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Pulse radiolysis studies of the reactions of nitrogen dioxide with the vitamin B_{12} complexes cob(II)alamin and nitrocobalamin

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ABSTRACT

Although now recognized to be an important reactive nitrogen species in biological systems that modifies the structures of proteins, DNA and lipids, there are few studies on the reactivity of $^{\bullet}NO_2$, including the reactions between $^{\bullet}NO_2$ and transition metal complexes. We report kinetic studies on the reactions of $^{\bullet}NO_2$ with two forms of vitamin B_{12} — cob(II)alamin and nitrocobalamin. UV–visible spectroscopy and HPLC analysis of the product solution show that $^{\bullet}NO_2$ cleanly oxidizes the metal center of cob(II)alamin to form nitrocobalamin, with a second-order rate constant of $(3.5 \pm 0.3) \times 10^8\ M^{-1}\ s^{-1}$ (pH 7.0 and 9.0, room temperature, I = 0.20 M). The stoichiometry of the reaction is 1:1. No reaction is detected by UV–visible spectroscopy and HPLC analysis of the product solution when nitrocobalamin is exposed to up to 2.0 mol equiv. $^{\bullet}NO_2$.

1. Introduction

The nitrogen dioxide radical ($^{\bullet}NO_2$) is an important reactive nitrogen species (RNS) in biological systems. One of the main sources of $^{\bullet}NO_2$ in vivo is from peroxynitrite/peroxynitrous acid (ONOO(H), pK_a 6.8 [1]); a powerful oxidant formed by the diffusion controlled reaction between nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$) radicals under oxidative stress conditions. Peroxynitrous acid undergoes bond homolysis to generate $^{\bullet}OH$ and $^{\bullet}NO_2$ with ~30% yields [2,3]. In biological systems peroxynitrite also reacts with CO_2 to form nitrosoperoxocarboxylate ($ONOOCO_2^-$), which undergoes homolytic peroxo bond cleavage to produce $^{\bullet}NO_2$ and the carbonate radical anion ($CO_3^{\bullet-}$) in ~35% yields [4]. Other pathways that potentially lead to $^{\bullet}NO_2$ generation in vivo include enzymatic oxidation of nitrite by peroxidases [4–7] and auto-oxidation of nitric oxide which produces nitrite as the final product [4].

$^{\bullet}NO_2$ is a strong one-electron oxidant ($E(^{\bullet}NO_2, NO_2^-) = 1.03$ V vs. NHE (normal hydrogen electrode) [8] and has a rich chemistry, including reacting rapidly with other radical species and $1e^-$ oxidation of reductants, and slower addition to double bonds and H atom abstraction [4,9–11]. $^{\bullet}NO_2$ reacts with lipids, DNA, and proteins [4,6,12,13] and plays a key role in cellular nitrosative stress [4]. Nitrosative stress has been implicated in various diseases associated with chronic inflammation including Alzheimer's disease (AD), multiple sclerosis (MS), atherosclerosis and amyotrophic lateral sclerosis (ALS) [14]. Therefore, identifying molecules that can scavenge $^{\bullet}NO_2$ is of interest [9,15,16], although still relatively unexplored compared with other ROS (reactive oxygen species)/RNS. Fluorescent probes for detecting $^{\bullet}NO_2$ in cells are also of interest [17].

Vitamin B_{12} derivatives (also commonly known as cobalamins, Cbls, Fig. 1) are important cofactors for mammalian adenosylcobalamin (5′-deoxy-5′-adenosyl (AdoCbl)) – dependent methylmalonyl-CoA mutase and methylcobalamin (MeCbl) – dependent methionine synthase [18,19]. Cbl is required in every cell in mammals and Cbl deficiency is associated with pernicious anemia and neurological diseases [20,21]. Upon uptake into cells, all cob(III)alamins (Co^{3+}) are reduced to the pentacoordinate cob(II)alamin (Cbl(II), Cbl(II)$^{\bullet}$, Co^{2+}) [22]. Given that Cbl(II) is a radical complex, Cbl(II) would be expected to react rapidly with radicals including $^{\bullet}NO_2$, and indeed, cell studies have shown that B_{12} protects against $O_2^{\bullet-}$, H_2O_2 and homocysteine — induced oxidative stress [23–25]. Furthermore Cbl supplementation has been used to treat a wide range of chronic inflammatory diseases [26,27].

