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Kinetic Studies on the Reaction between Cob(I)alamin and Peroxynitrite: Rapid Oxidation of Cob(I)alamin to Cob(II)alamin by Peroxynitrous Acid

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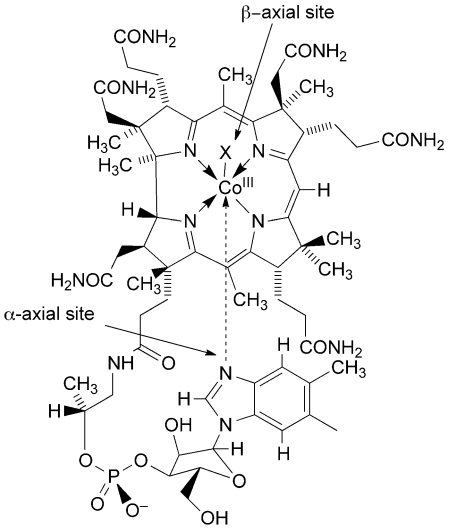
There is currently much interest in the biochemical reactivity of peroxynitrite/peroxynitrous acid ($ONOO^-$/ONOOH; $pK_{a(ONOOH)}=6.8$),[1] a strong oxidizing, hydroxylating and/or nitrating agent formed by the diffusion controlled reaction of nitric oxide with superoxide.[1] Elevated peroxynitrite levels are associated with chronic inflammatory disorders including neurological and vascular diseases, and circulatory shock.[1,2] ONOO(H) or its decomposition products react rapidly in vivo with amino acids, bases of nucleotides, lipids, circulatory CO_2, metalloproteins, thiols and antioxidants.[1,2] Nitration of tyrosine protein residues is widely used as a biomarker for elevated peroxynitrite levels.[1,2b]

Cobalamins (Cbls, vitamin B_{12} derivatives, Scheme 1) are essential Co^{III} macrocyclic cofactors for two mammalian enzymes: adenosylcobalamin-dependent L-methylmalonyl-CoA mutase and methylcobalamin-dependent methionine synthase.[3] Importantly, reduced Cbl cofactors (cob(I)alamin, Cbl(I), Co^+ and cob(II)alamin, Cbl(II), Co^{2+}) are involved in the catalytic cycles of these enzymes in addition to being intracellular biosynthetic cofactor precursors.[3] Both B_{12}-dependent enzymes are inactivated under oxidative/nitrosative stress conditions.[4] To our knowledge no studies have yet been reported on the reactions of Cbl(I) with peroxynitrite. We now present evidence showing that Cbl(I) reacts extremely rapidly with ONOOH ($k\sim2\times10^8\,M^{-1}s^{-1}$) to ultimately generate cob(II)alamin and nitrogen.



Scheme 1. Structure of vitamin B_{12} derivatives (cob(III)alamins, Cbl(III); X: Ado, Me, H_2O/HO, CN, etc.). Ligand X is lost and the bond to the 5,6-dimethylbenzimidazole moiety at the α-axial site is broken upon reduction of Cbl(II) to tetracoordinate cob(I)alamin, Cbl(I).

Kinetic studies on the reaction of Cbl(I) with ONOO(H) were carried out under strictly anaerobic conditions by using stopped-flow spectroscopy. Peroxynitrite ($ONOO^-$) is stable in strongly basic solution, whereas peroxynitrous acid, ONOOH, rapidly spontaneously decomposes to $^{\bullet}NO_2$, $^{\bullet}OH$ and nitrate.[2a,5] Sequential mixing and low buffer concentrations were used to prevent the decomposition of the reagent solutions prior to collecting kinetic data. Peroxynitrite solutions were prepared in NaOH (0.01 M). A typical plot of absorbance at 388 nm versus time for the reaction of Cbl(I) (5.50×10^{-5} M) with ONOO(H) (7.15×10^{-5} M) at pH 9.24 is shown in Figure 1a. The color of the solution changed from charcoal to brown, consistent with oxidation of Cbl(I) to Cbl(II) by ONOO(H). The data fit well to a single first-order rate equation, and give an observed rate constant, $k_{obs}=66.4\pm0.2\,s^{-1}$. Note that a Cbl(I)/ONOO(H) reaction stoichiometry of 5:1 (see below) allows the use of lower concentrations of ONOO(H) whilst still maintaining pseudo-first-order conditions with respect to the ONOO(H) concentration.

