ChemPubSoc Europe

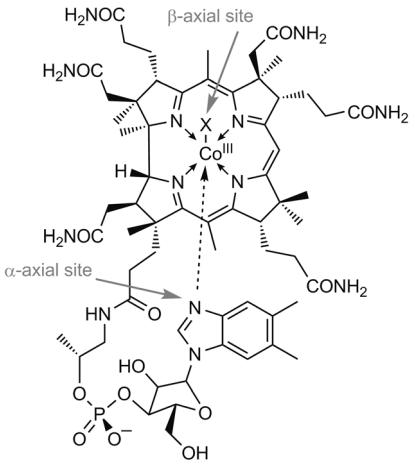
DOI: 10.1002/cbic.201300229

Pulse Radiolysis Studies on the Reaction of the Reduced Vitamin B_{12} Complex Cob(II)alamin with Superoxide

Rohan S. Dassanayake,[a] Diane E. Cabelli,*[b] and Nicola E. Brasch*[a]

Reactive oxygen and nitrogen species (ROS/RNS) including hydroxyl radicals, nitric oxide, hydrogen peroxide, peroxynitrite and superoxide, are important signaling molecules and are also generated at high concentrations as part of the innate immune response to pathogens.[1] However, elevated levels of reactive species can lead to oxidative stress and result in irreversible damage to biomolecules.[1] Oxidative stress has been implicated in numerous diseases including cardiovascular disease, cancer, neurological diseases and aging in general.[1–2] This has led to considerable interest in the development of ROS-/RNS-scavenging therapeutics, including porphyrin-based complexes.[3]

The structurally related vitamin B_{12} derivatives, the cobalamins (Cbls; X = Me or Ado, Scheme 1), are cofactors for two enzymes in humans—cytosolic methionine synthase (MS) and mitochondrial L-methylmalonyl-CoA mutase (MM-CoA mutase).[4] Cbl supplementation has been shown to be beneficial for treating chronic inflammatory diseases including chronic fatigue syndrome, sepsis, asthma, rheumatoid arthritis, neurological diseases such as Alzheimer's disease, and viral-based diseases such as AIDS.[5] Recently Scalabrino et al. demonstrated that Cbl attenuates the pro-inflammatory immune response in a rat model for chronic Cbl deficiency by modulating levels of cytokines and growth factors including TNF-α, nerve growth factor, epidermal growth factor, and IL-6.[6]



Scheme 1. Structure of vitamin B_{12} derivatives (cobalamins). X = CH_3, Ado, H_2O/OH, NO, etc.

The important ROS superoxide, $O_2^{\bullet-}$, is produced as a by-product of cellular metabolism by mitochondrial and reticular membrane electron-transport systems and by enzymes such as NADPH oxidase and xanthine oxidase.[7] $O_2^{\bullet-}$ is a precursor of two of the most potent ROS/RNS, peroxynitrite and the hydroxyl radical.[8] Superoxide dismutases, enzymes that catalyze the destruction of $O_2^{\bullet-}$ by converting it to O_2 and H_2O_2, are up-regulated in oxidative stress-associated diseases.[1] In vitro cell studies on human aortic endothelial cells show that vitamin B_{12} prevents $O_2^{\bullet-}$-induced oxidative stress at physiologically relevant (~10^{-9} M) concentrations.[9] With supplementation, intracellular Cbl levels can reach 0.7 μM.[10] Upon entering cells, vitamin B_{12} derivatives (cob(III)alamins) are reduced by the cblC protein to cob(II)alamin, Cbl(II).[11] We have previously shown that $O_2^{\bullet-}$ generated by the xanthine oxidase/acetaldehyde system reacts rapidly with the radical complex Cbl(II) by an indirect competition method.[9b] Given the importance of this reaction in human health, we now report the direct determination of rate constants for the reaction between Cbl(II) and $O_2^{\bullet-}$ by using pulse radiolysis to generate $O_2^{\bullet-}$.

