site can be readily accessed by solvent and small molecules[21]) that is, the amount of each of these species may reflect the concentrations and binding constants to aquacobalamin of the various inorganic ligands present. As such, GSCbl would be expected to be the major intracellular non-alkylcob(III)alamin, given that intracellular GSH concentrations are mM,[14] and only CN^- binds stronger than GSH to H_2OCbl^+($K_{CNCbl} \sim 10^{14}$ M^{-1} [22]). Finally, at 0.5 mM GSH the rate constant for the reaction between NO_2Cbl and GSH is ~ 8×10^{-3} s^{-1} at pH 7.0 (25 °C), corresponding to a half life of ~ 1.4 min. Hence formation of GSCbl is one possible reaction pathway by which NO_2Cbl decomposes in biological systems.

Experimental Section

General

Hydroxycobalamin hydrochloride (HOCbl•HCl, 98% stated purity by manufacturer) was purchased from Fluka. Glutathione (98%), acetic acid (sodium salt, ≥99%), sodium nitrite (97%) and CF_3SO_3H (99%) were obtained from Acros Organics. TES buffer (98%) was purchased from MP Biomedicals Inc. Potassium dihydrogen phosphate was purchased from Sigma. $NaCF_3SO_3$ was prepared by neutralizing a concentrated, aqueous solution of CF_3SO_3H with NaOH, reducing it to dryness by rotary evaporation, and drying it overnight in a vacuum oven at 70.0 °C. Nitrocobalamin was synthesized and characterized according to a published procedure.[15] The purity was ≥ 95%, as determined by ^1H NMR spectroscopy.

UV-visible spectra and kinetic data for slower reactions 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). Reactant solutions were thermostated for 15 min prior to measurements. Kinetic data for rapid reactions were obtained 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. Data were 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. All data were analyzed using Microcal Origin version 8.0.

pH measurements were carried out using an Orion model 710A pH meter equipped with a Wilmad 6030–02 pH electrode. The electrode was filled with a 3 M KCl/saturated AgCl solution (pH 7.0) and standardized with standard BDH buffer solutions at pH 6.98, 4.01 and 2.02. Solution pH was adjusted using 50% v/v aqueous $CF_3SO_3H$and NaOH (~ 5 M).

^1H NMR spectra was recorded on a Bruker Avance 400 MHz spectrometer equipped with 5 mm probe. Solutions for NMR measurements were prepared in D_2O. ^1H NMR spectra were internally referenced to TSP (0 ppm).

Kinetic measurements

The rates of the reaction between NO_2Cbl and glutathione (GSH) were determined under pseudo-first-order conditions with excess GSH. Stock solutions of GSH (0.500 M) in the presence or absence of sodium nitrite (5.00×10^{-4} M) were prepared in the appropriate buffer (0.020 M) at pH 4.00 and 7.00 and diluted as appropriate. A small aliquot of concentrated NO_2Cbl (final concentration 5.0×10^{-5} M) in water was added to initiate the reaction and the absorbance at 354 nm was recorded as a function of time.

Kinetic data for the reaction between H_2OCbl^+ (5.0×10^{-5} M) with varying concentrations of NO_2^- were obtained at pH 4.00 and 7.00. Stock solutions of $NaNO_2$ (0.010 M) were prepared in the appropriate buffer (0.020 M) and diluted as needed. Data were collected at 354 nm. Kinetic data for the reaction of H_2OCbl^+ (5.0×10^{-5} M) with varying concentrations of glutathione at pH 4.00 were collected at 354 nm in acetate buffer (0.020 M).

The rate of decomposition of NO_2Cbl to H_2OCbl^+ was determined in the pH 3.5–6.0 range by adding solid NO_2Cbl directly to the appropriate buffer solution (0.200 M; the solution was thermostated at 25.0 °C for 10 min prior to the addition of $NaNO_2$). The solution was quickly filtered through a micropore filter (0.45 μm) and data collection initiated at 354 nm.

The rate of decomposition of GSCbl to H_2OCbl^+ was determined at pH 4.00. An aliquot of GSCbl (4.0×10^{-5} M) was added to pH 4.00 acetate buffer (0.020 M; the buffer solution was thermostated at 25.0 °C for 10 min prior to the addition of GSCbl) and data were collected at 354 nm.

