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The Reaction of Nitric Oxide with Glutathionylcobalamin

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Since it was found that vitamin B_{12a}, aquocobalamin, can be used to reverse several biological effects caused by nitric oxide (NO)[1] considerable attention has been given by chemists to the interactions of cobalamin, Cbl, species with NO.[2] An early explanation of these effects was attributed[1e−g] to the formation of nitrosylcob(III)alamin, Cbl(III)−NO. However, there is no convincing chemical evidence for this species in aqueous solution. Recent kinetic studies based on stopped-flow UV−vis absorption showed that the spectral shifts observed with Cbl(III) and NO in aqueous solution are due to nitrite impurities, and it was concluded that Cbl(III) does not bind NO.[2a] Conversely, the UV−vis spectral evidence did indicate NO binding with vitamin B_{12r}, Cbl(II),[2] and we provided convincing evidence of a Co−NO bond based on a Raman vibrational band shift upon isotopic ^{15}NO substitution.[2b] The kinetics and mechanism of this reversible NO binding reaction:

$$\text{Cbl(II)} + \text{NO} \leftrightarrows \text{Cbl(II)}-\text{NO} \leftrightarrow \text{Cbl(III)}-\text{NO}^- \qquad (1)$$

have been further investigated by laser flash photolysis and stopped-flow techniques.[2c] Since in both of the latter two studies, a formation constant of the order of 10^8 M^{-1} was measured for reaction 1,[2b,c] it is reasonable to suggest that the Cbl(II) species plays the key role in the biological effects of NO involving exogenous cobalamin. On the other hand, most of these biological effects were initiated by adding Cbl(III) to the biological system under study.[1] It would appear that there are chemical processes whereby Cbl(III) can be utilized in a manner similar to Cbl(II) for binding NO in biological systems.

In fact, the NADH or NADPH-linked Cbl(III) reductases could catalyze the reduction of Cbl(III) to Cbl(II).[3] However, these reductases have been located in microsomes and in the inner mitochondrial membrane in mammalian and human tissues and may not be available at the sites of biological NO production. On the other hand, glutathione, GSH, is a major intracellular reducing agent present in almost all biological tissues. In fact, glutathionylcob(III)alamin, GS−Cbl(III), has been considered to be the substrate for Cbl(III) reductase,[4a] and it is likely that GS−Cbl(III) is one of the major forms of vitamin B_{12} in mammalian cells.[4b] Indeed, this species appears to be the natural precursor of the cobalamin coenzymes.[3b,4] Very recent reports show that GS−Cbl(III) has a unique stability in comparison with other thiolatocobalamins.[5] Considering that GSH is present in most biological tissues at higher concentrations (1−8 mM) [6] than that of Cbl and that it has a high binding affinity for Cbl(III), [5a,7] it may well be a reservoir for Cbl(III) in biological systems. All of the above evidence suggests that GS−Cbl(III) is an important intermediate for biological processes involving vitamin B_{12} species. These considerations led us to study the reaction of NO with GS−Cbl(III).

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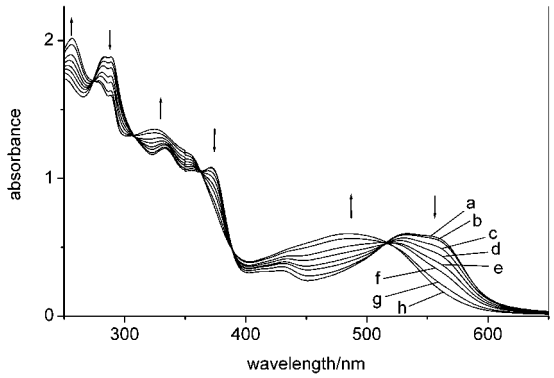

Figure 1. UV−vis spectra of the reaction of NO with GS−Cbl(III) in pH 7 buffer solution in a 2-mm cuvette. Cbl concentration is 4×10^{-4} M, GSH concentration is 4×10^{-3} M. The amount of NO is gradually increased from a to h. Arrows show the direction of absorbance change.

We first verified the formation of the GS−Cbl(III) complex by UV−vis spectrophotometry. GS−Cbl(III) forms on adding GSH to Cbl(III) in pH 7 buffer solution. Stoichiometric studies[7] have shown that a one-to-one complex forms:

$$\text{H}_2\text{O}-\text{Cbl}^+ + \text{GSH} \leftrightarrows \text{GS}-\text{Cbl} + \text{H}_3\text{O}^+ \qquad (2)$$

Figure 1 illustrates the change in absorbance spectra as NO is gradually added to the GS−Cbl(III) system in a pH 7 phosphate buffer solution. A color change from violet to brown is observed, and finally the spectrum changes in the presence of excess NO to that of Cbl(III)−NO^- with absorption peaks at 257, 277, 288, 325, and 487 nm.[2] Five isosbestic points, at 274, 308, 363, 389, 516 nm, are observed for the reaction, indicating that only two absorbing species are involved in the conversion of GS−Cbl(III) into Cbl(III)−NO^-. At pH 4, the isosbestic points are even sharper and easier to obtain; however, at pH 10 they can no longer be observed, indicating that more than two species are involved in the reaction at higher pH.