Figure 1 gives a plot of change of absorbance at 530 nm versus time for the reaction between Cbl(II) (7.3×10^{-5} M) and $O_2^{\bullet-}$ (9.0×10^{-6} M) at pH 7.42. Irradiating aqueous solutions generates $^{\bullet}OH$, e_{aq}^-, and $H^{\bullet}$.[12] In O_2-saturated aqueous solutions containing ethanol or formate, these primary radicals are rapid-

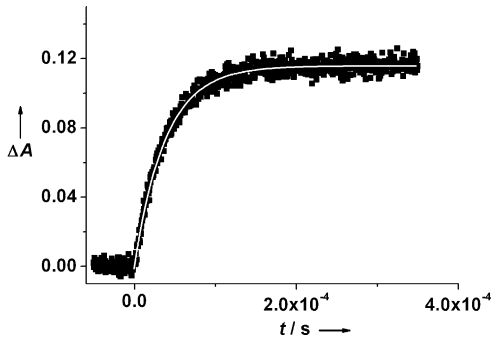


Figure 1. Plot of change in absorbance at 530 nm versus time for the reaction of $O_2^{\bullet-}$ (9.0×10^{-6} M) with excess Cbl(II) at pH 7.42 (0.500 M EtOH, 5.00×10^{-3} M phosphate buffer, I = 0.010 M, RT). The best fit of the data to a first-order rate equation is superimposed on the data and gives an observed rate constant, $k_{obs} = (2.70\pm0.02)\times10^4$ s^{-1}.

[a] R. S. Dassanayake, Dr. N. E. Brasch
Department of Chemistry & Biochemistry and School of Biomedical Sciences
Kent State University
Kent, OH 44242 (USA)
E-mail: nbrasch@kent.edu
Homepage: http://www.brasch-group.com/

[b] Dr. D. E. Cabelli
Department of Chemistry, Brookhaven National Laboratory
Upton, NY 11973 (USA)
E-mail: cabelli@bnl.gov

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201300229.

ly converted to $O_2^{\bullet-}$.[12] An anaerobic aqueous Cbl(II) solution was rapidly mixed with an O_2-saturated aqueous ethanol solution by using a stopped-flow mixing device. The resulting mixture was subsequently exposed to an intense ionizing pulse (30–300 ns), and data were collected. The time between mixing and pulsing was typically <2 s, and the Cbl(II) concentration was kept at least five times higher than the $O_2^{\bullet-}$ concentration in order to achieve essentially pseudo-first-order conditions. Our earlier stoichiometry experiments showed that the reaction between Cbl(II) and $O_2^{\bullet-}$ proceeds according to Equation (1), with clean oxidation of the metal center of Cbl(II) to give aquacobalamin/hydroxycobalamin, H_2OCbl^+/HOCbl ($pK_a(H_2OCbl^+)=7.8$[13]).[9b]

$$Cbl(II) + O_2^{\bullet-} + 2\,H_3O^+ \rightarrow H_2OCbl^+ + H_2O_2 + H_2O \qquad (1)$$

The data in Figure 1 fit well to a first-order rate equation to give an observed rate constant, $k_{obs}=(2.70\pm0.02)\times10^4\ s^{-1}$. The rate constant is unchanged when data are collected at 435 nm ($k_{obs}=(2.69\pm0.05)\times10^4\ s^{-1}$; Figure S1, Supporting Information).

Rate constants for the reaction between Cbl(II) and $O_2^{\bullet-}$ at pH 7.42 were also determined at other Cbl(II) concentrations, and the data are summarized in Figure 2. 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 $O_2^{\bullet-}$. From the slope, the second-order rate constant (k_{app}) of the reaction was $(3.78\pm0.07)\times10^8\ M^{-1}s^{-1}$. Separate pulse radiolysis experiments showed that H_2OCbl^+/HOCbl does not react with $O_2^{\bullet-}$ (pH 7.42, 0.500 M EtOH, $I=0.01$ M (phosphate buffer)). Analyzing these latter data at 260 nm gave a rate constant for $O_2^{\bullet-}$ decomposition similar to that reported in the literature[12] at this pH value (decomposition of superoxide is pH dependent). Values of k_{app} were also determined at pH 5.50 and 8.72 at a single Cbl(II) concentration to give $k_{app}=(4.23\pm0.18)\times10^8$ and $(4.40\pm0.22)\times10^8\ M^{-1}s^{-1}$, respectively (Figures S2 and S3). Given the similarity in these rate constants to the value obtained at pH 7.42 ($(3.78\pm0.07)\times10^8\ M^{-1}s^{-1}$), k_{app} is essentially independent of pH over this pH range.

