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Mechanistic Studies on the Reaction between Cob(II)alamin and Peroxynitrite: Evidence for a Dual Role for Cob(II)alamin as a Scavenger of Peroxynitrous Acid and Nitrogen Dioxide

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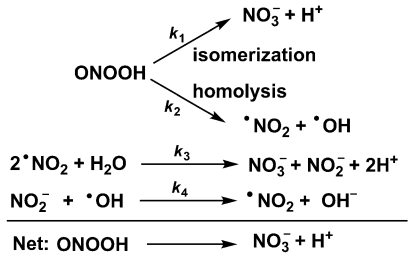
Abstract: Peroxynitrite/peroxynitrous acid ($ONOO^-$/ONOOH; $pK_{a(ONOOH)}$ = 6.8) is implicated in multiple chronic inflammatory and neurodegenerative diseases. Both mammalian B_{12}-dependent enzymes are inactivated under oxidative stress conditions. We report studies on the kinetics of the reaction between peroxynitrite/peroxynitrous acid and a major intracellular vitamin B_{12} form, cob(II)alamin (Cbl(II)), using stopped-flow spectroscopy. The pH dependence of the reaction is consistent with peroxynitrous acid reacting directly with Cbl(II) to give cob(III)alamin (Cbl(III)) and $^{\bullet}NO_2$, followed by a subsequent rapid reaction between $^{\bullet}NO_2$ and a second molecule of Cbl(II) to primarily form nitrocobalamin. In support of this mechanism, a Cbl(II)/ONOO(H) stoichiometry of 2:1 is observed at pH 7.35 and 12.0. The final major Cbl(III) product observed (nitrocobalamin or hydroxycobalamin) depends on the solution pH. Analysis of the reaction products in the presence of tyrosine—a well-established $^{\bullet}NO_2$ scavenger—reveals that Cbl(II) reacts with $^{\bullet}NO_2$ at least an order of magnitude faster than tyrosine itself. Given that protein-bound Cbl is accessible to small molecules, it is likely that enzyme-bound and free intracellular Cbl(II) molecules are rapidly oxidized to inactive Cbl(III) upon exposure to peroxynitrite or $^{\bullet}NO_2$.

Keywords: cobalt · kinetics · oxidative stress · peroxynitrite · vitamin B_{12}

Introduction

There is currently considerable interest in exploring the reactivity of the potent reactive nitrogen species peroxynitrite (peroxynitrite/peroxynitrous acid; $ONOO^-$/ONOOH; $pK_{a(ONOO(H))} = 6.8$),[1] an isomer of nitrate. ONOO(H) formation is implicated in oxidative/nitrosative stress-initiated pathologies, including shock, chronic inflammation, chemical sensitivity, chronic fatigue syndrome, vascular disease, and neurodegeneration.[2] ONOO(H) is a powerful oxidizing ($E°$ ($ONOO^-$, $2H^+$/$^{\bullet}NO_2$, H_2O) = 1.6 V, pH 7)[3] nitrating and/or hydroxylating agent.[2b,4] The anionic form, $ONOO^-$, is stable in strongly basic solutions,[5] whereas the acidic form, ONOOH, spontaneously decomposes to $^{\bullet}NO_2$ and $^{\bullet}OH$ (30% maximum yield in the presence of an effective trap at pH 7.4) or isomerizes to nitrate (Scheme 1).[2a,6] In vivo, ONOO(H) is formed by the diffusion-controlled reaction of nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$) in a variety of cells, including vascular endothelial cells, neurons, and immune cells.[7] Macrophages and neutrophils produce considerable concentrations of $O_2^{\bullet-}$ and $^{\bullet}NO$ (and hence ONOO(H)) during the inflammation-triggered "oxidative burst" response to invading pathogens.[7a–c] $O_2^{\bullet-}$ is a byproduct of normal, aerobic, cellular metabolism[7c] and all three nitrogen oxide synthase (NOS) isoforms produce $^{\bullet}NO$ and $O_2^{\bullet-}$ simultaneously ("NOS uncoupling") under certain conditions.[4,8]



Scheme 1. Decomposition pathways for ONOOH. Rate constants have been reported ($k_1 = 0.90 \pm 0.05\ s^{-1}$, $k_2 = 0.35 \pm 0.03\ s^{-1}$, $k_3 = 0.65 \times 10^8\ M^{-1}s^{-1}$, $k_4 = 5.3 \times 10^9\ M^{-1}s^{-1}$, in phosphate buffer, 25 °C).[6]

ONOO(H) or decomposition products thereof react rapidly in vivo with numerous species, including amino acids, nucleic bases, lipids, circulatory CO_2 (to give 33% $CO_3^- + {}^{\bullet}NO_2$; $^{\bullet}NO_2$ itself is a potent oxidizing and nitrating agent),[9] metal centers of metalloproteins, thiols (proteins and low molecular weight thiols), and antioxidants.[2b,4] Nitration of tyrosine residues of proteins occurs in many pathological conditions associated with oxidative/nitrosative stress, in-

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cluding cardiovascular and neurodegenerative diseases.[10] Protein-tyrosine nitration is therefore a widely used biomarker for peroxynitrite formation,[11] although it should be noted that peroxynitrite-independent mechanisms also exist for protein-tyrosine nitration in vivo, such as those mediated by myeloperoxidase.[12] Given the strong link between peroxynitrite production and oxidative/nitrosative stress-associated pathologies, studies on the reactions of biomolecules with peroxynitrite and the development of peroxynitrite scavengers to attenuate peroxynitrite levels in vivo are active areas of research.[2a,13] Some of the most promising ONOO(H) scavengers include Mn or Fe porphyrins, which catalyze the conversion of ONOO(H) to NO_3^- or NO_2^- ($2e^-$ reduction, Scheme 2).[2a,14]

$$M^{n+} + ONOO(H) + H^+ \xrightarrow{1e^- \text{ redox}} M^{(n+1)+} + {}^{\bullet}NO_2 + OH^-/H_2O$$

$$M^{n+} + ONOO(H) + H^+ \xrightarrow{2e^- \text{ redox}} M^{(n+2)+} + NO_2^- + OH^-/H_2O$$

$$M^{n+} + ONOO(H) \xrightarrow{\text{isomerization}} M^{n+} + NO_3^- (+ H^+)$$

Scheme 2. Reactions proposed in the literature for metal-assisted decomposition and isomerization of ONOO(H).