The total ionic strength was maintained at 1.0 M using $NaCF_3SO_3$ for all solutions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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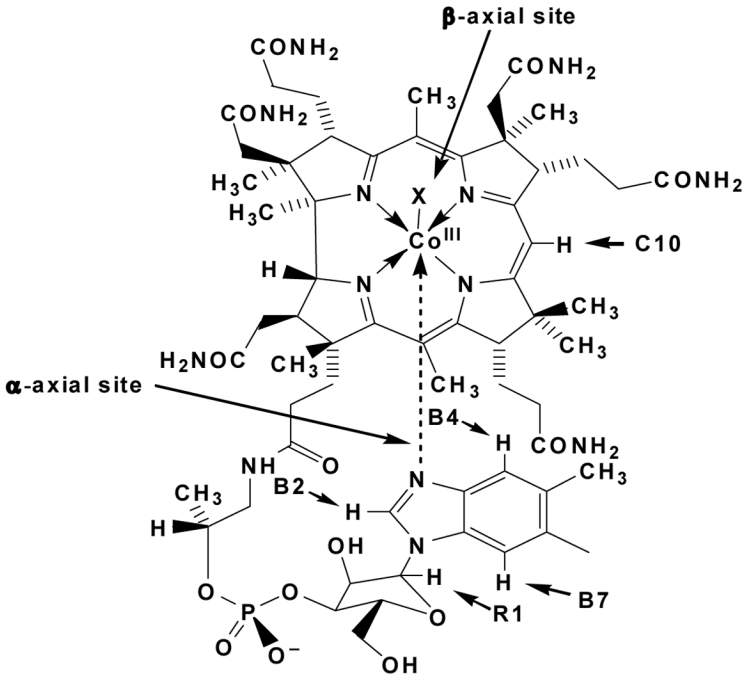


Figure 1.
Structure of cobalamins showing the two axial sites (upper = β, lower = α) with respect to the corrin ring.

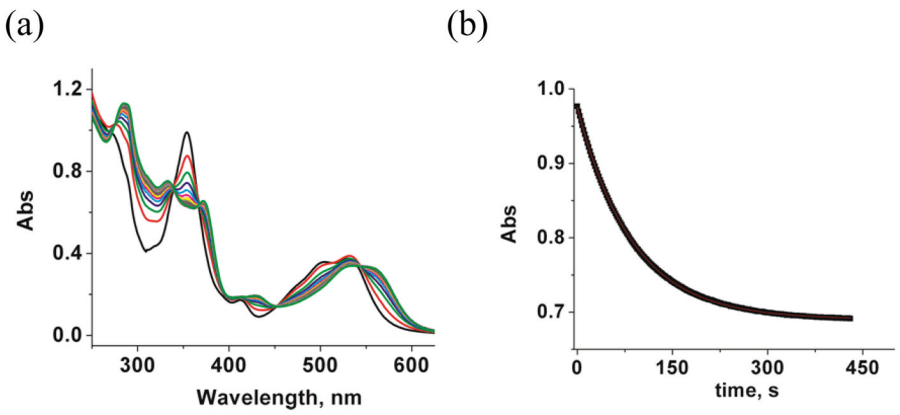


Figure 2.

(a) UV-vis spectra for the reaction of GSH (5.00×10^{-2} M) with NO_2Cbl (5.0×10^{-5} M) at pH 4.00 (25.0 °C, 0.020 M NaOAc, I = 1.0 M, $NaCF_3SO_3$). Selected spectra for the reaction are shown every 1.00 min. (b) Plot of absorbance at 354 nm versus time for the experiment shown in 2(a). Data were fitted to a first-order rate equation, giving $k_{obs} = (1.15 \pm 0.07) \times 10^{-2}$ s^{-1}.

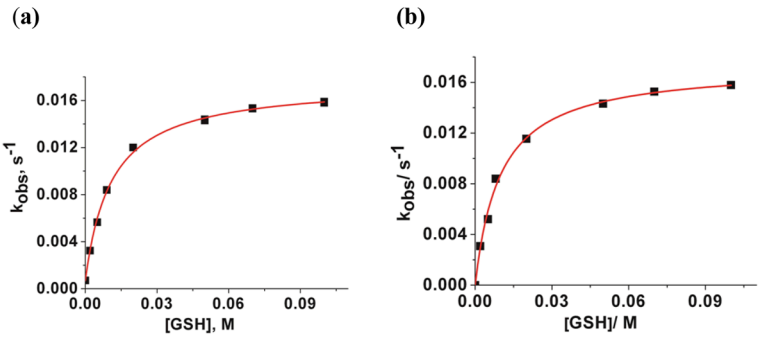


Figure 3.