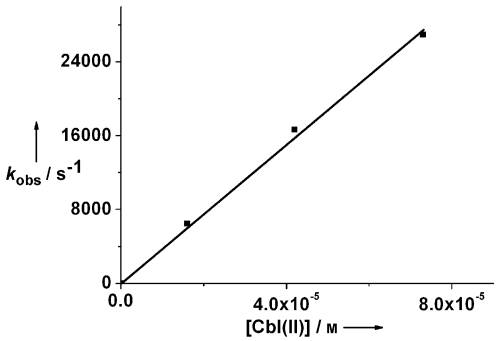


Figure 2. Plot of k_{obs} versus Cbl(II) concentration for the reaction between excess Cbl(II) and $O_2^{\bullet-}$ ($(1.0–9.0)\times10^{-6}$ M) at pH 7.42 (0.500 M EtOH, 5.00×10^{-3} M phosphate buffer, $I=0.01$ M, RT). Points represent the mean value of at least three independent measurements at three different wavelengths. Data were fitted to a line passing through the origin to give $k_{app}=(3.78\pm0.07)\times10^8\ M^{-1}s^{-1}$.

It is well established that Cbl(II) is oxidized to H_2OCbl^+/HOCbl by air.[9b] In order to confirm that Cbl(II) is also oxidized to H_2OCbl^+/HOCbl by $O_2^{\bullet-}$ in our pulse radiolysis experiments, a plot of change in extinction coefficient versus wavelength for the reaction of Cbl(II) with $O_2^{\bullet-}$ was generated and compared with the change in extinction coefficient for the Cbl(II)→(H_2OCbl^+/HOCbl conversion (Figure 3). (Note that it was not possible to obtain full spectra as a function of time from our experimental setup for the pulse radiolysis experiments.) There is excellent agreement between the two sets of data and the isosbestic points observed at 375, 490, and 578 nm correspond to the expected values reported for the Cbl(II)→(H_2OCbl^+/HOCbl conversion.[9b] The corresponding plots of absorbance of the reaction product solution versus wavelength for both sets of data are also in excellent agreement with each other (Figure S4). The formation of H_2OCbl^+/HOCbl from the reaction between Cbl(II) and $O_2^{\bullet-}$ was additionally confirmed by HPLC analysis of the product solution from the pulse radiolysis experiments and by using the xanthine oxidase/acetaldehyde system to generate $O_2^{\bullet-}$ (details are given in the Supporting Information).

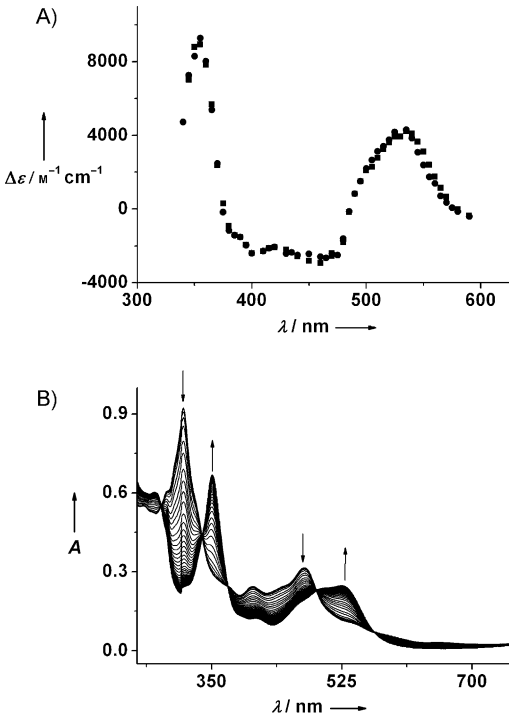


Figure 3. A) Change in the molar absorption coefficient versus wavelength for the reaction of (■) excess Cbl(II) with $O_2^{\bullet-}$ ($(1.0–9.0)\times10^{-6}$ M) at pH 7.42 (0.500 M EtOH, 5.00×10^{-3} M phosphate buffer, $I=0.01$ M, RT) and (●) Cbl(II) (5.00×10^{-5} M) with O_2 (from air) to form H_2OCbl^+/HOCbl (5.00×10^{-3} M phosphate buffer, 0.500 M EtOH, $I=0.01$ M, RT). B) UV/Vis spectra for the oxidation of Cbl(II) (3.00×10^{-5} M) by O_2 to form H_2OCbl^+/HOCbl (5.00×10^{-3} M phosphate buffer, 0.500 M EtOH, $I=0.01$ M, RT).