Furthermore, catalysis can even occur in the absence of an added reducing agent in a few special cases.[2a] However, a few metalloporphyrins (e.g., $[Mn^{III}(TMPyP)]$, TMPyP = tetrakis(*N*-methyl-4-pyridyl)porphyrin) actually enhance the production of $^{\bullet}NO_2$, thus increasing the risk of cell death.[15] Hemoglobin and myoglobin catalyze the conversion of ONOO(H) to NO_3^-.[16] Co^{III} porphyrins also catalyze peroxynitrite decomposition ($\approx 10^2$–$10^3\ M^{-1}s^{-1}$), although the mechanism was not investigated.[17]

The structurally related, tetrapyrroline cobalt complexes, cobalamins (Cbls, vitamin B_{12} derivatives; Figure 1, X = CN, 5′-deoxyadenosyl (Ado), Me, H_2O/HO, NO_2, etc.) are essential cofactors for the metabolism of homocysteine by methionine synthase and the isomerization of L-methylmalonyl-CoA to succinyl-CoA by L-methylmalonyl-CoA mutase in mammals.[18] Impaired homocysteine metabolism is associated with an increased risk of cardiovascular disease,[19] neural tube defects,[20] and neurodegenerative diseases.[21] B_{12} deficiency is common in the elderly.[22]

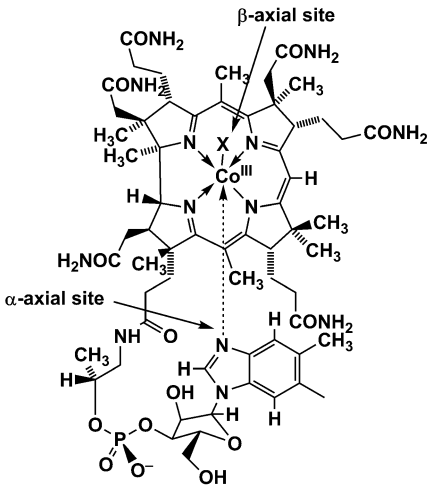


Figure 1. Structure of vitamin B_{12} derivatives (Cob(III)alamins; Cbl(III); X = Ado, Me, H_2O/HO, NO_2, CN, etc.). Ligand X is lost upon reduction of Cbl(III) to give pentacoordinate Cbl(II).

Upon entering cells Cbl(III) is reduced to Cbl(II) prior to binding to the Cbl-dependent enzymes.[23] Reduced Cbl cofactors are also involved in the methionine synthase and L-methylmalonyl-CoA mutase enzyme cycles and are sensitive to oxidation. Both B_{12}-dependent enzymes are inactivated under oxidative stress conditions.[24]

Importantly, a nonenzymatic role for Cbl in biology as a modulator of the immune response has also been suggested. Specifically, Cbl regulates the production of the proinflammatory cytokines TNF-α and IL-6, epidermal growth factors and nerve growth factors, and suppresses production of the inducible transcription factor NF-κB.[25] Furthermore Cbl therapy normalizes levels of TNF-α and epidermal growth factors in Cbl deficient patients.[26] Considerable amounts of free (nonprotein-bound) Cbl are also achievable upon Cbl supplementation,[27] and an ABC transporter protein that effluxes free Cbl from cells has been identified.[27b] Recent studies in our laboratory support Cbl(II) as an efficient intracellular scavenger of superoxide.[28] This led us to propose that scavenging of superoxide is a mechanism by which Cbl modulates the immune response and is beneficial in treating chronic inflammation.[28]

We now present mechanistic studies on the reaction between Cbl(II) and peroxynitrite, which reveal a novel dual role for Cbl(II) as a peroxynitrite and a $^{\bullet}NO_2$ scavenger. The latter reaction occurs at a rate at least one order of magnitude faster than the capture of $^{\bullet}NO_2$ by tyrosine.

Results and Discussion

Determination of the reaction stoichiometry: Experiments were carried out to determine the stoichiometry and products of the reaction of Cbl(II) with ONOO(H). Because the spontaneous decomposition of ONOO(H) is acid-catalyzed, the stoichiometry of the reaction was initially determined at pH 12.3; that is, under conditions for which spontaneous decomposition is negligible. Preliminary experiments showed that the reaction between Cbl(II) and $ONOO^-$ is completed in seconds. Figure 2 gives UV/Vis spectra of equilibrated anaerobic solutions of Cbl(II) (1.41×10^{-4} M, λ_{max} = 312, 405, and 475 nm) with $ONOO^-$ (0.21–0.51 mol equiv, 25.0 °C, 0.10 M phosphate buffer, pH 12.3, I = 0.40 M). Cbl(II) is cleanly converted to Cbl(III) (i.e., hydroxycobalamin, HOCbl; λ_{max} = 355, 410, 508, and 537 nm),[29] with isosbestic points at 339, 374, 493, and 577 nm. Comparison of the observed absorbance with that of HOCbl (1.41×10^{-4} M, 0.10 M phosphate buffer, pH 12.3, I = 0.40 M) gave a Cbl(II)/$ONOO^-$ ratio of 2:1 (final column, Table 1).