The spontaneous decomposition of ONOO(H) in the buffer was always several orders of magnitude slower than the reaction between Cbl(I) and ONOO(H) under all experimental conditions (e.g., at pH 9.2, $k_{spont}\sim6\times10^{-3}\,s^{-1}$);[6a] hence, ONOO(H), rather than its decomposition products, reacts with Cbl(I). Furthermore, to confirm that ONOO(H) is indeed required for the reaction to occur, ONOO(H) was allowed to fully decompose prior to treating it with Cbl(I). No reaction was observed within the time frame of these experiments (see the Supporting Information for further details). Finally, the $ONOO^-$ stock solution unavoidably contains nitrite (see the Experimental Section in the Supporting Information). However, control experiments showed that the reaction of Cbl(I) with nitrite (or nitrate) is several orders of magnitude slower than the rate-determining step

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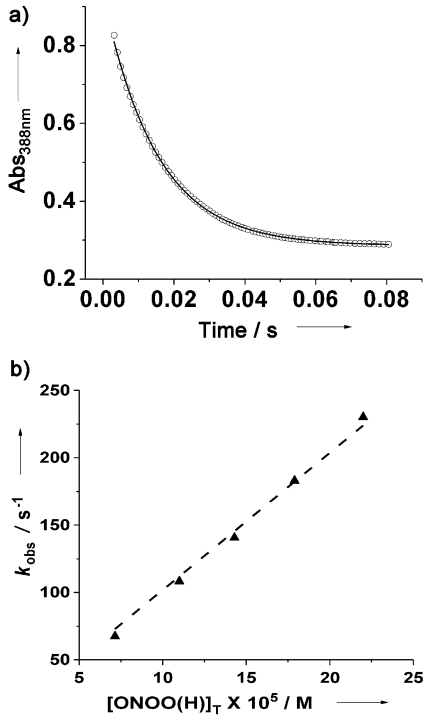


Figure 1. a) Plot of absorbance at 388 nm versus time for the reaction of Cbl(I) (5.50×10^{-5} M) with ONOO(H) (7.15×10^{-5} M) at pH 9.24 (0.070 M CHES buffer, 25.0 °C, $I=0.20$ M (Na_2HPO_4)). The data fit well to a first-order rate equation to give $k_{obs}=66.4\pm0.2\ s^{-1}$. b) Plot of observed rate constant, k_{obs}, versus total ONOO(H) concentration for the reaction between Cbl(I) (5.50×10^{-5} M) and ONOO(H) (7.15×10^{-5}–2.20×10^{-4} M) at pH 9.24 (0.070 M CHES buffer, 25.0 °C, $I=0.20$ M (Na_2HPO_4)). Data were fitted to a line passing through the origin to give: $k_{obs}/[ONOO(H)]_T=(1.02\pm0.01)\times10^6\ M^{-1}s^{-1}$.

of the Cbl(I)/ONOO(H) reaction under all the pH conditions of this study.[6b]

Similar experiments were carried out at other ONOO(H) concentrations at pH 9.24. The results are summarized in Figure 1 b, which shows a plot of k_{obs} versus total ONOO(H) concentration. The data fit well to a straight line passing through the origin; this indicates that a single, irreversible reaction occurs and that the reaction is first-order with respect to Cbl(I) and ONOO(H). From the slope of the line, the second-order rate constant for the reaction, $k_{obs}/[ONOO(H)]_T$, was found to be $(1.02\pm0.01)\times10^6\ M^{-1}s^{-1}$ at pH 9.24.

Kinetic data were also collected at other pH values (see the Supporting Information for k_{obs} vs. $[ONOO(H)]_T$ plots at other pH conditions). Figure 2 summarizes the dependence of the apparent second-order rate constant, k ($=k_{obs}/[ONOO(H)]_T$), for the treatment of Cbl(I) with ONOO(H) as a function of pH.

