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Spectroscopic Studies of Nitric Oxide (NO) Interactions with Cobalamins: Reaction of NO with Superoxocobalamin(III) Likely Accounts for Cobalamin Reversal of the Biological Effects of NO[1]

Title of paper

The authors

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ABSTRACT

Recent reports indicate that oxidized cobalamin, Cbl(III), can interfere with the biological effects of nitric oxide (NO) on vascular and visceral smooth muscle and in other systems. In attempting to elucidate the mechanism of these effects of Cbl(III), we reported that a Cbl(III)NO complex could be detected by electron paramagnetic resonance (EPR) spectroscopy, but not by ultraviolet/visible spectroscopy. Subsequently, others concluded that the alleged Cbl(III)NO complex is detectable by ultraviolet/visible, but not by EPR spectroscopy and provided ultraviolet/visible evidence for an alleged Cbl(II)NO complex. We report further investigation of the interaction of NO with Cbl, using both techniques, Fourier transform infrared (FTIR) spectroscopy and mass spectrometry. Our EPR results and the UV/VIS results of others appear to be experimental artifacts that can now, at least in part, be explained. Under conditions where FTIR measurements readily detect a N-O stretching frequency of NO bound to Fe(II), we do not detect a similar signal that can be ascribed to either Cbl(III)NO or Cbl(II)NO, indicating that neither Cbl(III) nor Cbl(II) form a stable complex with NO. Loss of the Cbl(II) EPR signal and mass spectral detection of N_2O upon addition of NO to Cbl(II) solutions, demonstrates that Cbl(II), which is present in aerobic Cbl(III) solutions, reduces NO; however, this reaction does not appear to be fast enough to account for the observed biological effects in aerated media. Nitric oxide also reacts rapidly and irreversibly with the superoxo complex of Cbl(III), $Cbl(III)O_2^-$, which is always present in aerated solutions of Cbl(III). We believe that this latter reaction accounts for the observed inactivation of NO by Cbl(III) in biological systems. Because $Cbl(III)O_2^-$ is spontaneously regenerated from Cbl(II) and O_2 in aerated solutions, this may constitute a cyclic mechanism for the rapid elimination (oxidation) of NO. Thus, several physicochemical techniques fail to provide convincing evidence for the existence of stable Cbl(III)NO or Cbl(II)NO complexes but do provide evidence that Cbl species participate in redox reactions with NO under aerobic conditions, thereby inhibiting its physiological roles.

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Recent reports indicate that Cbl(III) can interfere with the biological effects of NO under some conditions. In intact, conscious dogs, large i.v. doses of Cbl(III) significantly increased systemic vascular resistance (Riou *et al.*, 1991), possibly by reducing endogenous NO levels. *In vitro* Cbl(III) reverses the relaxation of isolated vascular and visceral smooth muscle by NO and NO-donating compounds (Rajanayagam *et al.*, 1993; Rand and Li, 1993). Injection of Cbl(III) in rodents prevented and reversed endotoxin-induced hypotension and mortality, presumably by inactivation of NO (Greenberg *et al.*, 1995). Finally, it was reported that Cbl(III) quenches NO-mediated inhibition of cell proliferation (Brouwer *et al.*, 1996).

All of these effects were ascribed to the formation of a Cbl(III) complex with NO, by analogy with the well characterized Hb complex with NO (*e.g.,* Kruszyna *et al.*, 1987). However, NO binds to the reduced Co(II) of Co-substituted Hb and Mb (Yonetani *et al.*, 1972), and a number of stable NO complexes of Co(II) porpyrins, but not Co(III) porphyrins,

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ABBREVIATIONS: Cbl(III), oxidized cobalamin; Cbl(II), reduced cobalamin; AdoCbl, adenosylcobalamin; MeCbl, methylcobalamin; NO, nitric oxide; Hb, deoxyhemoglobin; Mb, myoglobin; MetHb, methemoglobin; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared; GSH, reduced glutathione; GSSG, oxidized glutathione; UV/VIS, ultraviolet/visible; NO_2^-, nitrite; O_2^-, superoxide; N_2O, nitrous oxide.

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have been prepared and characterized (Fujita *et al.*, 1985). A "nitro-cobalamin" species was apparently isolated from an acidic solution of Cbl(III) and NO_2^- although the only proof of its identity were small UV/VIS spectral shifts (Kaczka *et al.*, 1951). However, the lack of UV/VIS changes was cited as evidence that NO does not bind to either Cbl(III) or Cbl(II) (Firth *et al.*, 1969). Nevertheless, a recent study claimed that UV/VIS spectral shifts indicated the formation of NO complexes with Cbl(III) and Cbl(II) (Brouwer *et al.*, 1996).