All peroxynitrite solutions contain significant concentrations of NO_2^- (see the Experimental Section) and the UV/

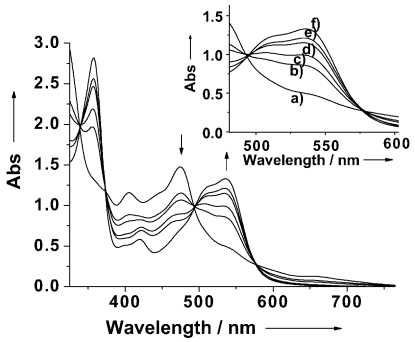


Figure 2. UV/Vis spectra for equilibrated anaerobic solutions of Cbl(II) (1.41×10^{-4} M) with 0, 0.21, 0.32, 0.43, and 0.51 mol equiv $ONOO^-$ (traces a)–e), inset) at pH 12.3 (25.0 °C, 0.10 M phosphate buffer, $I=0.40$ M). Cbl(II) ($\lambda_{max}=312$, 405, and 475 nm) is converted to HOCbl ($\lambda_{max}=355$, 410, 508, and 537 nm) with isosbestic points at 339, 374, 493, and 577 nm. UV/Vis spectrum of 1.41×10^{-4} M HOCbl (trace f)), inset; in 0.10 M phosphate buffer, pH 12.3, $I=0.40$ M).

Table 1. Determination of the stoichiometry of the reaction between Cbl(II) and $ONOO^-$ at pH 12.3 (25.0 °C, 0.10 M phosphate buffer, $I=0.40$ M). Absorbances were measured at 537 nm. i = initial concentration.

$[Cbl(II)]_i$ [10^{-4} M]	$[ONOO^-]_i$ [10^{-5} M]	$[ONOO^-]_i/[Cbl(II)]_i$	$Abs_{Cbl(II)}$ [a.u.]	Abs_{HOCbl} [a.u.]	Abs_{obs} [a.u.]	Fraction Cbl(II) reacted[a]	$ONOO^-$ required [mol equiv][b]
1.41	3.00	0.21	0.493	1.30	0.853	0.446	2.1
1.41	4.50	0.32	0.493	1.30	0.986	0.611	1.9
1.41	6.00	0.43	0.493	1.30	1.15	0.814	1.9
1.41	7.20	0.51	0.493	1.30	1.20	0.876	1.7

[a] Fraction of Cbl(II) reacted $=\frac{Abs_{Cbl(II)}-Abs_{obs}}{Abs_{Cbl(II)}-Abs_{Cbl(III)}}$. [b] Molar equivalents of $ONOO^-=\frac{\text{Fraction of Cbl(II) reacted}}{[ONOO^-]_i/[Cbl(II)]_i}$.

summarized in Table 2. Once again Cbl(II) was completely oxidized to Cbl(III) upon the addition of ONOO(H) (0.5 mol equiv), with isosbestic points at 334, 381, 488, and 576 nm. Under these pH conditions the Cbl(III) product was shown, by ^{1}H NMR spectroscopy, to be NO_2Cbl (Figure S4b in the Supporting Information).

Kinetic studies on the reaction between Cbl(II) and ONOO(H): Kinetic studies on the reaction of Cbl(II) with ONOO(H) were carried out under strictly anaerobic conditions under pseudo-first-order conditions by stopped-flow spectroscopy. Experiments were carried out in phosphate and borate buffers at low buffer concentrations, because the spontaneous decomposition of ONOO(H) is accelerated at high buffer concentrations and the biological buffers (HEPES, MOPS, etc.) also significantly accelerate the rate of spontaneous decomposition of ONOO(H).[31] Typical experimental data is shown in Figure 3, which gives rapid spectral scans obtained every 5.00 ms for the reaction between Cbl(II) (5.00×10^{-5} M) and ONOO(H) (1.00×10^{-5} M) at pH 7.35 (25.0 °C, 0.09 M phosphate buffer, $I=0.20$ M). Cbl(II) rather than ONOO(H) was used in excess, to minimize the concentration of NaOH added and hence maintain the pH of the buffered solution (peroxynitrite is stabilized in NaOH in the drive syringe of the stopped-flow instrument to prevent spontaneous decomposition; $[OH^-]_T\cong0.012$–0.014 M; T = total concentration, which includes OH^- from the stock solution of peroxynitrite). The corresponding plot of absorbance at 475 nm versus time is shown in the inset to Figure 3. The data fits a single first-order rate equation, giving $k_{obs}=18.4\pm0.5$ s^{-1}.

Table 2. Determination of the stoichiometry of the reaction between Cbl(II) and ONOO(H) at pH 7.35 (25.0 °C, 0.18 M phosphate buffer, $I=0.40$ M). Absorbances were measured at 537 nm.