Plots of k_{obs} vs [GSH] at pH 4.00 (a) and 7.00 (b) for the reaction between NO_2Cbl (5.00×10^{-5} M) and glutathione (25.0 °C, 0.020 M NaOAc (a) or 0.020 M KH_2PO_4 (b), I = 1.0 M ($NaCF_3SO_3$)). Data in (a) were fitted to eq (2) in the text fixing $k_{-2} = 7.4 \times 10^{-4}$ s^{-1}, giving $k_1 = (1.75 \pm 0.02) \times 10^{-2}$ s^{-1} and $K = 94.5 \pm 3.7$ M^{-1} at pH 4.00. Data in (b) were fitted to eq (2) fixing $k_{-2} = 0$ s^{-1}, giving $k_1 = (1.73 \pm 0.05) \times 10^{-2}$ s^{-1} and $K = 102.1 \pm 9.7$ M^{-1} at pH 7.00.

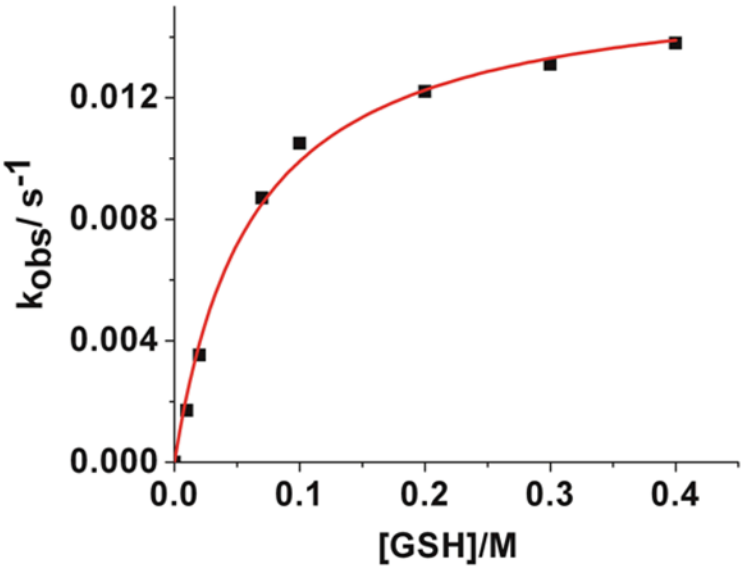
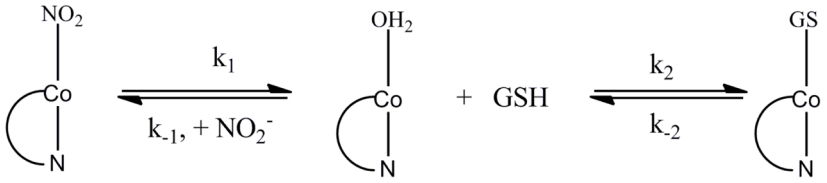


Figure 4.

Plot of k_{obs} vs [GSH] for the reaction between NO_2Cbl (5.0×10^{-5} M) and GSH in the presence of 5.00×10^{-4} M $NaNO_2$ at pH 7.00 (25.0°C, 0.020 M KH_2PO_4, 5.00×10^{-4} M $NaNO_2$, I = 1.0 M, $NaCF_3SO_3$). Data were fitted to eq (2) in the text fixing $k_{-2} = 0$, giving $k_1 = (1.60 \pm 0.05) \times 10^{-2} s^{-1}$ and $K = 16.2 \pm 0.2\ M^{-1}$.



Scheme 1.

Proposed reaction pathway for the reaction of NO_2Cbl with GSH. Note that in aqueous solution H_2OCbl^+ exists in equilibrium with HOCbl ($pK_a(H_2OCbl^+) = 7.8$ [17]); however at the pH conditions of our kinetic experiments HOCbl formation is unimportant, since the values of the rate constants k_{-1} and k_2 are the same at pH 4.00 and 7.00.