As we have recently confirmed (Rochelle *et al.*, 1995), the UV spectrum of NO overlaps that of all Cbl species and assignment of NO-Cbl interaction based on this spectral region is suspect. In the visible region, changes in the Co oxidation state produce distinct shifts in the Cbl absorption spectrum, but addition of NO to solutions of Cbl(III) produces only equivocal shifts, which are comparable to those found with small changes in pH or the reaction of NO with other Cbl species that are always found in equilibrium with Cbl(III) in aerated solutions (Rochelle *et al.*, 1995).

Cbl(III) is diamagnetic, but Cbl(II) is paramagnetic and has a characteristic and well studied electron paramagnetic resonance (EPR) signal (*e.g.,* Rochelle *et al.*, 1995). Although NO is also paramagnetic and has a well understood EPR signal in the gas phase (Whittaker 1991), it is typically not detected in solution by EPR spectroscopy (Wilcox and Smith, 1995; Singel and Lancaster, 1996), apparently because of fast electron spin relaxation. However, a Cbl(III)NO complex is expected to be EPR detectable, by analogy to the stable Cbl(III) complex with O_2^-, Cbl(III)O_2^-, that is formed by the reaction of Cbl(II) with O_2 (Bayston *et al.*, 1969). Previously the only EPR signal we could ascribe to Cbl(III)NO was a weak signal at $g = 1.99$ that was found in solutions of Cbl(III) and excess NO, and that was absent for similar NO solutions lacking Cbl(III) (Rochelle *et al.*, 1995). However, a similar $g = 1.99$ signal has been reported for solutions containing high concentrations of NO in the absence of Cbl (Galpin *et al.*, 1978; Stevens *et. al.*, 1979; Brudvig, *et. al.*, 1980; Martin, *et. al.*, 1981; Arciero *et al.*, 1985; Nelson, 1987; Musci *et al.*, 1991; Rubbo *et al.*, 1995).

Thus, both UV/VIS and EPR evidence for a Cbl(III)NO complex are controversial at best. But, if NO does not form a stable complex with Cbl(III), what is the Cbl species responsible for the reported biological activity? Both UV/VIS and EPR results appear to indicate that NO slowly oxidizes Cbl(II) to Cbl(III) under anaerobic conditions (Brouwer *et al.*, 1996; Rochelle *et al.*, 1995). Thus, Cbl(III) interference with the biological effects of NO *in vitro* might be due to a reduction in free NO by this redox reaction with Cbl(II), which is present at low levels in aerobic solutions of Cbl(III) or can be generated by reduction of Cbl(III) by biological tissues (*e.g.,* Rochelle *et al.*, 1995). However, the significance of such a reaction under necessarily aerobic *in vivo* conditions is unknown.

Therefore, we have used UV/VIS, EPR and FTIR spectroscopy and mass spectrometry to further characterize the interaction of NO with various Cbl species. We find evidence lacking for an NO complex with either Cbl(III) or Cbl(II), but present results that implicate Cbl(III)O_2^-, which is found in neutral aerobic solutions of Cbl(III), as the species responsible for Cbl(III) reversal of the biological effects of NO.

Methods

Unless otherwise stated, all chemicals are Fisher Certified ACS grade. Hydroxocobalamin acetate (Cbl(III)OH), methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl) were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Cbl(II) was prepared either by reduction of Cbl(III) by sodium borohydride ($NaBH_4$; Sigma) or by photolysis of AdoCbl or MeCbl. For the latter method, anaerobic solutions of AdoCbl or MeCbl in septum-capped quartz EPR tubes or a septum-capped 3-ml quartz cuvette were exposed to light from a 500 W Wyco DEK projector bulb at a distance of 4 inches. The extent of Cbl(II) formation was determined by EPR analysis (see below). Solutions containing increased concentrations of superoxocobalamin, Cb(III)O_2^-, were prepared by partially reducing Cbl(III) with $NaBH_4$, and then bubbling air through the solution for 2 min.

Anaerobic solutions were prepared by repeated evacuation and back-filling with O_2-free N_2; aerobic solutions were exposed to ambient atmosphere. Unless otherwise noted, all solutions were prepared in 0.1 M phosphate buffer, pH 7.4. NO was introduced into Cbl solutions by gas tight syringes from stock NO gas (99%; Matheson, Gloucester, MA) that had been passed through 5 M NaOH solutions and stored in a rigorously O_2-free tonometer. For some experiments NO was introduced by addition of sodium nitrite ($NaNO_2$) under acidic conditions, because NO exists in equilibrium with nitrite in solution. In certain of these experiments, 98% ^{15}N-$NaNO_2$, which also contained 5 to 10% $NaNO_3$, (Cambridge Isotopes, Andover, MA) was used to prepare samples.

UV/VIS absorption spectra were obtained with a Hitachi U-3000 recording spectrophotometer. For experiments under anaerobic conditions O_2-free N_2 gas was bubbled through the solutions in 1-cm cuvettes sealed with a rubber septum.