$[Cbl(II)]_i$ [10^{-4} M]	$[ONOO(H)]_i$ [10^{-5} M]	$[ONOO(H)]_i/[Cbl(II)]_i$	$Abs_{Cbl(II)}$ [a.u.]	Abs_{NO_2Cbl} [a.u.]	Abs_{obs} [a.u.]	Fraction Cbl(II) reacted[a]	ONOO(H) required [mol equiv][b]
1.36	3.00	0.22	0.483	1.19	0.812	0.465	2.1
1.36	4.50	0.33	0.483	1.19	0.952	0.663	2.0
1.36	6.00	0.44	0.483	1.19	1.12	0.901	2.1
1.36	7.50	0.55	0.483	1.19	1.25	1.09	2.0

[a] Fraction of Cbl(II) reacted $=\frac{Abs_{Cbl(II)}-Abs_{obs}}{Abs_{Cbl(II)}-Abs_{Cbl(III)}}$. [b] Molar equivalents of ONOO(H) $=\frac{\text{Fraction of Cbl(II) reacted}}{[ONOO(H)]_i/[Cbl(II)]_i}$.

Vis spectrum of HOCbl is very similar to that for nitrocobalamin (NO_2Cbl, $\lambda_{max}=354$, 413, 532 nm).[29] However each Cbl(III) complex has a unique set of resonances in the aromatic region,[30] and the ^{1}H NMR spectrum of an equilibrated solution of Cbl(II) (2.62×10^{-3} M) with $ONOO^-$ (0.55 mol equiv) at pD 13.2 clearly showed that HOCbl ($\delta=7.17$ (s), 6.73 (s), 6.50 (s), 6.24 (d), and 6.07 ppm (s)),[30] and not NO_2Cbl ($\delta=$ 7.20 (s), 6.74 (s), 6.42 (s), 6.28 (d) and 6.20 ppm (s)),[29] is the Cbl(III) product in strongly alkaline solution (Figure S4a in the Supporting Information). Control experiments also showed that NO_2Cbl is decomposed to HOCbl in alkaline solution.

The stoichiometry of the reaction between Cbl(II) and ONOO(H) was also determined at pH 7.35 using the same procedure. Preliminary kinetic experiments showed that the rate of the reaction between Cbl(II) and ONOO(H) is well over an order of magnitude faster than the rate of spontaneous decomposition of ONOO(H) under these conditions. Figure S5 in the Supporting Information gives UV/Vis spectra of equilibrated anaerobic solutions of Cbl(II) with ONOO(H) (0.22–0.55 mol equiv) at pH 7.35. The results are

Because the $ONOO^-$ stock solution unavoidably contains considerable NO_2^-, control experiments were carried out on the reaction between Cbl(II) and NO_2^-. The rate of this reaction is several orders of magnitude slower than the reaction of interest under the conditions of this study, in agreement with literature.[32] Control experiments also showed that NO_3^- (from peroxynitrite decomposition) does not react with Cbl(II).

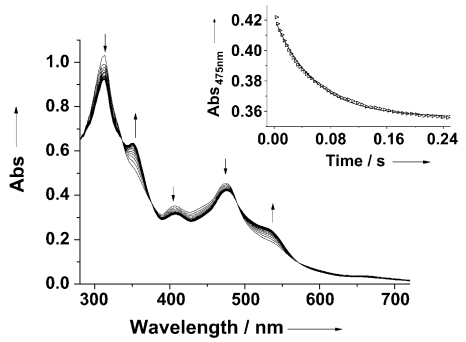


Figure 3. Plot of absorbance versus wavelength for the reaction of Cbl(II) (5.00×10^{-5} M) and ONOO(H) (1.00×10^{-5} M) at pH 7.35 (0.09 M phosphate buffer, $I=0.20$ M Na_2HPO_4, 25.0 °C). Spectra were collected every 6.00 ms for 0.240 s. Approximately 40% conversion of Cbl(II) to Cbl(III) is observed, consistent with the reaction stoichiometry. Isosbestic points at 336, 376, 490, and 572 nm indicate that Cbl(II) is cleanly converted to Cbl(III) ($=NO_2$Cbl). Inset: The best fit of absorbance data at 475 nm versus time to a first-order rate equation, giving $k_{obs}=18.4\pm0.5\ s^{-1}$.

Similar experiments were carried out at other Cbl(II) concentrations at pH 7.35 (5.00×10^{-5}–5.00×10^{-4} M Cbl(II), 1.00×10^{-5} M ONOO(H)) and the results are summarized in Figure 4. The data was fitted to a line passing through the origin, which indicates that a single, irreversible reaction occurs and that the reaction is first-order with respect to Cbl(II) and ONOO(H). From the slope of the line, the second-order rate constant for the reaction, $k_{obs}/[Cbl(II)]_T$, was found to be $(3.70\pm0.05)\times10^5\ M^{-1}s^{-1}$ at pH 7.35. Typical values reported for free (nonprotein-bound) porphyrins reacting with ONOO(H) at pH 7.4 ± 0.1 in phosphate buffer are 1.0×10^5 (25 °C), 1.8×10^6 (24 °C), 1.6×10^7 (37 °C), 3.8×10^6 (37 °C), and 3.7×10^6 (37 °C) $M^{-1}s^{-1}$ for $[Fe^{III}(TMPS)]$,[33] $[Mn^{III}(TMPyP)]$,[34] $[Mn^{III}(TM\text{-}2\text{-}PyP)]$, $[Mn^{III}(TM\text{-}3\text{-}PyP)]$, and $[Mn^{III}(TM\text{-}4\text{-}PyP)]$,[35] respectively. Protein-bound porphyrins react slower with ONOO(H) (pH 7.4 ± 0.1 in phosphate buffer): 8.8×10^4 (pH 7.0, 20 °C), 5.4×10^4 (20 °C), 1.7×10^3 (37 °C), and $1.0\times10^4\ M^{-1}s^{-1}$ (temperature not given) for oxyhemoglobin,[36] oxymyoglobin,[36] methemoglobin,[15b] and metmyoglobin,[16a] respectively.