About 0.96 mol equiv of H_2O_2 was found to be produced in the reaction between Cbl(II) and $O_2^{\bullet-}$ [Eq. (1)] by using the H_2O_2-specific Amplex Red assay from a calibration plot of fluorescence intensity of oxidized Amplex Red versus H_2O_2 concentration.[14] $O_2^{\bullet-}$ was generated by using the xanthine oxidase/acetaldehyde system for these experiments. Details are given in the Supporting Information.

To summarize, the rate constant of the reaction between Cbl(II) by $O_2^{\bullet-}$ has been directly determined by using pulse radiolysis and was found to be $3.8\times10^8\,M^{-1}s^{-1}$ (pH 7.42, RT). This value is in reasonable agreement with our earlier rate constant value ($7.0\times10^8\,M^{-1}s^{-1}$, 0.010 M phosphate buffer, pH 7.40, 25 °C),[9a] which was determined indirectly by using a competitor and generating $O_2^{\bullet-}$ by using the xanthine oxidase/acetaldehyde system. Cbl(II) is cleanly oxidized to H_2OCbl^+/HOCbl, and the rate of the reaction is independent of pH over the pH 5.52–8.72 region, as expected, because neither Cbl(II) nor $O_2^{\bullet-}$ ionizes in this region. The rate constant for the reaction between Cbl(II) and $O_2^{\bullet-}$ is within an order of magnitude of that reported for cytosolic Cu/Zn superoxide dismutase (Cu/Zn SOD; $2.0\times10^9\,M^{-1}s^{-1}$, pH 5.0–9.5, 25.0 °C[15]) and mitochondrial manganese superoxide dismutase (MnSOD; $1.1\times10^9\,M^{-1}s^{-1}$, pH 8.2, RT[16]). Cob(II)alamin is a major intracellular cobalamin form, and both intracellular cobalamin and SOD concentrations can reach micromolar levels.[11b, 17] Intracellular reductases subsequently reduce H_2OCbl^+/HOCbl to Cbl(II).[11] Cell studies also show that Cbl prevents $O_2^{\bullet-}$-induced oxidative stress.[9] These results combined with the similarity of intracellular concentrations of Cbl and SOD and the rate constants for the reactions of SOD or Cbl(II) with $O_2^{\bullet-}$ support a role for Cbl as a $O_2^{\bullet-}$ scavenger in addition to acting as a cofactor for the two mammalian B_{12}-dependent enzyme reactions. Because a significant proportion (up to 30–40%) of the elderly population are vitamin B_{12} deficient[18] and vitamin B_{12} is nontoxic even at high doses,[18] B_{12} supplementation might be especially beneficial for treating chronic inflammatory diseases associated with aging. Enzyme-bound Cbl(II) is also likely to be rapidly oxidized to its inactive H_2OCbl^+/HOCbl form by $O_2^{\bullet-}$; this is consistent with inactivation of the B_{12} enzymes under oxidative stress conditions.[19]

Experimental Section

Experimental details are given in the Supporting Information.

Acknowledgements

The authors thank Dr. Edward Suarez-Moreira for his valuable suggestions. This research was funded by the US National Institute of General Medical Sciences of the National Institutes of Health under award number 1R15GM094707-01A1. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The work at Brookhaven National laboratory was carried out at the Accelerator Center for Energy Research, which is supported by the U.S. DOE Office of Science, Division of Chemical Sciences, Geosciences and Biosciences under Contract No. DE-AC02-98CH10886.