EPR spectra were obtained with a Bruker ESP-300 spectrometer operating at 100 KHz modulation frequency, 20 G modulation amplitude and 4.9 mW microwave power, unless otherwise indicated. Samples were in septum-capped 4 mm o.d. × 3 mm i.d. quartz EPR tubes immersed in liquid N_2 in a Wilmad (Buena, NJ) EPR dewar. Concentrations of EPR-detectable species were determined by quantitative comparison (double integration) to EPR spectra, recorded under identical non-saturating conditions (4.9 mW microwave power), of 50% aqueous glycerol solutions containing known concentrations of cupric sulfate. For power saturation analysis, relative signal intensity was determined by the difference between the maximum and minimum of the second derivative EPR signal.

FTIR measurements were obtained on a Perkin-Elmer 1605 spectrometer equipped with a lithium tantalate detector and a sample compartment that was constantly purged with N_2 to remove water vapor and CO_2. IR spectra (64 scans) were collected at 4.0 cm^{-1} resolution in the 2000 to 1500 cm^{-1} range, and a buffer IR spectrum, obtained under identical conditions, was subtracted. Samples were pumped under a N_2 atmosphere from a 3-ml quartz reaction vessel into a sealed flow-through micro CIRCLE Cell (SpectraTech, Shelton, CT) IR cell; subsequent to FTIR analysis at room temperature, samples were pumped into evacuated quartz EPR tubes for EPR analysis. All FTIR samples were in phosphate buffer made with D_2O (99.9% D; Cambridge Isotopes).

Headspace gases from reactions of NO and AdoCbl were analyzed by mass spectrometry with a Hewlett Packard G 1800B GCD system equipped with a 25-m volatile organic column (0.2 mm i.d.) and an electron ionization detector. The system was operated with a helium flow rate of 1.0 ml/min. Head space gas (5 μl) was injected directly on the column, and the selective ion monitoring mode was set to detect masses of 30 (NO) and 44 (N_2O).

Results

As we (Rochelle *et al.*, 1995) and others have reported, the EPR spectrum of Cbl(III) solutions (prepared with

Cbl(III)OH) has weak signals at g = 2.33 and g = 2.01 that are attributed to Cbl(II) and Cbl(III)O_2^-, respectively, which are in equilibrium with Cbl(III) under aerobic conditions. Addition of NO, under anaerobic conditions, to Cbl(III) solutions that exhibit these signals results in immediate loss of the Cbl(III)O_2^- signal, but a ~3-fold slower reduction of the g = 2.33 Cbl(II) signal.

The weak g = 1.99 EPR signal that we observe at 77 K in anaerobic solutions of Cbl(III) and excess NO and that we have attributed to Cbl(III)NO is part of an anisotropic EPR spectrum with g⊥ = 1.99 and g‖ ~ 1.79, as shown in figure 1. No signal is detected for similar NO concentrations in the absence of Cbl(III) under the same conditions. This signal disappears when the solution is purged with air or N_2 and reappears upon introduction of NO under anaerobic conditions. For comparison, figure 1 also shows the well characterized EPR spectrum of Cbl(III)O_2^-, which is centered at g = 2.01 and exhibits hyperfine splitting by the ^{59}Co nucleus (I = 7/2). These two EPR signals from ostensibly similar species have quite different power saturation behavior at 77 K (fig. 1, inset), with no saturation of the former at microwave power up to 158 mW (log P = 2.2) but saturation of the latter at microwave power > 20 mW (log P = 1.3).

Novel EPR signals with g values and line widths indicative of Co(II) species were found upon prolonged 37°C anaerobic incubation of Cbl(III) or Cbl(II) with excess NO, introduced either as NO gas or by incubation with $NaNO_2$ under acidic conditions. The first of these signals that appears, Cbl(II)* (fig. 2), eliminates or obscures the weak g = 1.99 signal (fig. 1), and has a large three-line hyperfine splitting in the g‖ = 2.38 region and small resolved hyperfine splitting in the g⊥ = 2.05 region. Upon longer incubation this signal is replaced by a similar one that lacks resolved g hyperfine splitting and is attributed to another species, Cbl(II)** (fig. 2). These EPR spectra have been simulated (table 1) and both are characterized by an unusually large three-line g‖ nitrogen hyperfine coupling of ~170 × 10^{-4} cm^{-1}. To further elucidate the origin of this unusual hyperfine coupling, Cbl(II)** was prepared with $Na^{15}NO_2$ and the three-line g‖ hyperfine coupling was replaced by two broad lines (fig. 2), as is expected for substituting ^{15}N (I = 1/2) for ^{14}N (I = 1); in addition, there is some broadening in the g⊥ region. Clearly the nitrogen that is responsible for the large g‖ hyperfine splitting in these spectra originates from NO, and the nature of these signals (g‖ > g⊥ > 2.00) indicates a different structure than that of