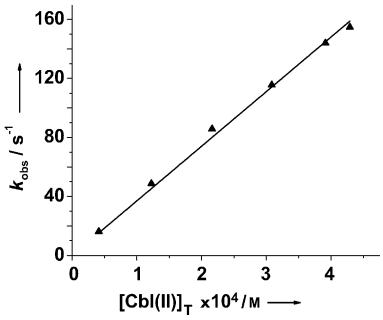


Figure 4. Plot of the observed rate constant, k_{obs}, versus total Cbl(II) concentration for the reaction between Cbl(II) (5.00×10^{-5}–5.00×10^{-4} M) and ONOO(H) (1.00×10^{-5} M) at pH 7.35 (0.09 M phosphate buffer, 25.0 °C, $I=0.20$ M Na_2HPO_4). Data were fitted to a line passing through origin, giving $k_{obs}/[Cbl(II)]_T=(3.70\pm0.05)\times10^5\ M^{-1}s^{-1}$.

Kinetic data was also collected at other pH values. Kinetic data for the reaction between $ONOO^-$ (5.00×10^{-5} M) and Cbl(II) (5.00×10^{-4} M) at the highest pH value used in this study, pH 12.0, is shown in Figure S6 in the Supporting Information, giving $k_{obs}=2.21\pm0.12\ s^{-1}$. Rate data as a function of the Cbl(II) concentration (5.00×10^{-5}–5.00×10^{-4} M, 1.00×10^{-5} M $ONOO^-$), at pH 12.0 can again be fitted to a line passing through the origin (see the Supporting Information, Figure S7; $k_{obs}/[Cbl(II)]_T=(4.75\pm0.12)\times10^3\ M^{-1}s^{-1}$). By studying the reaction with $ONOO^-$, rather than Cbl(II), in excess at pH 12.0 ($[ONOO^-]\geq10\times[Cbl(II)]$) the same rate constant, within experimental error, was received ($(4.72\pm0.05)\times10^3\ M^{-1}s^{-1}$; Figure S8 in the Supporting Information). Figure 5 summarizes the dependence of the second-order rate constant, $k_{obs}/[Cbl(II)]_T$, for the reaction of Cbl(II) with ONOO(H) as a function of pH.

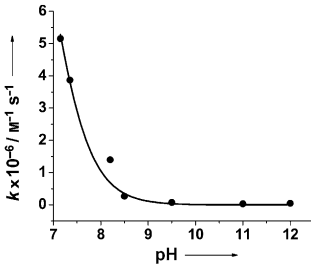
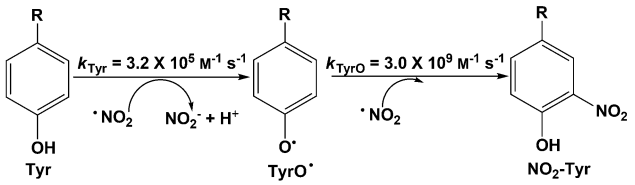


Figure 5. Plot of $k\times10^{-6}$ (i.e., $k_{obs}\times10^{-6}/[Cbl(II)]_T$) versus pH for the reaction of Cbl(II) with ONOO(H) (phosphate or borate buffer, 25.0 °C, $I=0.20$ M (Na_2HPO_4)). Data is fitted to Equation (1) fixing $K_a=1.34\times10^{-7}$ M, giving $k_{Cbl(II)}=(1.57\pm0.08)\times10^6\ M^{-1}s^{-1}$.

Studies on the products of the reaction between Cbl(II) and ONOO(H) in the presence of tyrosine: Given that a $1e^-$ oxidation of Cbl(II) by ONOO(H) to form Cbl(III) occurs, it is likely that ONOO(H) is reduced to $^{\bullet}NO_2$. To probe whether $^{\bullet}NO_2$ is a reaction intermediate, the reaction between Cbl(II) and peroxynitrite was investigated in the presence of the $^{\bullet}NO_2$ trap tyrosine (Tyr). It is well established that Tyr reacts rapidly with $^{\bullet}NO_2$ obtained from the spontaneous homolytic decomposition of ONOOH to ultimately form 3-nitrotyrosine (NO_2–Tyr, Scheme 3). The products of the reaction between Tyr (6.00×10^{-3} M) and ONOO(H) (1.00×10^{-3} M) were initially determined under anaerobic conditions in the absence of Cbl(II) (0.40 M phosphate buffer, room temperature, pH 7.4 ± 0.1). The HPLC chromatogram of the product solution (Figure 6a) shows peaks at 15.1 (3-hydroxytyrosine, HO–Tyr), 17.1 (Tyr), and 24.0 min (NO_2–Tyr, peaks were assigned by spiking with authentic samples). HO–Tyr is a product of the reaction of Tyr with $^{\bullet}OH$,[38] the latter species produced by the homolytic decomposition of ONOOH (Scheme 1). To quantify the amount of NO_2–Tyr and HO–Tyr formed, standard calibration curves for these two compounds were generated under the same conditions (see the Supporting Information, Figures S9 and S10). From the calibration curve, the yield of NO_2–Tyr was



Scheme 3. Oxidation and nitration of Tyr by $^{\bullet}NO_2$.[37] R = $H_2CCH(NH_3^+)CO_2^-$.