Keywords: cobalamins · kinetics · pulse radiolysis · superoxides · vitamins

[1] a) S. V. Avery, *Biochem. J.* **2011**, *434*, 201–210; b) H. Cui, Y. Kong, H. Zhang, *J. Signal Transduction* **2012**, *2012*, DOI:10.1155/2012/646354.
[2] a) M. Jang, H. Kim, K. Kang, T. Yokozawa, J. Park, *Pharmacal. Res.* **2009**, *32*, 341–345; b) F. J. Pashkow, *Int. J. Inflam.* **2011**, *2011*, 514623.
[3] I. Batinic-Haberle, Z. Rajic, A. Tovmasyan, J. S. Reboucas, X. Ye, K. W. Leong, M. W. Dewhirst, Z. Vujaskovic, L. Benov, I. Spasojevic, *Free Radical Biol. Med.* **2011**, *51*, 1035–1053.
[4] B. Kräutler in *Water Soluble Vitamins, Vol. 56* (Ed.: O. Stanger), Springer, Dordrecht, **2012**, pp. 323–346.
[5] a) W. Carmen, *Med. Hypotheses* **2006**, *67*, 124–142; b) S. S. Greenberg, J. Xie, J. M. Zatarain, D. R. Kapusta, M. J. Miller, *J. Pharmacol. Exp. Ther.* **1995**, *273*, 257–265; c) M. Weil, R. Abeles, A. Nachmany, V. Gold, E. Michael, *Cell Death Differ.* **2004**, *11*, 361–363; d) C. Wheatley, *J. Nutr. Environ. Med.* **2007**, *16*, 181–211.
[6] a) G. Scalabrino, *Prog. Neurobiol.* **2009**, *88*, 203–220; b) G. Scalabrino, D. Veber, E. Mutti, *Brain Res. Rev.* **2008**, *59*, 42–54.
[7] J. M. McCord, B. A. Omar, *Toxicol. Ind. Health* **1993**, *9*, 23–37.
[8] I. B. Afanas'ev, *Curr. Med. Chem.* **2005**, *12*, 2731–2739.
[9] a) E. S. Moreira, N. E. Brasch, J. Yun, *Free Radical Biol. Med.* **2011**, *51*, 876–883; b) E. Suarez-Moreira, J. Yun, C. S. Birch, J. H. H. Williams, A. McCaddon, N. E. Brasch, *J. Am. Chem. Soc.* **2009**, *131*, 15078–15079.
[10] R. Banerjee, *ACS Chem. Biol.* **2006**, *1*, 149–159.
[11] a) L. Hannibal, J. Kim, N. E. Brasch, S. Wang, D. S. Rosenblatt, R. Banerjee, D. W. Jacobsen, *Mol. Genet. Metab.* **2009**, *97*, 260–266; b) R. Banerjee, C. Gherasim, D. Padovani, *Curr. Opin. Chem. Biol.* **2009**, *13*, 484–491.
[12] B. H. J. Bielski, D. E. Cabelli, R. L. Arudi, A. B. Ross, *J. Phys. Chem. Ref. Data* **1985**, *14*, 1041–1100.
[13] L. Xia, A. G. Cregan, L. A. Berben, N. E. Brasch, *Inorg. Chem.* **2004**, *43*, 6848–6857.
[14] a) M. Zhou, Z. Diwu, N. Panchuk-Voloshina, R. P. Haugland, *Anal. Biochem.* **1997**, *253*, 162–168; b) T. V. Votyakova, I. J. Reynolds, *Arch. Biochem. Biophys.* **2004**, *431*, 138–144.
[15] I. Fridovich, *J. Biol. Chem.* **1989**, *264*, 7761–7764.
[16] a) I. A. Abreu, D. E. Cabelli, *Biochim. Biophys. Acta Proteins Proteomics* **2010**, *1804*, 263–274; b) J. J. P. Perry, A. S. Hearn, D. E. Cabelli, H. S. Nick, J. A. Tainer, D. N. Silverman, *Biochemistry* **2009**, *48*, 3417–3424.
[17] J. Lee, J. A. Hunt, J. T. Groves, *J. Am. Chem. Soc.* **1998**, *120*, 6053–6061.
[18] R. Green, J. W. Miller in *Handbook of Vitamins*, 4th ed. (Eds.: R. B Rucker, J. Zempleni, J. W. Suttie, D. B. McCormick), CRC Press, Boca Raton, **2007**, pp. 413–457.
[19] D. Padovani, R. Banerjee, *Biochemistry* **2009**, *48*, 5350–5357.

Received: April 11, 2013
Published online on May 13, 2013