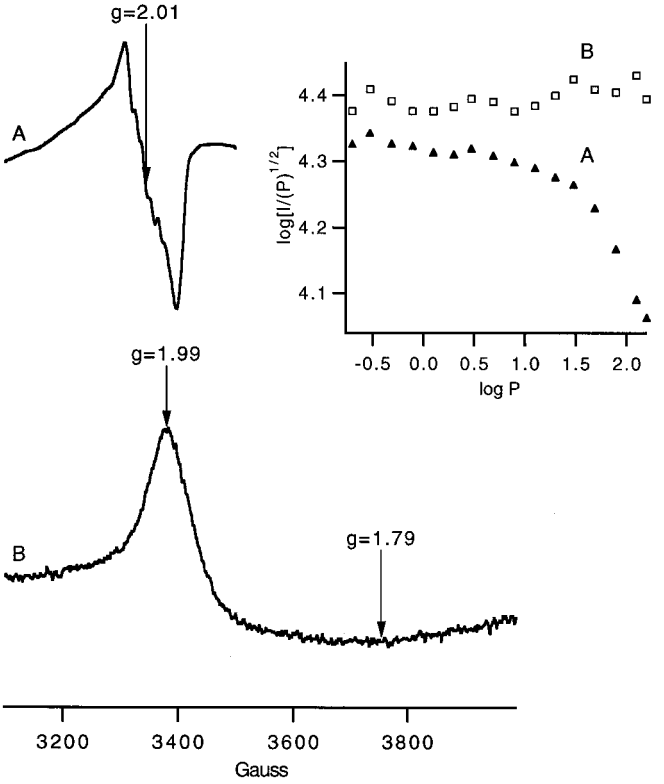

Fig. 1. Frozen solution (77 K) EPR spectra of: (A) Cbl(III)O_2^- and (B) a mixture of Cbl(III) and excess NO. The insert shows power saturation data for the same two frozen solutions, with only Cbl(III)O_2^- showing saturation at higher powers. See "Methods" for instrument settings.

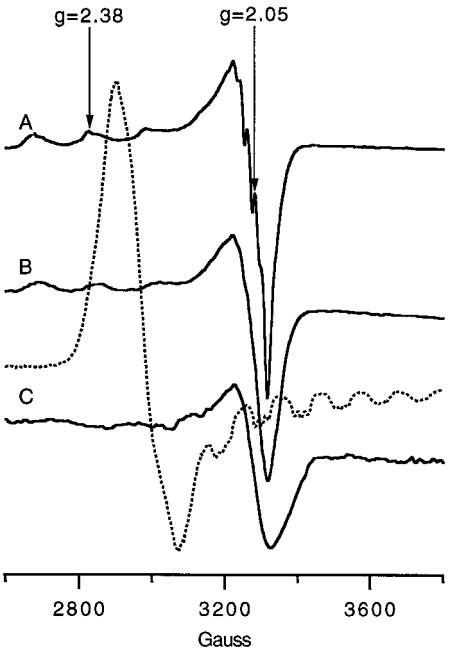

Fig. 2. Frozen solution (77 K) EPR spectra of: (A) Cbl(II)*, (B) Cbl(II)** and (C) Cbl(II)** prepared with $Na^{15}NO_2$. The 77 K EPR spectrum of Cbl(II) is superimposed with a dashed line. See "Methods" for instrument settings.

TABLE 1
Simulation values that best fit the experimental EPR spectra

	x[a]	y[a]	z[a]
CbI(II)*			
g[b]	2.047	2.047	2.379
A(N)1[c]	5	5	167
A(N)2[c]	23	23	10
A(N)3[c]	4	4	12
A(Co)[c]	19	19	0.1
W[d]	6	6	50
CbI(II)**			
g[b]	2.043	2.043	2.354
A(N)[c]	0.1	0.1	169
A(Co)[c]	10	10	0.1
W[d]	39	39	41

[a] Direction axes with z normal to and x, y in the corrin plane.
[b] The g factor; measured relative to g = 2.0023 of the free electron; indicates the orbital that contains the unpaired electron and, thus, the molecular structure.
[c] Hyperfine coupling value for nucleus in parentheses; units of cm^{-1} × 10^4; measures the magnitude of the splitting of the g factor and indicates the extent of interaction of the unpaired electron with the indicated nucleus.
[d] Line width.

Cbl(II), which has $g\perp > g\|$ 2.00 and whose EPR spectrum is also shown in figure 2.

EPR spectroscopy was also used to monitor the reaction of NO with Cbl(II), which we and others have studied with UV/VIS spectroscopy (Brouwer *et al.*, 1996; Rochelle *et al.*, 1995). In initial experiments, Cbl(II) was generated by borohydride reduction of Cbl(III). However, to avoid possible complications of excess borohydride, Cbl(II) was also prepared by photolysis of anaerobic solutions of AdoCbl or MeCbl in quartz EPR tubes. The extent of photolytic formation of Cbl(II) was first assessed by EPR measurements, and then 100 μl (4 μmol) of NO, N_2 or O_2 was added, which was sufficient to provide 0.2 atm of gas above each 0.3 ml solution. After 2 min of incubation at room temperature, the tubes were immersed in liquid N_2 and EPR spectra were taken again. In a typical experiment, merely thawing and refreezing the sample reduced the Cbl(II) concentration from 0.58 to 0.45 mM. The addition of N_2, NO and O_2 reduced the Cbl(II) concentration from 0.58 mM to 0.23, 0.08 and 0.06 mM, respectively; as expected, a large Cbl(III)O_2^- signal appeared at g = 2.01 for the O_2 sample. After accounting for recombination of the adenosyl radical with Cbl(II) and for Cbl(II) oxidation by adventitious air during introduction of the gases and freezing and thawing the samples, these results suggest that NO reacts with Cbl(II) at a rate similar to the O_2 oxidation of Cbl(II) under these conditions.