It is clear from Figure 2 that the apparent rate constant increases with lowering the pH, and becomes pH independent at pH > 10.5. The reaction rate was too rapid to be determined at pH < 8.4 with our sequential mixing setup. Assuming that both ONOOH and $ONOO^-$ react with Cbl(I), the rate-determining step can be expressed as:

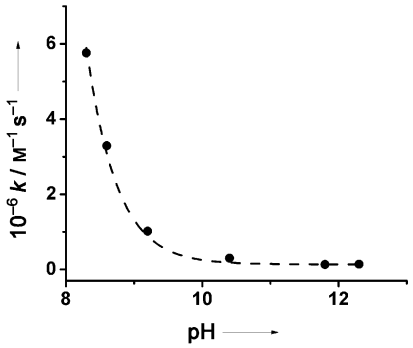


Figure 2. Plot of $10^{-6}k$ ($=10^{-6}k_{obs}/[ONOO(H)]_T$) versus pH for the reaction of Cbl(I) with ONOO(H) (phosphate, TAPS, CAPS or CHES buffer, 25.0 °C, $I=0.20$ M (Na_2HPO_4)). Data were fitted to Equation (2) given in the text; $K_a=1.34\times10^{-7}$ M and $k_{ONOO^-}=1.36\times10^5\ M^{-1}s^{-1}$ were fixed (mean value of k at pH 11.67 and 12.23) to give $k_{ONOOH}=(1.60\pm0.03)\times10^8\ M^{-1}s^{-1}$.

$$\text{rate}=k_{ONOO^-}[Cbl(I)][ONOO^-]+k_{ONOOH}[Cbl(I)][ONOOH] \quad (1)$$

The data in Figure 2 can be fitted to the equation:

$$k_{obs}/[ONOO(H)]_T=(k_{ONOOH}\times[H^+])+(k_{ONOO^-}\times K_{a(ONOOH)})/([H^+]+K_{a(ONOOH)}) \quad (2)$$

The $pK_{a(ONOOH)}$ was determined independently ($=6.87\pm0.06$, 0.08 M phosphate buffer, 25.0 °C)[6a] and is in excellent agreement with values reported by others ($pK_{a(ONOOH)}=6.8$, 0.1 M phosphate buffer, 25 °C).[7] Fitting the data in Figure 2 to Equation (2), fixing $K_{a(ONOOH)}=1.34\times10^{-7}$ M and $k_{ONOO^-}=1.36\times10^5\ M^{-1}s^{-1}$ (mean value of k at pH 11.67 and 12.23) gives $k_{ONOOH}=(1.60\pm0.03)\times10^8\ M^{-1}s^{-1}$. It is well established that stronger oxidants are obtained upon protonation of oxyanions, since the protonated oxyanion is more electron deficient.[8] Using this model the apparent second-order rate constant (k) of the reaction at pH 7.4 was calculated to be $3.6\times10^7\ M^{-1}s^{-1}$. Second-order rate constants for the reactions of the structurally related protein-free porphyrins with ONOO(H) at physiological pH are typically one to two orders of magnitude smaller (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 $3.7\times10^6\ M^{-1}s^{-1}$ (37 °C) for Fe^{III}TMPS, Mn^{III}TMPyP, Mn^{III}TM-2-PyP, Mn^{III}TM-3-PyP and Mn^{III}TM-4-PyP, respectively).[8a,9] Protein-bound porphyrins react slower ($\sim10^3$–$10^4\ M^{-1}s^{-1}$) with ONOO(H).[10]

Alternatively, one can assume that only ONOOH reacts with Cbl(I) to give $k_{ONOOH}=(1.78\pm0.05)\times10^8\ M^{-1}s^{-1}$; however, the fit of the data to this model is significantly worse (Figure S2 in the Supporting Information).

In order to identify the Cbl reaction product(s), complete UV/Vis spectra for the reaction of Cbl(I) with $ONOO^-$ were collected, at pH 12.25, immediately (within 2–3 min) after the addition of $ONOO^-$ to a Cbl(I) solution; that is, under pH conditions in which the reaction is the slowest. The data are shown in Figure 3, and confirm that Cbl(I) is