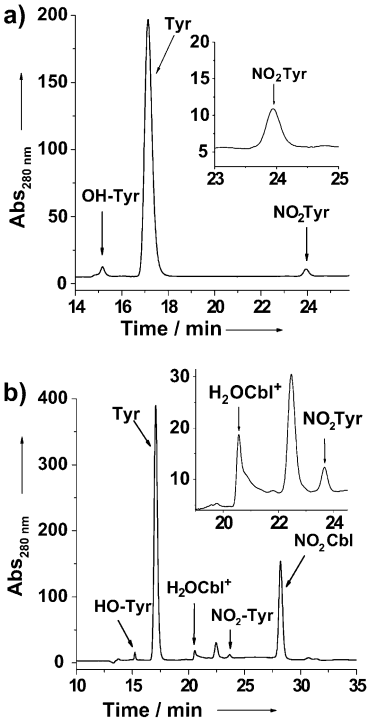


Figure 6. HPLC chromatogram (280 nm) for the reaction of Tyr (6.00×10^{-3} M) and ONOO(H) (1.00×10^{-3} M) in the absence (a) and presence (b) of 5.00×10^{-4} M Cbl(II) at pH 7.4 ± 0.1 (room temperature, 0.40 M phosphate buffer). Peaks at 15.1, 17.1, 20.6, 24.0, and 28.6 min correspond to HO–Tyr, Tyr, H_2OCbl^+, NO_2–Tyr, and NO_2Cbl, respectively. Insets: Larger images of the NO_2–Tyr region. The peak at 22.5 min in (b) is attributed to a second minor corrinoid product of the reaction between Cbl(II) and $^{\bullet}NO_2$.

found to be 4.8 ± 0.3% (mean value of six experiments) with respect to the ONOO(H) reactant. Others have reported NO_2–Tyr yields in the 5–8% range for the reaction of Tyr with ONOO(H) (room temperature, pH 7.4, phosphate buffer).[1b,15b,38a,39] The yield of HO–Tyr was 9.0% (Table 3).

The experiment was repeated in the presence of Cbl(II). Figure 6b gives a HPLC chromatogram of the products of the reaction upon the addition of ONOO(H) (final concentration 1.00×10^{-3} M) to a solution of Cbl(II) (5.00×10^{-4} M) and Tyr (6.00×10^{-3} M) at pH 7.4 ± 0.1 in phosphate buffer (0.40 M). Peaks were observed at 20.6 (H_2OCbl^+) and 28.6 min (NO_2Cbl) (confirmed by spiking with authentic samples of these complexes), in addition to peaks from HO–Tyr, Tyr, and NO_2–Tyr. By using the calibration curve for NO_2–Tyr, the yield of NO_2–Tyr was reduced from 4.8 to

Table 3. Yield of NO_2–Tyr and HO–Tyr formed for the reaction of ONOO(H) (1.00×10^{-3} M) with Tyr (6.00×10^{-3} M) in the presence of varying concentrations of Cbl(II) (room temperature, pH 7.4 ± 0.1, 0.40 M phosphate buffer).

[Cbl(II)] [M]	[Cbl(II)]/[ONOO(H)]	Yield of NO_2–Tyr formed [%]	Yield of HO–Tyr formed [%]
0	–	4.8 ± 0.3	9.0 ± 0.4
5.00×10^{-4}	0.50	3.0 ± 0.4	5.9 ± 0.4
1.00×10^{-3}	1.0	2.0 ± 0.3	1.9 ± 0.2
2.00×10^{-3}	2.0	negligible	negligible

3.0 ± 0.4% (mean value of six experiments) with respect to ONOO(H) (Table 3). By increasing the Cbl(II) concentration to 1.00×10^{-3} M, while keeping everything else constant, a further decrease in the yield of NO_2–Tyr to 2.0 ± 0.3% was achieved (mean value of 12 experiments). Moreover, negligible NO_2–Tyr was formed when the Cbl(II) concentration was further increased to 2.00×10^{-3} M (ONOO(H) = 1.00×10^{-3} M, Tyr = 6.00×10^{-3} M; 0.40 M phosphate buffer, pH 7.4 ± 0.1). A similar trend was observed for HO–Tyr as the Cbl(II) concentration increased (Table 3) with negligible HO–Tyr formed at the highest Cbl(II) concentration (2.00×10^{-3} M).

One-electron oxidation of the metal center by $^{\bullet}NO_2$ to form the corresponding oxidized nitro complex has been reported previously for Fe^{II} porphyrins.[40] Importantly, an additional product peak was observed at 22.5 min in the HPLC chromatogram (Figure 6b). The area of this peak at 280 nm increased linearly with increasing Cbl(II) concentration (Table S1 in the Supporting Information), suggesting the formation of a second minor corrinoid product from the reaction between Cbl(II) and $^{\bullet}NO_2$ in addition to NO_2Cbl. Control experiments in which Cbl(II) was directly reacted with $^{\bullet}NO_2$(g) showed that this is indeed the case (see the Supporting Information); that is, whereas NO_2Cbl is the major species formed when Cbl(II) reacts with $^{\bullet}NO_2$, small amounts of a putative NO_2–corrinoid complex are also produced, presumably by attack of $^{\bullet}NO_2$ on the corrin ring itself. Others have reported $^{\bullet}NO_2$ addition at the *meso* (β-pyrolic) carbons of porphyrins.[41]