Because oxidation of Cbl(II) by O_2 results in O_2^-, oxidation of Cbl(II) by NO should result in NO^-, which spontaneously dimerizes and dehydrates to form N_2O in aqueous solution. To determine if NO oxidizes Cbl(II), the head-space gases of anaerobic reactions of Cbl(II), generated by photolysis of AdoCbl, and NO were analyzed by mass spectrometry. The AdoCbl solution was initially red but changed to a brown color, characteristic of a mixture of Cbl(II) and Cbl(III), upon photolysis. Injection of NO into this solution resulted in a rapid color change back to the red color of Cbl(III). Only NO (mass 30) was detected in the gas sample before it was injected into the reaction vessel but a substantial amount of N_2O (mass 44), in addition to excess NO, was detected in the headspace gas after NO injection and reappearance of the red color (data not shown).

FTIR spectroscopy was used in attempts to identify the characteristic N-O stretching frequency of NO bound to Cbl. Initially, we used sample conditions identical to those reported by Kaczka *et al.* (1951) for the preparation of "nitrocobalamin," and $Na^{15}NO_2$ was employed to identify the N-O stretching frequency by its isotopic shift, as was done to identify NO bound to Hb (Maxwell and Caughey, 1976). These samples exhibited the g = 1.99 EPR signal, indicating high concentrations of NO. The 2000 to 1500 cm^{-1} IR spectrum of Cbl(III) in D_2O has prominent bands at 1630 and 1570 cm^{-1}, which are assigned as the amide I band of the peptide bond joining the corrin and the axial benzimidazole and a corrin ring vibrational mode, respectively (Puckett *et al*, 1996). After subtraction of the Cbl(III) spectrum, broad bands centered at 1623 cm^{-1} for $^{14}NO_2^-$ and 1582 cm^{-1} for $^{15}NO_2^-$ were observed (data not shown); however, similar IR features were obtained with ^{14}N- and ^{15}N-nitrite in the absence of Cbl(III), indicating that these are vibrational bands of the excess nitrite. We also find no FTIR evidence for N-O vibrational bands upon introduction of 0.5 atm of NO gas to anaerobic 8 mM Cbl(III) solutions (fig. 3A). In another experiment, 7.9 mM Cbl(III) was reduced by $NaBH_4$ to a concentration of 4.0 mM Cbl(II) and 0.5 atm of NO gas was then added anaerobically. Other than small shifts in the 1550 to 1570 cm^{-1} region, independently shown to be associated with changes in the Co oxidation state, no IR bands indicative of NO bound to Cbl(II) were observed (fig. 3B). As a positive control, the FTIR spectrum of 4 mM sodium nitroprusside, $Na_2Fe(CN)_5NO$, obtained with identical sample and instrument conditions, showed a strong characteristic N-O stretching frequency at 1935 cm^{-1} (spectrum not shown), which is close to the reported value of 1938 cm^{-1} for nitroprusside in aqueous solution (Bor, 1961). This band was absent from the FTIR spectrum of 4 mM $K_4Fe(CN)_6$, obtained under identical conditions, and we used this N-O stretching band to estimate a 0.5 mM limit of detection. Although borohydride has no spectral interference in the 2000 to 1500 cm^{-1} region, we also generated 0.5 mM Cbl(II) photolytically from 3 mM AdoCbl but, as before, anaerobic addition of excess NO gas resulted in no new IR features attributable to NO bound to Cbl(II). In this experiment, addition of NO resulted in 80% loss of the Cbl(II) EPR signal through NO oxidation of Cbl(II). Thus, we were unable to obtain FTIR spectroscopic evidence for a NO complex with either Cbl(III) or Cbl(II).