It has been reported[8] that in alkaline media, thiolates can reduce Cbl from Co^{III} to Co^{I} through reaction 3:

$$\text{RS}^- + \text{RS}-\text{Cbl(III)} \leftrightarrows \text{Cbl(I)} + \text{RS}-\text{SR} \qquad (3)$$

Since the pK_a value for GSH is about 9.2, Cbl(I) formation could be the reason the isosbestic points are no longer observed in pH 10 buffer. Eventually, Cbl(III)−NO^- would be formed since we find that Cbl(I) can be oxidized by NO to Cbl(II) which goes to Cbl(III)−NO^- in excess NO.[9] Thus, with excess NO all the final product of cobalamin is Cbl(III)−NO^-.

GS−Cbl(III) has been reported to have unique stability in comparison to other thiolatocobalamins,[5b] and the formation

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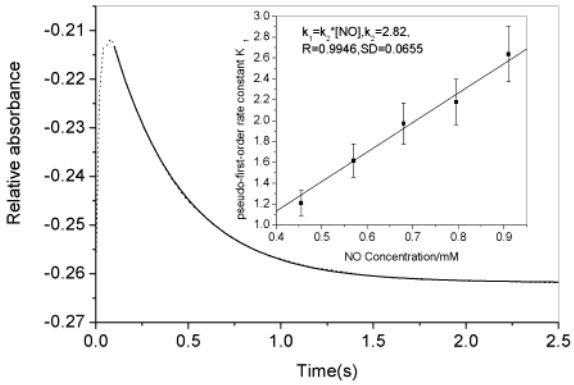


Figure 2. Time course for the reaction of 0.1 mM aquocobalamin plus 0.5 mM glutathione with saturated NO (1.82 mM) in pH 7 buffer solution. The dotted lines represent the experimental data, measured at 560 nm in a 2-mm cuvette, and the full lines correspond to the fit to the data.

constant for reaction 2 is $1.1 \pm 0.3 \times 10^5$ M^{-1} at 25 °C in pH 5.5.[5a] Furthermore, we have shown [2b] that the binding constant of NO with Cbl(II) is as high as $1.0 \pm 0.5 \times 10^8$ M^{-1}. On the basis of the above considerations, we propose a radical substitution reaction:

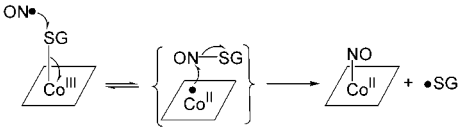
$$\text{GS}-\text{Cbl(III)} + \text{NO} \rightarrow \text{Cbl(III)}-\text{NO}^- + \text{GS}\bullet \qquad (4)$$

which could yield Cbl(III)$-NO^-$ by a variety of ligand substitution mechanisms, that is, (i) dissociative (D) to form Cbl(II) and GS• followed by NO complexation of Cbl(II), (ii) an associative (A) path to form an intermediate with an increased coordination number, or (iii) an interchange reaction I which forms a transition state in which NO attacks GS$-$Co(III) in an associative (I_a) or dissociative (I_d) manner. *S*-nitrosoglutathione is not a stable intermediate since its absorbance would interfere with the observed isosbestic points. Our attempts to verify thiyl radical formation with ESR spectroscopy by trapping the GS• radical with radical traps (DMPO, PBN) failed presumably because of the competition of NO with these traps.

We have studied the mechanism of reaction 4 by stopped-flow UV$-$vis measurements made by following the absorbance decrease at 560 nm for the reaction under nearly pseudo-first-order conditions with NO from about 10 to 20 times higher than GS$-$Cbl. The decay fits quite well to a single-exponential expression (Figure 2), indicating the reaction is first-order with respect to GS$-$Cbl. A calculated second-order rate constant $k_2 = 2.82 \times 10^3$ M^{-1} s^{-1} is obtained from the change in pseudo-first-order rate constant with concentration of nitric oxide which is linear (insert in Figure 2), indicating that the reaction is also first-order in NO. The pseudo-first-order rate constant is generally constant from pH 4 to 10 with a slight increase for pH higher than 9. Since a seventh coordination position has not been reported for Cbl, an associative (A) mechanism is unlikely. On the other hand, GS$-$Cbl(III) is very stable; its half-life for decomposition to Cbl(II) is estimated to be longer than 6×10^4 min at pH 7.2.[5b] Because of the relative fast nature of the reaction, it is highly unlikely that a dissociative (D) mechanism is occurring. Thus, we suggest that an interchange (I) mechanism occurs which could go by either an associative (I_a) or a dissociative (I_d) transition state. Scheme 1 shows the latter where a (Cbl(II) + *S*-nitrosoglutathione) cage is formed which is similar to a previously suggested cobalamin cage structure.[10]

The above results show that Cbl(III) after complexation by GSH can be converted relatively rapidly to Cbl(III)$-NO^-$ in the absence of a reductase. We calculate a $t_{1/2}$, assuming GSH is present, of about 1.2 s for the same conditions used in a typical physiological

Scheme 1



experiment [1h] where the inhibitory effect of Cbl was observed in isolated guinea-pig basilar arteries on the vasodilatations induced by NO. Our results show that Cbl(III) in the presence of GSH would have the same general ability as Cbl(II) to reverse the biological effects caused by NO. However, the above rate constant shows that the process is relatively slow in comparison with the direct binding of Cbl(II) with NO, which was found to be nearly diffusion-controlled.[2c]

In preliminary in vitro experiments on the effect of added Cbl on the NO-induced relaxation of the rabbit aorta ring, we found relaxation of the aorta ring was inhibited by both Cbl(III) and Cbl-(II) but with a faster response time for Cbl(II), Figure S3 (Supporting Information). This is consistent with a slower process for Cbl(III) to Cbl(II) conversion.