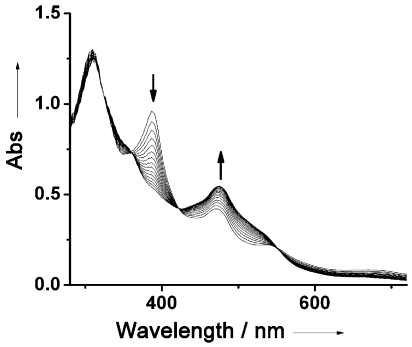


Figure 3. UV/Vis spectra for the reaction of Cbl(I) (7.50×10^{-5} M) with $ONOO^-$ (9.60×10^{-5} M) at pH 12.25 (25.0 °C, 0.050 M phosphate buffer, $I=0.20$ M (Na_2HPO_4)). Spectra are shown every 10 ms for 140 ms. Cbl(I) ($\lambda_{max}=387$, 460 and 560 nm)[11] was converted to Cbl(II) ($\lambda_{max}=312$, 405 and 475 nm).[12] Note that nitrite present in the $ONOO^-$ solution absorbs at $\lambda<400$ nm.

indeed oxidized to Cbl(II). Note that at much longer (two orders of magnitude larger) reaction times a further reaction is observed (see the Supporting Information for details), corresponding to the reaction of Cbl(II) with a second molecule of ONOO(H). Detailed studies on this reaction have been reported elsewhere.[6a]

The stoichiometry of the reaction between Cbl(I) and ONOO(H) was also determined. Since ONOOH rapidly spontaneously decomposes, the stoichiometry of the reaction was determined at pH 12.25, that is, under conditions in which spontaneous decomposition is negligible. Under these conditions the reaction between Cbl(I) and $ONOO^-$ is completed in seconds, and control experiments also showed that the reaction between Cbl(I) and nitrite (an impurity in the $ONOO^-$ solution) is negligible within the time frame of the experiments. Figure 4 gives UV/Vis spectra of equilibrated anaerobic solutions of Cbl(I) with $ONOO^-$ (0–0.30 mol equiv) at pH 12.25. A relatively high Cbl(I) concentration was used to maximize the stability of Cbl(I) against oxidation to Cbl(II). Cbl(I) was cleanly converted to Cbl(II) ($\lambda_{max}=475$ nm) with isosbestic points at approximately 417 and 542 nm, in agreement with literature values.[11] Figure 4b gives the corresponding plot of absorbance at 489 nm versus the mole ratio of $ONOO^-$ to Cbl(I) for the data shown in Figure 4a. This wavelength (an isosbestic wavelength for Cbl(II)/hydroxycobalamin)[13] was chosen to ensure no interference from the subsequent Cbl(II)+peroxynitrite reaction, in which Cbl(II) is oxidized to hydroxycobalamin.[6a] Figure 4b clearly shows that the reaction was complete upon the addition of approximately 0.2 equiv of $ONOO^-$ to Cbl(I); that is, $ONOO^-$ acts as a $5e^-$ oxidant in the reaction of Cbl(I) with $ONOO^-$.

As expected, a similar conclusion is reached by comparison of the observed absorbance change for each experiment with the absorbance difference between Cbl(I) and authentic Cbl(II) (Table S1 in the Supporting Information). It was not possible to determine the stoichiometry of the reaction at lower pH values (e.g., pH 7) since the reaction between Cbl(I) and NO_2^-, which is in the NaONOO stock solution,