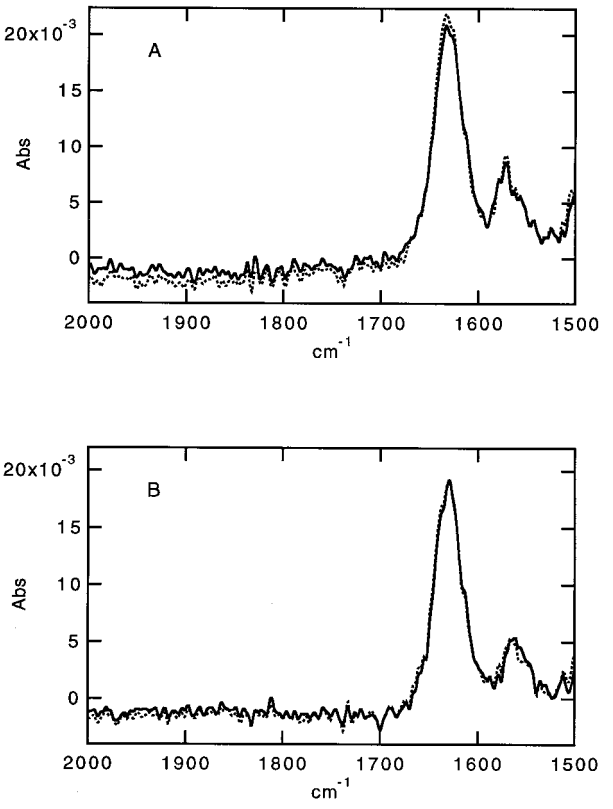

Fig. 3. Room temperature FTIR spectra of: (A) 8 mM Cbl(III) (——) and 8 mM Cbl(III) with excess NO (- - - -) and (B) a solution containing 4 mM each of Cbl(II) and Cbl(III) (——) and a similar solution containing excess NO (- - - -).

The high affinity of Hb for NO suggests that it might compete effectively with Cbl(III) or Cbl(II) for NO, and this could be shown by detection of the characteristic HbNO EPR signal. Initially, we found that anaerobic addition of freshly prepared Hb quantitatively reduced Cbl(III) to Cbl(II) (data not shown). When 40 mmol of NO were introduced into a solution consisting of 3.6 mmol Hb, 8 mmol Cbl(III) and 1.35

mmol Cbl(II), the EPR-detectable products were 0.5 mmol HbNO, 3 mmol MetHb and 5 mmol Cbl(II).

As shown in figure 4, addition of 1 mM GSH to an anaerobic 60 μM Cbl(III) solution results in a decrease in intensity and a splitting of the 351-nm absorption band and a decrease in intensity and shifting to longer wavelengths of the visible absorption bands within 15 min. The resulting spectrum matches that reported for the glutathione complex with Cbl(III), GSCbl(III) (Scheuring *et al.*, 1994). A similar result is obtained, albeit at a slower rate, after the anaerobic addition of 1 atm of NO to a 60 μM Cbl(III) solution, followed by evacuation and backfilling with N_2 as described by Brouwer *et al.* (1996), and finally anaerobic addition of 1 mM GSH (fig. 4). EPR analysis of an anaerobic sample consisting of 10 mM GSH and 8 mM Cbl(III), and an identical sample where the Cbl(III) was initially treated with 1 atm of NO, followed by evacuation and backfilling with N_2, showed that after 45 min the reaction that had been treated with NO contained 3 mM Cbl(II), although the reaction lacking exposure to NO had 1.5 mM Cbl(II).

Discussion

Previous investigations of NO interaction with Cbl have relied heavily on UV/VIS spectral data. The first of these reported the isolation of a "nitro-cobalamin" from an acidic solution of Cbl(III) and nitrite (Kaczka *et al.*, 1951), which is in equilibrium with NO under these conditions. However, proof of the identity of this species rested entirely on small shifts in the Cbl(III) UV/VIS spectrum. In an extensive and quantitative analysis of ligand binding to Cbl, no UV/VIS spectral evidence was found for NO binding either to Cbl(III) at pH 5.5 or to Cbl(II), prepared by electrochemical reduction of Cbl(III) at pH 8 (Firth *et al.*, 1969). However, competition with thiocyanate indicated that NO_2^- binds to Cbl(III) with a binding constant of 2.3×10^5 M^{-1}. More recently, UV spectral data were used to suggest the formation of a Cbl(III)NO complex and the isolation of this complex from the urine of rats treated with Cbl(III) and lipopolysaccharide (Greenberg *et al.*, 1995), which is known to induce endogenous NO production. However, the key data of this study primarily show the UV spectrum of NO in solution, and does not constitute evidence for a Cbl(III)NO species or its formation *in vivo*. Finally, the visible spectrum of Cbl was used to characterize the interaction of NO with Cbl(III) (Brouwer *et al.*, 1996). Although oxidation and reduction of Cbl are readily determined from these data, the spectral shifts supposedly associated with NO binding are equivocal. We have observed and previously ascribed these small shifts to reactions of NO with traces of Cbl(II) and Cbl(III)O_2^- (Rochelle *et al.*, 1995), species always found in equilibrium with Cb(III) in aerated solutions. We are also able to produce similar spectral changes by acidification of Cbl(III) solutions (data not shown). Clearly, more definitive data are necessary to unambiguously characterize the interaction of NO with Cbl, and in this study we have used EPR and FTIR spectroscopy and mass spectrometry for this purpose.