In this study we report kinetic and mechanistic studies on the reaction of $^{\bullet}NO_2$ with cob(II)alamin and nitrocobalamin (X = NO_2^-, Fig. 1), using pulse radiolysis of solutions containing nitrite to generate $^{\bullet}NO_2$.

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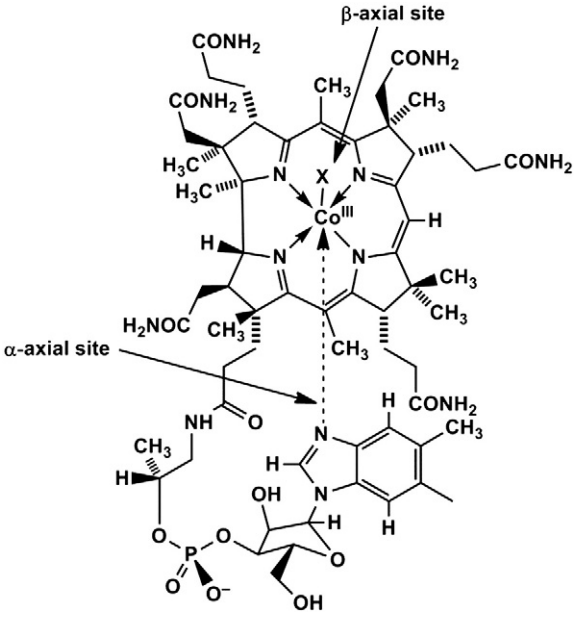

Fig. 1. Structure of cob(III)alamins X = CH_3, Ado, H_2O/OH, CN^-, NO_2^-, etc. The ligand "X" is lost upon reduction of cob(III)alamins to give pentacoordinate cob(II)alamin.

2. Experimental section

2.1. Chemicals

Hydroxocobalamin hydrochloride, HOCbl·HCl (·nH_2O) (≥96%, 10–15% water, batch dependent [28]) was purchased from Fluka and sodium borohydride (≥98%) and acetic acid were obtained from Acros Organics. Potassium dihydrogen phosphate, sodium hydroxide, ammonia, acetonitrile (HPLC grade), water (HPLC grade) and potassium cyanide (≥99%) were purchased from Fisher Scientific. Potassium bicarbonate (≥99%), sodium hydrogen phosphate (≥99%), potassium hydroxide, potassium nitrite (≥99%) and sodium hydroxide were obtained from J.T. Baker Chemical Company. Water was purified using a Barnstead Nanopure Diamond or Millipore water purification system.

2.2. Synthesis of cob(II)alamin (Cbl(II))

Cbl(II) was prepared by reducing HOCbl·HCl with $NaBH_4$ (1.2 mol equiv.) under anaerobic conditions using a procedure reported in the literature [25]. In a typical synthesis, HOCbl·HCl (~25 mg, 1.6×10^{-5} mol (10–15% H_2O)) was dissolved in anaerobic water (0.75 ml) in a vial. An aqueous, anaerobic stock solution of $NaBH_4$ (~10 mg in 1.00 ml) was prepared and $NaBH_4$ (1.2 mol equiv.) was added to the HOCbl·HCl solution. The vial was shaken vigorously for ~1 min and the reaction was allowed to proceed for 15–30 min. After the reaction was complete, excess $NaBH_4$ was quenched by the addition of acetone (0.200 ml). Cbl(II) was characterized by UV–visible (UV–vis) spectroscopy (λ_{max} 312, 405, 475 nm) [29], and solutions were stored under anaerobic conditions at −24 °C.