Negligible NO_2–Tyr is formed upon the stoichiometric addition (2.0 equiv) of Cbl(II) to a solution of Tyr + ONOO(H) (Table 3). This suggests that the rate constant for the reaction between Cbl(II) and $^{\bullet}NO_2$ is considerably larger than that of the reaction of Tyr with $^{\bullet}NO_2$ (Scheme 3).[37] Attempts were made to obtain an estimate of this rate constant. NO_2–Tyr can either be formed by the reaction of $^{\bullet}NO_2$ or $^{\bullet}OH$ with Tyr to form TyrO$^{\bullet}$ followed by the rapid reaction of a second $^{\bullet}NO_2$ with TyrO$^{\bullet}$ to give NO_2–Tyr,[37,38b] or by the reaction of $^{\bullet}NO_2$ with Tyr followed by the rapid reaction of this intermediate with $^{\bullet}OH$[42] (Scheme S1 in the Supporting Information). It therefore occurred to us that by scavenging $^{\bullet}OH$, pathways involving $^{\bullet}OH$ would be prevented so that NO_2–Tyr formation would require $^{\bullet}NO_2$ exclusively (Scheme 3). The yield of NO_2–Tyr in the presence of Cbl(II) would therefore allow an estimation of the rate constant for the reaction between Cbl(II)

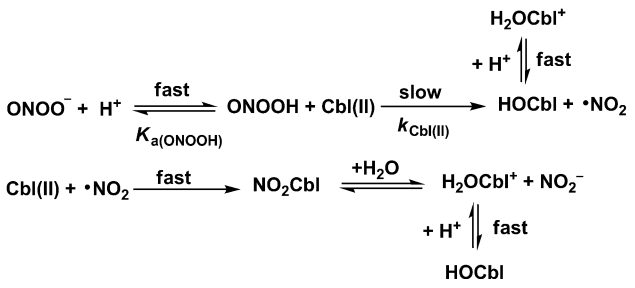
and $^{\bullet}NO_2$. Furthermore, the presence or absence of the HO–Tyr product provides a convenient way to check the efficiency of the $^{\bullet}OH$ scavenger, because HO–Tyr is a product of the reaction of Tyr with $^{\bullet}OH$[38] and should therefore not be formed if the $^{\bullet}OH$ scavenger scavenges all $^{\bullet}OH$ formed during the reaction. D-mannitol and ethanol were tested for their ability to compete with Tyr for $^{\bullet}OH$ in this system. Mannitol was found to be the most efficient $^{\bullet}OH$ scavenger. However, HO–Tyr was still formed in a saturated solution of mannitol (see the Supporting Information); hence the formation of NO_2–Tyr through pathways involving $^{\bullet}OH$ could not be excluded. The formation of HO–Tyr was also not prevented by ethanol (54% v/v, details given in the Supporting Information). An estimate of the rate constant for the reaction between Cbl(II) and $^{\bullet}NO_2$ was therefore not possible from this data.

To summarize, product studies on the reaction between Cbl(II) and ONOO(H) in the presence of Tyr show that $^{\bullet}NO_2$ reacts preferentially with Cbl(II) rather than with Tyr, with stoichiometric scavenging of $^{\bullet}NO_2$ by Cbl(II). An estimate of the rate constant of the Cbl(II)+$^{\bullet}NO_2$ reaction from the yield of NO_2–Tyr formed was not possible, because pathways leading to NO_2–Tyr formation involving $^{\bullet}OH$ could not be prevented even in the presence of the efficient $^{\bullet}OH$ scavengers D-mannitol and ethanol.

Proposed reaction mechanism: From Figure 5, it is clear that the rate of the reaction between Cbl(II) and ONOO(H) becomes slower with increasing pH to finally approach 0 above pH 9.5. This suggests that ONOOH, not $ONOO^-$, reacts with Cbl(II). It is well established that stronger oxidants are obtained upon protonation of oxyanions, because the protonated oxyanion is more electron deficient.[43] If we assume that only ONOOH reacts with Cbl(II), the proposed mechanism is given in Scheme 4, in which rate-determining oxidation of Cbl(II) by ONOOH, to yield $HOCbl/H_2OCbl^+$ and $^{\bullet}NO_2$, is followed by the rapid reaction of $^{\bullet}NO_2$ with a second molecule of Cbl(II) to form NO_2Cbl (Cbl(III)), consistent with the Cbl(II)/ONOO(H) stoichiometry of 2:1.

From Scheme 4, it can be shown that:

$$k_{obs}/[Cbl(II)]_T = (k_{Cbl(II)} \times [H^+])/([H^+] + K_{a(ONOOH)}) \quad (1)$$



Scheme 4. Proposed mechanism of the reaction of Cbl(II) with ONOO(H). Note that minor amounts of a second corrinoid(III) product were observed in addition to NO_2Cbl.

$pK_{a(ONOOH)}$ was determined independently ($=6.87\pm0.06$, 0.08 M phosphate buffer, 25.0 °C; details given in the Experimental Section) and is in excellent agreement with values reported by others ($pK_{a(ONOOH)}=6.8$, 0.1 M phosphate buffer, 25 °C).[1] By fitting the data in Figure 5 to Equation (1) and fixing $K_{a(ONOOH)}=1.34\times10^{-7}$ M, $k_{Cbl(II)}=(1.57\pm0.08)\times10^6\,M^{-1}s^{-1}$ is obtained. Of interest is the observation that the rate of oxidation of a series of Mn^{III} porphyrins by ONOO(H) increases with increasing pH in the pH range 5–8.5 (pK_a 6.15), leading the authors to propose that $ONOO^-$, not ONOOH, is the oxidant.[35] A change in the Mn^{III} speciation with respect to pH (the redox potential of metal complexes that form hydroxo complexes becomes more negative with increasing pH) was apparently not responsible for the increase in the observed reaction rate with increasing pH.[35,44]