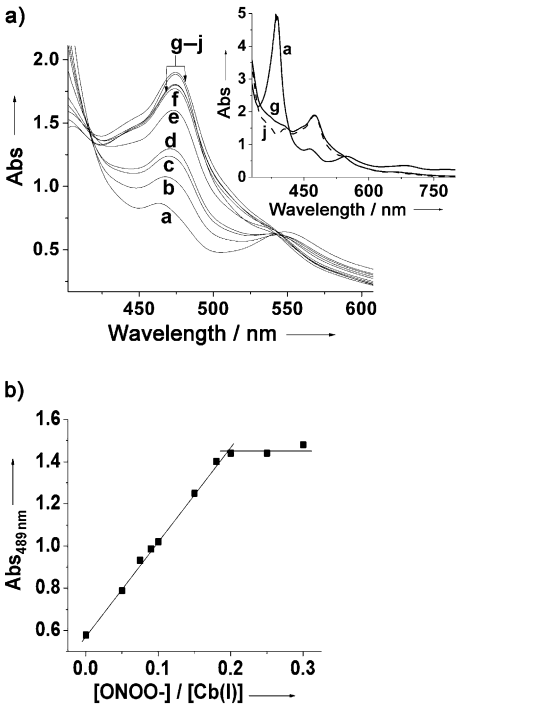


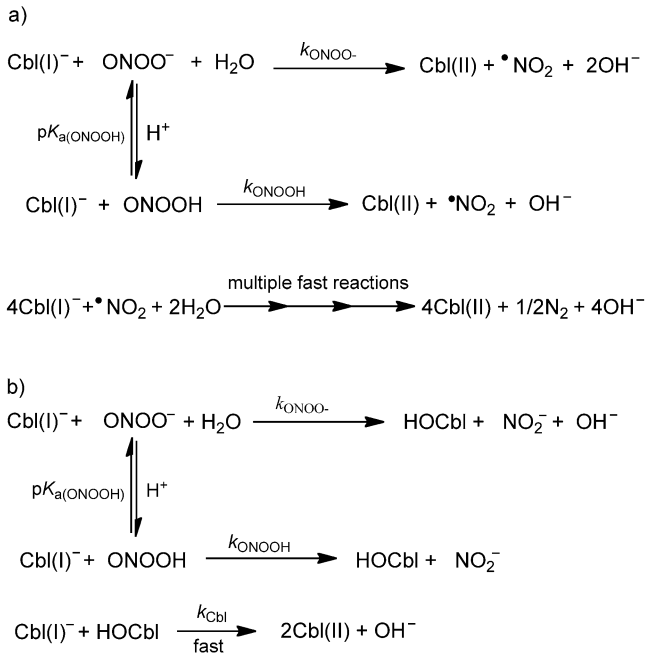
Figure 4. a) UV/Vis spectra for equilibrated anaerobic solutions of Cbl(I) (2.07×10^{-4} M) with 0, 0.05, 0.08, 0.10, 0.15, 0.18, 0.20, 0.25 and 0.30 mol equiv $ONOO^-$ (traces a–i) at pH 12.25 (25.0 °C, 0.10 M phosphate buffer, $I=0.40$ M). Trace j: UV/Vis spectrum of 2.00×10^{-4} M Cbl(II) under identical conditions (0.10 M phosphate buffer, $I=0.40$ M). Cbl(I) ($\lambda_{max}=388$, 463, 548 and 682 nm) is converted to Cbl(II) ($\lambda_{max}=312$, 405 and 475 nm) with isosbestic points at 417 and 542 nm. Inset: traces a and g (——) superimposed with trace j (– – –; authentic Cbl(II)). Note that nitrite present in the $ONOO^-$ solution absorbs at $\lambda<400$ nm. b) Plot of absorbance at 489 nm versus $[ONOO^-]/[Cbl(I)]$ for the data shown in (a). The reaction is complete upon the addition of approximately 0.2 mol equiv $ONOO^-$.

is much faster at lower pH values[6b] and interferes with these experiments.

In a $5e^-$ redox reaction peroxynitrite is oxidized to dinitrogen. The overall reaction is therefore:

$$5\,Cbl(I)^- + 2(3)\,H_2O + ONOO(H) \rightarrow 5\,Cbl(II) + {}^1/_2\,N_2 + 5(6)\,OH^- \quad (3)$$

Given that Cbl(I) is such a powerful reductant (E^0(Cbl(II)/Cbl(I)) = −0.61 V with respect to SHE[14]), it is not surprising that a $5e^-$ redox reaction occurs to ultimately produce the most thermodynamically stable product, N_2.[15] Control experiments were also carried out; these confirmed that a $6e^-$ reaction does not occur (NH_2OH would be formed; see the Supporting Information). A $4e^-$ reduction of ONOO(H) to N_2O can also be ruled out since others have shown that N_2O reacts with Cbl(I) to yield Cbl(II) and N_2.[16] It is well established by others that peroxynitrite is a $1e^-$ or $2e^-$ oxidant.[1,2] The proposed pathways for the reaction between Cbl(I) and ONOO(H) are given in Scheme 2a. Spectral changes for the rate-determining step corresponded to com-



Scheme 2. Possible reaction pathways for the reaction between Cbl(I) and ONOO(H). A rate constant for the reaction between Cbl(I) and $HOCbl/H_2OCbl^+$ to yield 2 Cbl(II) has been reported ($k = 3.2 \times 10^7\,M^{-1}s^{-1}$; pH independent).[16]

plete conversion of Cbl(I) to Cbl(II) at all pH values. Rate-determining $1e^-$ oxidation of Cbl(I) by ONOOH or $ONOO^-$ to give Cbl(II) and nitrogen dioxide ($^\bullet NO_2$) is, therefore, succeeded by multiple fast steps (elementary steps of molecularity greater than two are extremely rare); this leads ultimately to the oxidation of a further four Cbl(I) molecules to Cbl(II) and the formation of N_2.