2.3. Determination of Cbl concentrations

Cbl concentrations were determined by converting Cbls to dicyanocobalamin, $(CN)_2Cbl^-$. Cobalamins were allowed to react with KCN (0.10 M, pH 11.50) to produce $(CN)_2Cbl^-$ ($\varepsilon_{368\ nm}$ = 30,000 $M^{-1}\ cm^{-1}$ [30]).

2.4. pH measurements

pH measurements were carried out at room temperature using an Orion model 520A or 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 and 10.00. Solution pH was adjusted using H_3PO_4, NaOH, or KOH solutions as necessary.

2.5. Pulse radiolysis experiments

Pulse radiolysis experiments were carried out at Brookhaven National Laboratory with a 2 MeV Van de Graaff accelerator producing electron pulses (pulse width 30–300 ns) that resulted in 1–30 Gy ((1–30) × 10^{-6} M primary radicals) generated in aqueous solution. The optical path of the cell was 2 cm. $^{\bullet}NO_2$ was generated upon irradiation of buffered N_2O-saturated aqueous solutions containing 0.050 M $NaNO_2$ at pH 6.00, 7.40 and 9.00 (phosphate buffer, I = 0.20 M, room temperature (RT)). Radiolysis generates hydrated electrons (e_{aq}^-), $^{\bullet}OH$ and $^{\bullet}H$ [31–33] which oxidize NO_2^- to $^{\bullet}NO_2$ in N_2O-saturated nitrite solutions [31–33]. The dose per pulse was determined with a thiocyanate (0.010 M) dosimeter, saturated with N_2O (0.026 M), taking $G(SCN)_2^{\bullet-}$ = 6.13, where G is the number of molecules formed per 1.602×10^{-17} J of energy absorbed by the solution, and $\varepsilon_{472\ nm}$ = 7590 ± 230 $M^{-1}\ cm^{-1}$ [34].

Prior to irradiation, solid Cbl(II) was quickly added to the appropriate anaerobic buffer containing nitrite in the solution reservoir and the solution bubbled with argon for a further ~30 min. Then the solution was saturated with N_2O for 5–8 min prior to collecting data. Reported rate constants are the average values of at least three independent measurements at three different wavelengths. The data were collected and fitted using the Numerical Integration of Chemical Kinetics program in PRWIN (by H. Schwarz, BNL).

2.6. ^{60}Co γ-radiolysis

Steady-state ^{60}Co γ-radiolysis studies on the reaction between cob(II)alamin or nitrocobalamin with $^{\bullet}NO_2$ were carried out at pH 7.40 (0.068 M KH_2PO_4, 0.050 M $NaNO_2$, I = 0.20, 50.00 ml) in a buffered solution saturated with N_2O gas for 10–15 min. Cbl(II) (~1.66 mg) solid was quickly added and the solution bubbled for a further ~2–3 min with N_2O. The solution was transferred to a N_2O-flushed quartz cuvette, capped and the cuvette repeatedly exposed to a continuous flux of $^{\bullet}NO_2$ with a production rate of ~2.5×10^{-7} M $^{\bullet}NO_2$/s. The UV–vis spectrum was subsequently recorded after each irradiation. A similar experiment was carried out replacing Cbl(II) with HOCbl·HCl (pH 7.40, 0.068 M KH_2PO_4, 0.050 M $NaNO_2$, I = 0.20, RT), which rapidly reacts with nitrite to form nitrocobalamin in the presence of nitrite. ^{60}Co γ-radiolysis studies were carried out under anaerobic conditions using N_2O-saturated solutions.

