Spectroscopic Evidence for Nitric Oxide Binding with Cob(II)alamin

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Chemical interactions between cobalamin, Cbl, redox forms and nitric oxide, NO, appear to occur in view of several biological effects. Addition of i.v. doses of Cbl(III) to intact conscious dogs significantly increased resistance to blood flow,[1] which was suggested to result from interference with endogenous NO.[2] In vitro, Cbl(III) can reverse the relaxation of isolated smooth muscle by NO and NO-donating compounds.[3,4] Injection of Cbl(III) in rodents can prevent and reverse pathological results caused by an overproduction of NO.[5] Finally it was reported that Cbl(III) quenched NO-mediated inhibition of leukemia cell proliferation.[6] Such biological effects have been interpreted[5−7] to result from the formation of Cbl(III)-NO via the reaction:

$$H_2O\text{-Cbl(III)} + NO \leftrightarrows \text{Cbl(III)-NO} + H_2O \qquad (1)$$

However, the existence of Cbl(III)-NO has been questioned and chemical studies on this system have been contradictory. It was claimed that "nitrosylcobalamin" could be isolated from an acidic solution of NO_2^- (in equilibrium with NO) and Cbl(III);[8] however, later it was reported that NO does not bind to either Cbl(III) or Cbl(II).[9] Nevertheless, recently UV/visible spectral shifts were observed after NO was added to both Cbl(III) and Cbl(II).[6] Unfortunately, ESR and FTIR studies failed to provide convincing evidence for the existence of stable Cbl(III)-NO or Cbl(II)-NO complexes,[2,7] while on the other hand a dubious report claimed a solid nitrosylcobalamin compound had been synthesized by simply bubbling unpurified NO into a Cbl(III) solution.[10] More recently, 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.[11] Conversely, the UV−vis spectral evidence did indicate NO binding with vitamin B_{12r}, Cbl(II).[11]

Most of the previous research has been focused on the binding of NO to Co(III), which would be a direct explanation of the biological effects. However, there is no convincing physical evidence for a stable Cbl(III)-NO complex. Because of the contradictions in the literature, we have investigated Cbl(III) and Cbl(II) interactions with NO using both UV−vis and especially resonance Raman (RR) spectroscopy. We verified that UV−visible spectroscopy shows a slight shift after NO is added to aqueous hydroxocobalamin, HO-Cbl(III), solution, reported to be caused by traces of nitrite which form nitrito-Cbl(III).[11] On gradually adding NO to a Cbl(II) solution,[12,13] we find absorption peaks shift from 289, 311, and 475 nm to 258, 275, 289, 320, 352, and 480 nm, and five clear isosbestic points at 290, 329, 387, 483, and 547 nm are formed (Figure 1B). These spectra verify the previous data[6,11] and can be interpreted as a result of the binding of Cbl(II) with NO. Furthermore, we found that when a HO-Cbl(III) solution is irradiated with strong light (blue or green laser or white light) in the presence of excess NO for a given time (Figure 1A), a color change from red to brown occurs and the spectrum of the putative Cbl(II)-NO develops. On irradiation, UV−vis absorption peaks of HO-Cbl(III) shift from 352, 412, 505, and 531 nm to 256, 276, 288, 321, 351, and 481 nm, with three clear isosbestic points, 340, 370, and 497 nm (Figure 1A). In fact, both experiments give the same final UV−visible spectrum, indicating the same product has formed. These results strongly imply that the equilibrium reaction

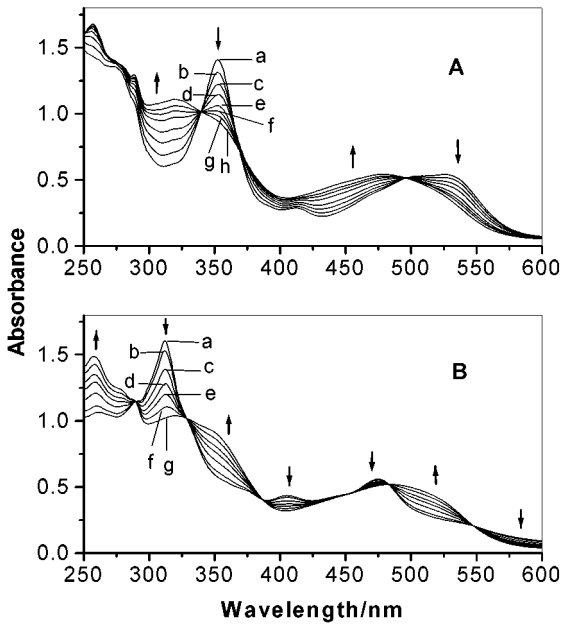

Figure 1. UV−visible spectra of Cobalamin and NO in pH 7.0 aqueous buffer solution. (A) Photolysis of about 10^{-4} M HO-Cbl in the presence of about 10^{-3} M NO with excitation at 514.5 nm. From a to h, each spectrum was recorded with about 5 min duration of irradiation at 1.0 W laser power. (B) Addition of NO to 10^{-4} M Cbl(II). From a to g, concentrations of NO in the solution were increased by injection of purified NO gas. Arrows show the trend in the absorption peaks.

$$\text{Cbl(II)} + NO \rightleftarrows \text{Cbl(II)-NO} \qquad (2)$$

occurs with formation of a stable Cbl(II)-NO species and also that Cbl(III) can be photoreduced to this complex in the presence of NO. The UV−vis spectrum of the Cbl(II)-NO complex in a pH 4 buffer is similar to the pH 7 buffer but the peak at 289 nm shifts to 285 nm indicating that the base-on complex at pH 7 has become the protonated base-off form at pH 4. A formation

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(12) NO was first collected with a gas container from a gas cylinder, then washed with deoxygenated 0.1 M NaOH solution.
(13) To avoid the presence of excess reducing agent, Cbl(II) was obtained by the photolysis of methylcobalamin.