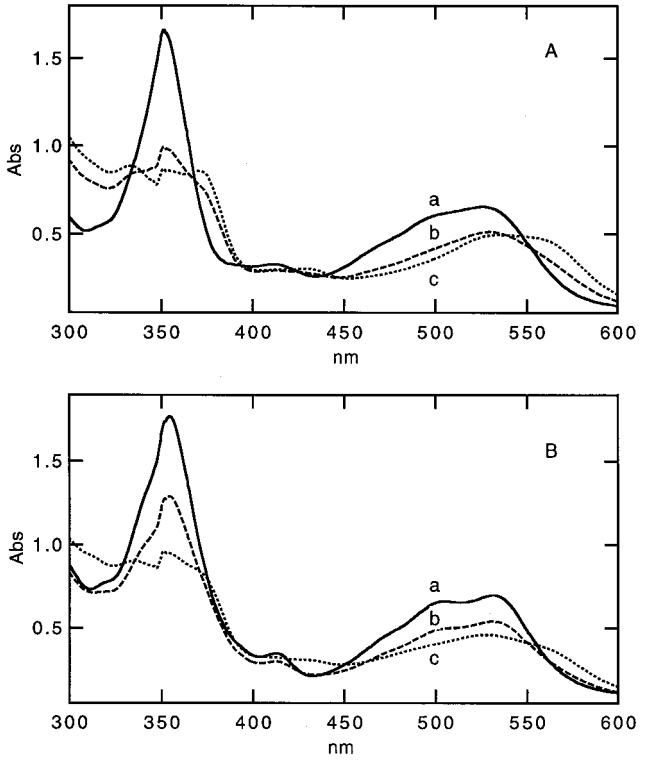

Fig. 4. Room temperature, anaerobic UV/VIS spectra of A: (a) 60 μM Cbl(III), and the same sample (b) 15 min and (c) 21 min after addition of 1 mM GSH; and B: (a) 60 μM Cbl(III) after addition of excess NO, followed by evacuation and backfilling with N_2, and the same sample (b) 30 min and (c) 150 min after addition of 1 mM GSH. In B, NO absorbance may be contributing to the spectra at wavelengths < 400 nm.

A complex of diamagnetic Cbl(III) and paramagnetic NO is iso-electronic with nitrosylhemoglobin (HbNO) and, like HbNO, should be detectable by EPR spectroscopy. Earlier we reported a weak EPR signal at g = 1.99 that appeared in Cbl(III) solutions upon addition of excess NO and seemed to correspond to Cbl(III)NO (Rochelle *et al.*, 1995). This signal has been reported previously for solutions containing high concentrations of NO and a protein, particularly a paramagnetic metalloprotein exhibiting EPR signal(s) (Galpin *et al.*, 1978; Stevens *et al.*, 1979; Brudvig *et al.*, 1980; Martin *et al.*, 1981; Arciero *et al.*, 1985; Nelson, 1987; Musci *et al.*, 1991; Rubbo *et al.*, 1995); further, this signal, reported as g = 1.97 and g = 1.7 (Brudvig *et al.*, 1980), has also been observed in buffer solutions containing only high NO levels (Stevens *et al.*, 1979). The intensity of the signal depends on NO concentration; it has been reported for solutions in equilibrium with 1 atm NO gas, and one study indicated that the signal disappeared when NO pressure was decreased to 0.05 atm (Nelson, 1987). Signal intensity also increases with decreasing temperature (typically observed at T < 77 K), and in the presence of protein, hence the term matrix- or protein-bound NO (Stevens *et al.*, 1979). We observe this signal in solutions containing excess NO and Cbl(III), which inevitably contains small percentages of paramagnetic (and EPR-detectable) Cbl(II) and Cbl(III)O_2^- that appear to affect the electron spin relaxation of NO, rendering it EPR-detectable. Thus, we believe we understand why addition of Cbl(III) to EPR-silent solutions of NO results in appearance of the g = 1.99 EPR signal and our misassignment of this signal. A quantitative power saturation analysis of this signal and that of Cbl(III)O_2^- at 77 K (fig. 1) indicates different electronic properties and little resemblance of the g = 1.99 species to a known complex of Cbl(III) and a radical ligand.

The EPR spectra we observe for Cbl(III) solutions after long periods of incubation with excess NO are novel but

result from irreversible reactions, probably involving the Cbl corrin ring. Thus, they probably have little relevance to the reported effects of Cbl(III) on the biological activity of NO. The nature of these EPR spectra ($g\| > g\perp > 2.00$; fig. 2) indicates that these species consist of Co(II) in a structure with two strong axial ligands (unpaired electron in the $d_x2_{-y}2$ orbital), one of which originates from NO, as shown by ^{15}N isotopic labeling. Clearly, this must involve significant changes in the corrin, which provides four strong equatorial pyrrole ligands for the Co(II) of Cbl(II), leading to its characteristic EPR spectrum with $g\perp > g\|$ 2.00 (unpaired electron in the d_z2 orbital), shown for comparative purposes in figure 2.