2.7. HPLC experiments

HPLC analyses were carried out using an Agilent 1100 series HPLC system equipped with a degasser, quaternary pump, autosampler, and a photodiode array detector (resolution of 2 nm), using an Alltech Alltima C_{18} semipreparative column (5 μm, 100 Å, 10 mm × 300 mm) thermostated to 25 °C. A mobile phase consisting of acetate buffer (1% v/v CH_3COOH, pH 3.5), **A**, and CH_3CN (1% v/v CH_3COOH), **B**, were used in the following method: 0–25 min isocratic elution of 85:15 **A**:**B**, 25–27 min 85:15 to 30:70 **A**:**B**, 27–34 min isocratic elution of 30:70 **A**:**B**, 34–36 min 30:70 to 85:15 **A**:**B**. All gradients were linear and a flow rate of 2 ml/min was used. Product peaks were monitored at 254 and 350 nm.

3. Results and discussion

3.1. Studies on the reaction of cob(II)alamin with $^{\bullet}NO_2$

The reaction between reduced vitamin B_{12} (cob(II)alamin (Cbl(II)) and $^{\bullet}NO_2$ was studied using pulse radiolysis in N_2O-saturated nitrite

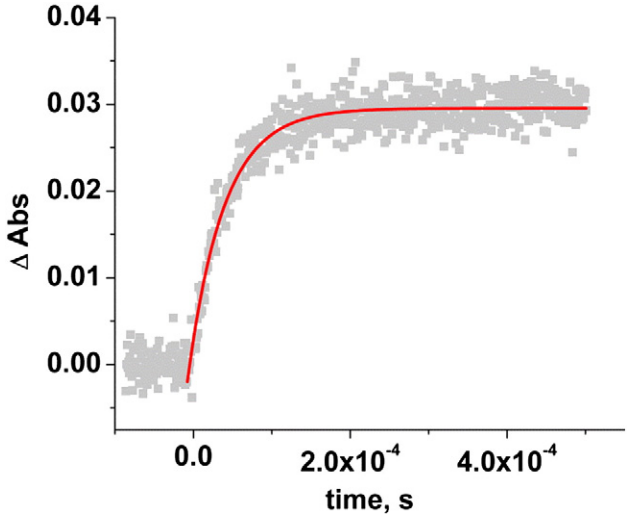

Fig. 2. Plot of change in absorbance at 355 nm versus time for the reaction of $^{\bullet}NO_2$ (3.0×10^{-6} M) with excess Cbl(II) (7.0×10^{-5} M) at pH 7.40 (0.050 M $NaNO_2$, 0.068 M KH_2PO_4, RT, $I = 0.20$ M, N_2O-saturated buffer). The best fit of the data to a first-order rate equation is superimposed on the data, giving $k_{obs} = (2.10 \pm 0.19) \times 10^4\ s^{-1}$.

solutions. Radiolysis of water generates hydrated electrons (e^-_{aq}), $^{\bullet}H$ and $^{\bullet}OH$ [31–33], the latter species oxidizing NO_2^- to $^{\bullet}NO_2$ [31–33].

$$H_2O \rightsquigarrow {}^{\bullet}OH + e^-_{aq} + H_2O_2 + H^+ + {}^{\bullet}H + H_2 \quad (1)$$