Note that like peroxynitrite, $^\bullet NO_2$ is a powerful oxidant ($E^0(^\bullet NO_2, N_2) = +1.36$ V with respect to SHE).[17] Control experiments also showed that Cbl(I) reacts instantly with $^\bullet NO_2$ (see the Supporting Information). For a $2e^-$ rate-determining step (Scheme 2b) a Cbl(I)/ONOO(H) reaction stoichiometry of 2:1 would be expected, which is not consistent with our experimentally observed stoichiometry of 5:1. At lower pH conditions a $2e^-$ pathway via NO_2^- can also be ruled out since control experiments (pH 6.5–10.8) showed that the reaction between NO_2^- and Cbl(I) is negligible at all pH conditions in this study on the time scale of these experiments.[6b] Furthermore, the reaction stoichiometry of the Cbl(I) + NO_2^- reaction is 4:1 Cbl(I)/NO_2^- (pH 7.0 and 9.5) with hydroxylamine, not N_2, being produced.[6b] Others have reported that NO reacts with Cbl(I) to give $^1/_2 N_2O$ and Cbl(II) (the rate of the reaction was not determined).[18] N_2O reacts with 2 Cbl(I) to yield N_2 and 2 Cbl(II); however, the second-order rate constant at pH 8 is approximately $200\,M^{-1}s^{-1}$,[16] which is four orders of magnitude slower than the rate-determining step of the Cbl(I) + ONOO(H) reaction at this pH. It is, therefore, unlikely that the reaction proceeds via NO_2^-, NO or N_2O intermediates. Further experiments are required to examine the reactivity of Cbl(I) with other nitrogen oxide species, which is beyond the scope of this communication.

The calculated apparent second-order rate constant for the reaction between Cbl(I) and ONOO(H) is $3.6 \times 10^7\,M^{-1}s^{-1}$ at pH 7.4 (25 °C). The total intracellular Cbl concentration is 30–700 nM,[19] and the intracellular concentration and half-life of ONOO(H) have been estimated to be nM and 10 ms, respectively.[2a] However, to our knowledge the fraction of intracellular Cbl existing as Cbl(I) has not yet been reported, hence it is difficult to assess the biological relevance of this reaction. Finally, of interest is the observation by Groves et al. that intracellular protein tyrosine nitration by ONOO(H) can occur despite calculations indicating that ONOO(H) should react preferentially with intracellular thiols based on the individual rate constants and intracellular concentrations of these species.[20]

To summarize, kinetic studies on the reaction between Cbl(I) and peroxynitrite/peroxynitrous acid show that both ONOOH and $ONOO^-$ rapidly oxidize Cbl(I) to Cbl(II), with the former species reacting much faster as expected (1.6×10^8 vs. $1.4 \times 10^5\,M^{-1}s^{-1}$, respectively) since ONOOH is a much stronger oxidant. The proposed reaction pathways involve rate-determining $1e^-$ oxidation of Cbl(I) by ONOO(H) to yield Cbl(II) and $^\bullet NO_2$ followed by multiple fast steps leading ultimately to the oxidation of 5 Cbl(I) to 5 Cbl(II) and the generation of N_2. Protein-bound Cbl is readily accessed by small molecules.[21] Note that in biological systems $^\bullet NO_2$ might not necessarily react with a second Cbl(I) center, but instead react with another biomolecule. Cbl(I) is a key intermediate in the biosynthesis of the B_{12} cofactors and Cbl-dependent methylation of homocysteine to methionine catalyzed by methionine synthase.[3] Furthermore, hyperhomocysteinemia is an independent risk factor for cardiovascular and neurological diseases.[22] Finally, note that although peroxynitrite rapidly oxidizes the metal center of cob(I)alamin, the corrin moiety of the cobalamin complex remains intact. This is important, given that others have reported elevated levels of "cobalamin analogues" (the corrin moiety of cobalamin is modified) in neurological diseases associated with oxidative stress.[23]

Experimental Section

Experimental details are provided in the Supporting Information.

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

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