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Note Added after ASAP. There were errors in the version posted ASAP 7/16/2002. The corrected version was posted 7/17/2002.

Supporting Information Available: UV$-$vis spectra of the formation of GS$-$Cbl(III) and its reaction with NO at pH 4 (Figure S1) and at pH 10 (Figure S2), dynamics of aorta ring relaxation (Figure S3), pseudo-first-order rate constant as a function of pH (Figure S4), calculation of $t_{1/2}$, and experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Riou, B.; Gérard J.-L.; LaRochelle, C. D.; Bourdon, R.; Berdeaux, A.; Giudicelli, J. F. *Anesthesiology* **1991**, *74*, 552−558.(b) Kruszyna, H.; Magyar, J. S.; Rochelle, L. G.; Russell, M. A.; Smith, R. P.; Wilcox, D. E. *J. Pharmacol. Exp. Ther.* **1998**, *285*, 665−671. (c) Rajanayagam, H. A. S.; Li, C. G.; Rand, M. J. *Br. J. Pharmacol.* **1993**, *108*, 3−5. (d) Rand, M. J.; Li, C. G. *Eur. J. Pharmacol.* **1993**, *241*, 249−254. (e) Greenberg, S. S.; Xie, J.; Zatarain, J. M.; Kapusta, D. R.; Miller, J. S. *J. Pharmacol. Exp. Ther.* **1995**, *273*, 257−265. (f) Brouwer, M.; Chamulitrat, W.; Ferruzzi, G.; Sauls, D. L.; Weinberg, J. B. *Blood* **1996**, *88*, 1857−1864. (g) Rochelle L. G.; Morana, S. J.; Kruszyna, H.; Russell, M. A.; Wilcox, D. E.; Smith, R. P. *J. Pharmacol. Exp. Ther.* **1995**, *275*, 48−52. (h) Jiang, F.; Li, C. G.; Rand, M. J. *Euro. J. Pharmacol.* **1997**, *340*, 181−186.

(2) (a) Wolak, M.; Stochel G.; Hamza M.; and van Eldik R. *Inorg. Chem.* **2000**, *39*, 2018−2019. (b) Zheng, D.; Birke, R. L. *J. Am. Chem. Soc.* **2001**, *123*, 4637−4638. (c) Wolak, M.,; Zahl. A.; Schneppensieper, T.; Stochel, G.; van Eldik, R. *J. Am. Chem. Soc.* **2001**, *123*, 9780−9791.

(3) (a) Watanabe, F.; Nakano, Y.; Tachikake, N.; Kitaoka, S.; Tamura, Y.; Yamanaka, S.; Haga, S.; Imai, S.; Saidao, H. *Int J. Biochem.* **1991**, *23*, 531−533. (b) Pezacka, E. H. *Biochim. Biophys. Acta*, **1993**, *1157*, 167−177 and references therein.

(4) (a) Pezacka, E.; Green, R.; Jacobsen, D. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 443−450. (b) Jacobsen, D. W.; Lee-Denison, C.; Luce, K.; Green, R. *Fed. Proc.* **1987**, *46*, 1005. (c) Pezacka, E. H.; Jacobson, D. W.; Luce, K.; Green, R. *Biochem. Biophys. Res. Commun.* **1992**, *184*, 832−839.

(5) (a) Brasch, N. E.; Hsu, T. C.; Doll, K. M.; Finke, R. G. J. *Inorg. Biochem.* **1999**, *76*, 197−209. (b) Suto, R. K.; Brasch, N. E.; Anderson, O. P.; Finke, R. G. *Inorg. Chem.* **2001**, *40*, 2686−2692.

(6) Griffin, O. W. *Free Radical Biol. Med.* **1999**, *27(9−10)*, 922−935.

(7) Alder, N.; Medwick, T.; Poznanski, T. J. *J. Am. Chem. Soc.* **1966**, *88*, 5018−5020.

(8) (a) Pratt, J. M.; Hamza, M. S. A.; Buist, G. J. *J. Chem. Soc., Chem. Commun.* **1993**, 701−702. (b) Schrauzer, G. N.; Windgassen, R. J. *J. Am. Chem. Soc.* **1967**, *89*, 3607−3612.

(9) Zheng, D.; Yan, L.; Birke, R. L. *Inorg. Chem.* **2002**, *41*, 2548−2555.

(10) Baldwin D. A.; Betterton, E. A.; Chemaly, S. M.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1985**, 1613−1618.

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