$$e^-_{(aq)} + N_2O + H_2O \rightarrow N_2 + OH^- + {}^{\bullet}OH \quad (2)$$

$$^{\bullet}H + N_2O \rightarrow N_2 + {}^{\bullet}OH \quad (3)$$

$$^{\bullet}OH + NO_2^- \rightarrow {}^{\bullet}NO_2 + OH^- \quad (4)$$

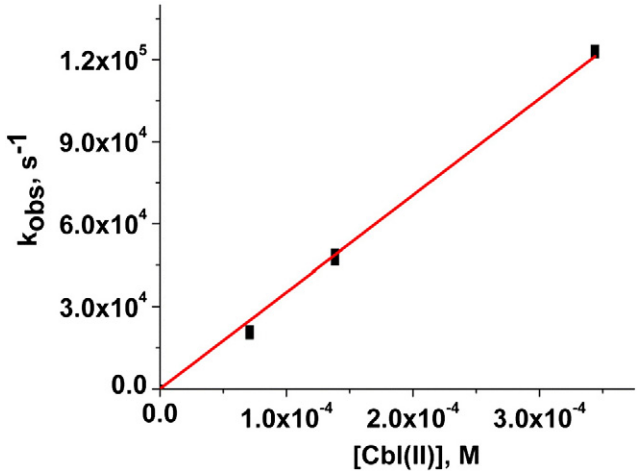

Fig. 3. Plot of observed rate, k_{obs}, versus Cbl(II) concentration for the reaction between Cbl(II) ($(0.7–3.4) \times 10^{-5}$ M) and $NO_2^{\bullet}$ ($(0.3–5.8) \times 10^{-6}$ M) at pH 7.40 (0.068 M KH_2PO_4, 0.050 M $NaNO_2$, $I = 0.20$ M, RT, N_2O-saturated buffer). Data have been fitted to a line passing through origin, giving $k_{app} = (3.5 \pm 0.3) \times 10^8\ M^{-1}\ s^{-1}$.

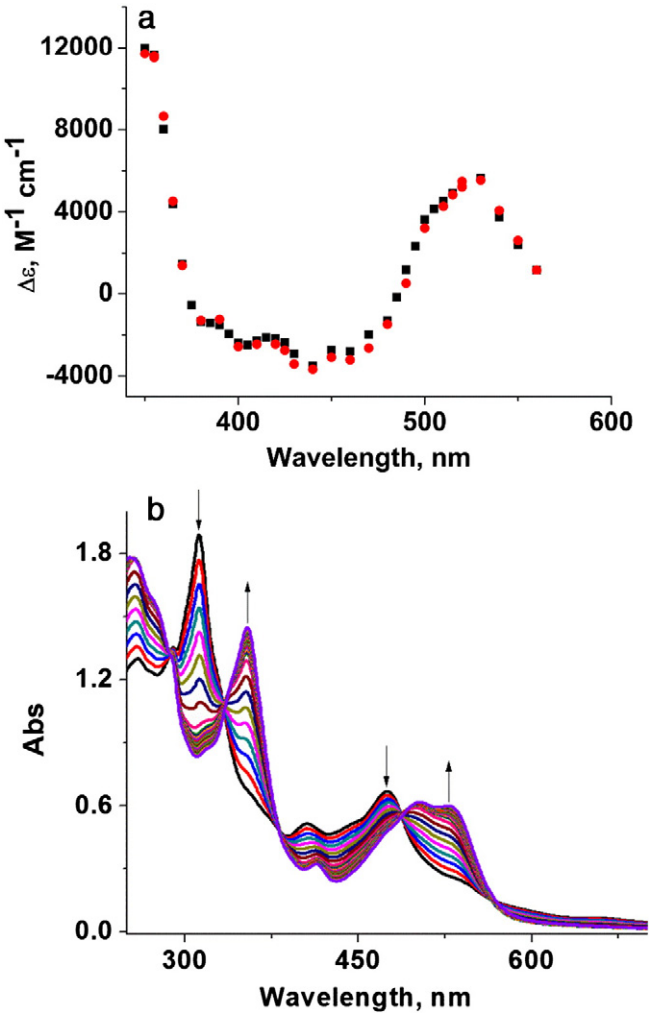

Fig. 4. (a) Plot of change in molar extinction coefficient versus wavelength for (black squares) the reaction of excess Cbl(II) (5.0×10^{-5} M) with $^{\bullet}NO_2$ ($(2.0–13.5) \times 10^{-6}$ M; produced by pulse radiolysis) at pH 7.40 (0.050 M $NaNO_2$, 0.068 M KH_2PO_4, RT, $I = 0.20$ M, N_2O-saturated buffer) and (red circles) an anaerobic solution of Cbl(II) (5.8×10^{-5} M) containing excess nitrite exposed to air, resulting in NO_2Cbl formation (pH 7.40, 0.050 M $NaNO_2$, 0.068 M KH_2PO_4, RT, $I = 0.20$ M). (b) UV–vis spectral change for the latter reaction. The arrows indicate the directions of absorbance changes.