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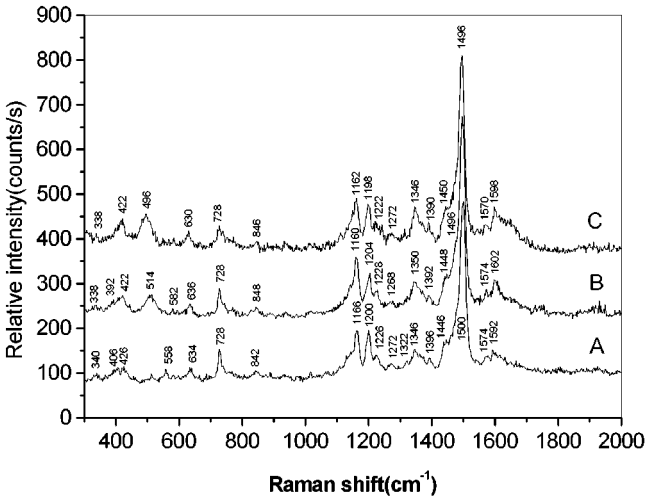

Figure 2. Resonance Raman spectra of 3×10^{-4}M HO-Cbl in unbuffered aqueous solution: (A) original solution; (B) after being irradiated with 514.5 nm laser light for 30 min in the presence of ^{14}NO; and (C) same condition as for spectra B except ^{15}NO was used instead of ^{14}NO. Laser excitation, 514.5 nm; power, 200 mW; slit width, 320 μm.

constant of $K_F = 1.0 \pm 0.5 \times 10^8$ M^{-1} was calculated for reaction 2 at pH 7.0.[14]

To confirm the formation of the Co(II)-NO bound species, RR spectroscopy was utilized to further investigate both of the above experiments, and as expected both experiments give the same final RR spectrum, Figure 2B. The RR spectrum of the new product is different from that of Cbl(II), but shows similarities to that of H_2O-Cbl(III), Figure 2A, suggesting that the complex involves a charge shift from the cobalt(II) ion to NO, Co(III)-NO$^-$, giving a bent Co−N═O bond. This result is consistent with the structure of the isoelectronic octahedral complex PipCo(II)-NOTTP, nitrosyl-piperidine-tetraphenylporphinatocobalt(II), which has a highly bent Co−N═O bond angle ≤128.5°.[15] Comparing the two spectra, A and B in Figures 2, a peak at 514 cm^{-1} comes out in the RR spectrum of the product when ^{14}NO was used for both of the above experiments. With ^{15}NO, the RR spectrum, Figure 2C, shows a shift of this band from 514 cm^{-1} to 496 cm^{-1} representing an isotopic shift of the expected magnitude.[16] A Co−N stretching vibration has been reported to appear in this low-frequency range for some cobalt-NO compounds.[17,18] Semiempirical PM3 MO frequency calculations that we have performed also show the possibility of a Co-NO bending mode in this region. Thus the new band can be assigned to either a stretching or bending vibration involving the Co−N bond. To rule out the possibility that this band is from a Co(III)-N vibration of a nitrite complex, we obtained the RR spectrum of NO_2^- -cob(III)alamin and found no shifts in any vibrational bands on using $^{15}NO_2^-$, showing that the 514 cm^{-1} band is definitely not from Co(III)-NO_2^- formation. Unfortunately, a new peak with an isotopic effect for an N═O stretching vibration of metal-bound NO was not found in the 1600−1700 cm^{-1} region, as reported for some heme containing NO complexes. This band could to be broad in RR scattering and its polarizibility component may be quite small. When oxygen or air was injected into the system, the Co−N vibrational band disappeared indicating that NO−Cbl(II) is unstable to oxygen, and addition of air saturated cyanide solution produces the UV−visible spectrum of dicyano-Cbl(III) showing that NO does not attack the corrin ring.

In summary, the above results confirm NO binding to Cbl(II), as indicated by the Co-^{15}N isotopic shift in the RR spectrum with no convincing evidence to support NO binding to Cbl(III). Thus it would appear that the Co(II) species, vitamin B_{12r}, is the cobalamin form that can bind NO in biological systems.

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Supporting Information Available: UV−vis spectra of nitrosyl-Cbl(II) at pH 4 and 7, absorbance data and its plot vs [NO] for calculation of K_F, RR spectrum of Cbl(II), and experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) This measurement was made by forming the reduced species, Cbl(II), electrochemically at a Hg electrode in an electrochemical cell, then pumping the solution into a temperature-controlled cell that contained a calibrated WPI electrode probe for measuring injected amounts[12] of NO, and finally pumping this solution into the spectrophotometer cell, all under anaerobic conditions. The absorbance data were plotted using a method similar to that in Hoshino et al. (Hoshino, M.; Maeda, M.; Konishi, R.; Seki, H.; Ford, P. C. *J. Am. Chem. Soc.* **1996**, *118*, 5702), except that the data were corrected for any Cbl(III) formation. The estimated standard error is at the 95% confidence level.

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