Conclusion

Spectroscopic and kinetic evidence is presented for a novel reaction between Cbl(II) and peroxynitrous acid, in which Cbl(II) reacts with peroxynitrous acid to give Cbl(III) and $^{\bullet}NO_2$ ($k=3.70\times10^5\,M^{-1}s^{-1}$ at physiological pH) followed by rapid scavenging of $^{\bullet}NO_2$ by a second molecule of Cbl(II) to form predominately NO_2Cbl. This mechanism differs from those previously proposed for the reactions between ONOO(H) and reduced porphyrins (Scheme 2). The rate constant for the reaction between Cbl(II) and $^{\bullet}NO_2$ is at least one order of magnitude larger than that reported for the reaction between Tyr and $^{\bullet}NO_2$ from analysis of the products of the latter reaction in the presence of Cbl(II) by HPLC. Importantly, both B_{12}-dependent enzymes are inactivated under oxidative stress conditions.[24] Given that protein-bound Cbl is readily accessible to small molecules,[45] it is likely that enzyme-bound Cbl(II) is also rapidly oxidized to inactive Cbl(III) upon exposure to peroxynitrite or $^{\bullet}NO_2$ in addition to free intracellular Cbl(II). Finally, kinetic studies on the reaction between Cbl(I) and ONOO(H) show that cob(I)alamin is even more rapidly oxidized by ONOO(H) ($k=3.6\times10^7\,M^{-1}s^{-1}$ at physiological pH, 25 °C), yielding Cbl(II) and dinitrogen.[46]

Experimental Section

Details concerning chemicals and synthesis of cob(II)alamin and peroxynitrite are given in the Supporting Information.

Solution preparation: Anaerobic solutions were degassed by bubbling through argon for 24 h and were stored and manipulated in an MBRAUN Labmaster 130 (1250/78) glove box (<1 ppm O_2), equipped with O_2 and H_2O sensors and a freezer. Solutions of tyrosine ($\varepsilon_{274}=1400\,M^{-1}cm^{-1}$ at pH 6.0),[47] 3-nitrotyrosine ($\varepsilon_{438}=4200\,M^{-1}cm^{-1}$ at pH 11.5),[48] Cbl(II) ($\varepsilon_{475}=1.05\times10^4\,M^{-1}cm^{-1}$; see the Supporting Information), and $ONOO^-$ ($\varepsilon_{302}=1670\,M^{-1}cm^{-1}$)[49] were standardized spectrophotometrically prior to use. The peroxynitrite stock solution was diluted with 1.00×10^{-2} M NaOH, to prevent spontaneous decomposition. Aliquots of peroxynitrite in NaOH(aq) were added with microsyringes to

buffered Cbl(II) and tyrosine solutions in vials closed with septa, while vortexing.

Determination of the acid dissociation constant and the rate constant for spontaneous decomposition of ONOOH: The spontaneous decomposition of ONOO(H) (5.00×10^{-5} M) was followed by stopped-flow spectroscopy in the pH range 5.53–8.37 (25.0 °C, 0.08 M phosphate buffer) at 302 nm. The phosphate concentration rather than the ionic strength was kept constant because $pK_{a(ONOOH)}$ is dependent on the phosphate concentration.[31b] A typical absorbance versus time trace for the decomposition of ONOO(H) at pH 7.31 is given in Figure S2 in the Supporting Information. The absorbance versus time traces fitted well to a single first-order reaction under all conditions. Figure S3 in the Supporting Information summarizes the dependence of k_{obs} on pH for the decomposition of ONOO(H). It is well established that ONOOH, not $ONOO^-$, undergoes spontaneous decomposition.[14g,50] Fitting the data to $k_{obs}=(k_d[H^+])/([H^+]+K_a(ONOOH))$[50] gave $K_{a(ONOOH)}=(1.34\pm0.12)\times10^{-7}$ M ($pK_{a(ONOOH)}=6.87\pm0.06$) and $k_d=1.23\pm0.03\ s^{-1}$ (see the Supporting Information), which agrees well with values reported by others ($k_d=1.25\ s^{-1}$, $pK_{a(ONOOH)}=6.8$, in phosphate buffer, 25 °C).[6]

Determination of the stoichiometry of the reaction between Cbl(II) and ONOO(H): Experiments were carried out using strictly anaerobic solutions. Cbl(II) solutions in either 0.10 M phosphate buffer, pH 12.0, $I=0.40$ M, or 0.18 M phosphate buffer, pH 7.35, $I=0.40$ M, were prepared. Solutions of $ONOO^-$ were prepared by diluting the stock $ONOO^-$ solution with 1.00×10^{-2} M NaOH.

Aliquots of $ONOO^-$ (100–500 μL) were added to Cbl(II) in phosphate buffer (4.00 mL), in the glove box, the mixtures were equilibrated for 5 min and the increase in absorbance was measured at 537 nm. The absorbance change was compared with the absorbance change upon aerial oxidation of the Cbl(II) solution to Cbl(III) (i.e., HOCbl at pH 12.0 and NO_2Cbl at pH 7.35). In the latter case, after exposing Cbl(II) to air, the resulting H_2OCbl^+ was converted to NO_2Cbl by the addition of excess solid $NaNO_2$.

Kinetic measurements on the reaction of Cbl(II) with ONOO(H): All solutions were prepared directly before use. Cbl(II) solutions were prepared in either anaerobic phosphate or borate/phosphate buffers (5.00×10^{-2} M borate) at a total ionic strength of 0.40 M (Na_2HPO_4). The pH of the buffered solutions was about 0.4 units lower than the desired final pH value. $ONOO^-$ solutions were prepared from the stock $ONOO^-$ solution by dilution with 1.00×10^{-2} M NaOH. Kinetic data were collected under pseudo-first-order conditions and were independent of the wavelength at which they were collected. The final solution pH was determined by measuring the pH of the solution in the stopped syringe of the stopped-flow instrument.

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