In kinetic experiments the Cbl(II) concentration was kept at least 5 times higher than the $^{\bullet}NO_2$ concentration throughout to achieve essentially pseudo-first order conditions. Fig. 2 gives a plot of absorbance at 355 nm versus time for the reaction between Cbl(II) (7.0×10^{-5} M) and $^{\bullet}NO_2$ (3.0×10^{-6} M) at pH 7.40. The data fit well to a first-order rate equation, giving an observed rate, $k_{obs} = (2.10 \pm 0.19) \times 10^4\ s^{-1}$. The rate is essentially the same when data are collected at 405 nm ($k_{obs} = (2.00 \pm 0.18) \times 10^4\ s^{-1}$; Fig. S1, Supplemental information).

Rates for the reaction between Cbl(II) and $^{\bullet}NO_2$ were determined at other Cbl(II) concentrations at pH 7.4; Fig. 3. The data fit well to a straight line passing through the origin, consistent with a single irreversible reaction. The linear relationship suggests that the reaction is first-order with respect to Cbl(II) and $^{\bullet}NO_2$. From the slope, the second-order rate constant (k_{app}) of the reaction was $(3.5 \pm 0.3) \times 10^8\ M^{-1}\ s^{-1}$.

The reaction was also studied at pH 9.00. The observed rate constant was determined at a range of Cbl(II) concentrations and the data are summarized in Fig. S2, Supplemental information. The reaction is once again irreversible, and, from the slope, the rate constant (k_{app}) of the

reaction was determined. This value is the same within experimental error as that obtained at pH 7.0 as expected, since there is no change in the ionization of the species in this pH range. It was not possible to determine k_{app} at values less than 7.0, since increasingly more HNO_2 is formed ($pK_a(HNO_2/NO_2^-) = 3.40$ [12]). (H)NO_2 is required in the solution for $^{\bullet}NO_2$ generation and others have shown that HNO_2 (not NO_2^-) rapidly oxidizes Cbl(II) to aquacobalamin [35]. At pH 6.0 the half-life for oxidation of Cbl(II) by (H)NO_2 (0.05 M) is ~30 s (Fig. S3, Supplemental information; 0.134 M KH_2PO_4, 0.050 M $NaNO_2$, $I = 0.20$ M).

A previous experiment in our lab suggested that Cbl(II) ($\lambda_{max} = 312$, 405, and 475 nm) reacts rapidly with $^{\bullet}NO_2$ to form the cob(III)alamin, nitrocobalamin (NO_2Cbl) [36]. It was not possible to obtain full spectra as function of time from our experimental setup for the pulse radiolysis experiments. In order to confirm that Cbl(II) is indeed converted to NO_2Cbl ($\lambda_{max} = 354$, 413, and 531 nm at pH 7.40) by $^{\bullet}NO_2$ [36,37], a plot of change in molar extinction coefficient versus wavelength for the reaction between Cbl(II) and $^{\bullet}NO_2$ was generated and compared with the change in molar extinction coefficient for the conversion of Cbl(II) to NO_2Cbl, Fig. 4. The latter data was obtained by exposing an anaerobic solution of Cbl(II) in pH 7.40 buffer containing excess nitrite (0.050 M $NaNO_2$) to air, since Cbl(II) is oxidized by air to aquacobalamin/hydroxocobalamin, which reacts rapidly with nitrite to form nitrocobalamin [37]. There is excellent agreement between the two sets of data, with both having isosbestic points at 378 and 489 as expected for the conversion of Cbl(II) to NO_2Cbl [36]. A similar result was also obtained at pH 9.00 (Fig. S4, Supplemental information); hence under the pH conditions of our study $^{\bullet}NO_2$ oxidizes Cbl(II) to NO_2Cbl.

The stoichiometry of the reaction between Cbl(II) and $^{\bullet}NO_2$ was investigated using a ^{60}Co γ-source to generate $^{\bullet}NO_2$. Cbl(II) (2.7×10^{-5} M) was repeatedly exposed to a continuous flux of $^{\bullet}NO_2$ (2.5×10^{-7} M $^{\bullet}NO_2$/s) for 32 s time intervals (0–2 mol equiv. $^{\bullet}NO_2$ added in total) with UV–vis spectra recorded after each exposure, Fig. 5. Cbl(II) is cleanly oxidized to NO_2Cbl ($\lambda_{max} = 354$, 413 and 531 nm) with sharp isosbestic points observed at 334, 378, 489, and 570 nm in agreement with literature values for the Cbl(II)/NO_2Cbl conversion [36,37]. The inset to Fig. 5 gives a plot of absorbance at 312 nm versus mol equiv. of $^{\bullet}NO_2$ added for the same data. The absorbance at 312 nm decreases linearly up to 1.0 mol equiv. $^{\bullet}NO_2$ and is unchanged upon the addition of further

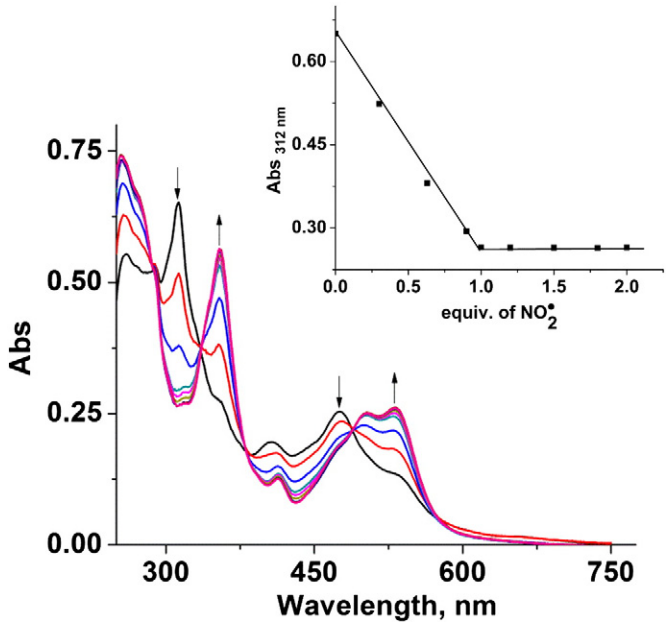

Fig. 5. UV–vis spectral change for the products of the reaction between cob(II)alamin (2.7×10^{-5} M) and $^{\bullet}NO_2$ (0–2 mol equiv.) at pH 7.40 (0.068 M KH_2PO_4, 0.050 M $NaNO_2$, $I = 0.20$ M, N_2O saturated buffer). *Inset*: Plot of absorbance at 312 nm versus mol equiv. $^{\bullet}NO_2$. The arrows indicate the directions of absorbance changes.

$^{\bullet}NO_2$. Therefore, the stoichiometry of the reaction between Cbl(II) and $^{\bullet}NO_2$ at pH 7.40 is 1:1.

In order to probe whether $^{\bullet}NO_2$ modifies the corrin ring of B_{12}, the product solution for the reaction between Cbl(II) and 2.0 mol equiv. $^{\bullet}NO_2$ at pH 7.40 was analyzed by HPLC. A single corrinoid product was observed in the HPLC chromatogram eluting with the same retention time as authentic NO_2Cbl, Fig. S5, Supplemental information. Hence $^{\bullet}NO_2$ reacts with Cbl(II) to form the cob(III)alamin complex nitrocobalamin, NO_2Cbl, Eq. (5).

$$\text{Cbl(II)}^{\bullet} + {}^{\bullet}NO_2 \rightarrow NO_2\text{Cbl} \quad (5)$$

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) and Co(II) porphyrins exposed to $^{\bullet}NO_2$ at low pressures [38]. Oxidation of the metal center has also been observed for the reactions of $^{\bullet}NO_2$ with nitrosylhemoglobin ($MbFe^{II}NO$) and nitrosylhemoglobin ($HbFe^{II}NO$), with rate constants of $(2.9 \pm 0.3) \times 10^7$ M^{-1} s^{-1} and $(1.8 \pm 0.3) \times 10^7$ M^{-1} s^{-1}, respectively, followed by dissociation of $^{\bullet}NO$ [39].

3.2. Studies on the reaction of nitrocobalamin with $^{\bullet}NO_2$: $^{\bullet}NO_2$ does not react with the corrin ring of B_{12}

The reaction between nitrocobalamin (NO_2Cbl) and $^{\bullet}NO_2$ was explored using the ^{60}Co γ source to generate $^{\bullet}NO_2$ at pH 7.40. Nitrocobalamin is rapidly formed upon the addition of H_2OCbl^+/HOCbl to a solution containing excess nitrite [37]. Cbl(II) (2.1×10^{-5} M) was repeatedly exposed to a continuous flux of $NO_2^{\bullet}$ (2.5×10^{-7} M $^{\bullet}NO_2$/s) for 32 s time intervals (0–2 mol equiv. $NO_2^{\bullet}$ added in total) with UV–vis spectra recorded after each exposure, Fig. S6 in the Supplemental information. No change in the UV–vis spectra was observed. Analyzing the product solution of the reaction between NO_2Cbl and $^{\bullet}NO_2$ using HPLC, Fig. S7, Supplemental information, confirmed that no reaction occurs — that is, $^{\bullet}NO_2$ does not modify the corrin macrocycle at the low concentrations of $^{\bullet}NO_2$ produced in these experiments. Interesting, reacting $^{\bullet}NO_2$ with Cbl(II) using Cu/HNO_3 to generate $^{\bullet}NO_2$ resulted in a small HPLC peak in addition to NO_2Cbl which was attributed to a nitrocorrinoid(III) species arising from modification of the corrin ring by $^{\bullet}NO_2$ [36]. It is possible that higher $^{\bullet}NO_2$ concentrations were achieved in solution in the latter experiments, resulting in corrin ring modification by $^{\bullet}NO_2$, although no experiments were carried out to probe this further. No reaction was observed between metmyoglobin ($MbFe^{III}$–OH_2) and $^{\bullet}NO_2$ [40]. Rate constants have also been reported for the reactions of $^{\bullet}NO_2$ with oxymyoglobin ($MbFe^{II}O_2$; $(4.5 \pm 0.3) \times 10^7$ M^{-1} s^{-1}) and ferrylmyoglobin ($MbFe^{IV} = O$; $(1.2 \pm 0.2) \times 10^7$ M^{-1} s^{-1}) at pH 7.4, with the reactions proceeding via $MbFe^{III}O_2NO_2$ and $MbFe^{III}ONO_2$ intermediates, respectively [40].

4. Conclusions

The second-order rate constant of the reaction between Cbl(II) and $^{\bullet}NO_2$ to form nitrocobalamin has been directly determined using pulse radiolysis and was found to be 3.5×10^8 M^{-1} s^{-1} (pH 7.4 and 9.0). Measurements at lower pH values were not possible since HNO_2 oxidizes Cbl(II) to aquacobalamin. Nitrocobalamin formation was shown by both UV–vis spectroscopy and HPLC. No reaction was observed between nitrocobalamin and $^{\bullet}NO_2$. Our results show that under physiological conditions although $^{\bullet}NO_2$ will rapidly oxidize the metal center of Cbl(II), it is unlikely to modify the corrin macrocycle. Given that GSH is present at such high (mM) concentrations in cells and that the second-order rate constant for the reaction between glutathione and $^{\bullet}NO_2$ is ~2×10^7 [9], GSH is likely to be a much more important intracellular scavenger of $^{\bullet}NO_2$.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2014.09.014.

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