Although not generally used as an analytical method, FTIR is valuable for characterizing molecular structure and bonding. In fact, the N-O stretching frequency of NO bound to metal complexes, which is typically found in the 2000 to 1500 cm^{-1} range, can be used to determine the metal oxidation state and the bonding mode of the NO (*i.e.,* whether it is coordinated in a linear or bent geometry). Relevant and representative Co-nitrosyl complexes have the following N-O stretching frequencies: CoMbNO, 1613 cm^{-1}, and CoHbNO, 1619 cm^{-1} (Hu, 1993); Co(OEP)NO, 1660 cm^{-1} (OEP = 2,3,7,8,12,13,17,18 octaethylporphyrin; Fujita, *et al.*, 1985); Co(TTP)NO, 1689 cm^{-1} (TTP = 5,10,15,20 tetraphenylporphyrin; Scheidt and Hoard, 1973). Under sample and instrumental conditions where we can readily observe the N-O stretching band of nitroprusside we observe no IR features that can be attributed to a N-O stretching band in solutions consisting of NO and Cbl(III) or Cbl(II). This includes the acidic solution of nitrite and Cbl(III) from which Kaczka *et al.* (1951) apparently isolated a "nitro-cobalamin" and for which we find the g = 1.99 EPR signal, indicative of high NO concentrations in the presence of a paramagnetic species.

Others have suggested the existence of Cbl(III)NO, based on apparent evidence for an unusual stability of the putative complex to prolonged incubation, elevated temperature, exposure to bright light or repeated cycles of nitrogen flushing and evacuation (Brouwer *et al.*, 1996). However, similar results would also be found for solutions containing only Cbl(III). Evidence purported to demonstrate reversibility of the Cbl(III)NO complex was obtained for samples incubated with Hb, which resulted in a solution containing both Cbl(III) and Cbl(II), as well as oxyHb but not HbNO. However, as reported here, Hb can reduce Cbl(III) to Cbl(II), whether or not NO is present. Furthermore, release of NO from putative Cbl(III)NO in the presence of Hb should result in HbNO, which we detect by its characteristic EPR signal upon anaerobic incubation of Cbl(III), NO and Hb. Brouwer *et al.* (1996) postulate that NO released from putative Cbl(III)NO was transferred to the cysteine thiolate of the globin protein, but provide no evidence in support of a thionitrosyl. Finally, these authors report that incubation of GSH with putative Cbl(III)NO slowly results in Cbl(II) formation. Glutathione binds to Cbl(III) to give the well-characterized GSCbl(III) complex (Brown *et al.*, 1993), and we show (fig. 4) that a similar result is found upon GSH incubation with Cbl(III) after it has supposedly been converted to Cbl(III)NO. This reaction is slower than the analogous reaction without prior NO exposure, but in our hands it is difficult to remove all the dissolved NO by evacuation and backfilling with nitrogen, and residual NO may affect the rate of GSCbl(III) formation. Subsequently, a slower reduction of Cbl(III) by excess GSH occurs, whether NO had been present or not, as shown by appearance of the Cbl(II) EPR signal. It is of some interest to note that this reaction is 2-fold faster in the presence of NO, in spite of the fact that NO reoxidizes the resulting Cbl(II). This may be explained by the following reactions,

$$\text{Cbl(III)} + \text{GSH} \rightleftharpoons \text{GSCbl(III)} + \text{H}^+ \qquad (1)$$

$$\text{GSCbl(III)} \longrightarrow \text{Cbl(II)} + 1/2\ \text{GSSG} \qquad (2)$$

$$\text{GSCbl(III)} + \text{NO} \longrightarrow \text{Cbl(II)} + \text{GSNO} \qquad (3)$$

where both reaction 2 and 3 result in EPR-detectable Cbl(II) in the presence of NO. Thus, we do not find the spectral shifts alleged to show formation of Cbl(III)NO convincing, and offer alternative explanations for all observations suggested to support the existence of such a complex. We conclude that Cbl(III)NO is not a stable complex, and therefore it cannot account for the inhibition/blockade of the biological effects of NO by Cbl(III).

It is well documented that addition of NO eliminates the EPR signal of Cbl(II). This could result from the formation of Cbl(II)NO, which should be EPR-silent as is the NO complex of Co(II)-substituted Hb or Mb (Hori *et al.*, 1982) or from a redox reaction leading to Cbl(III) and NO^-. Brouwer *et al.* (1996) present UV/VIS spectral data that supposedly supports the former assignment. However, our inability to detect an N-O stretching feature in the FTIR spectrum of anaerobic samples that consist of NO and Cbl(II) argues against the formation of Cbl(II)NO. More convincing, our detection of N_2O in the headspace gas above a solution containing Cbl(II) and NO indicates that loss of the Cbl(II) EPR signal is due, at least in part, to NO oxidation of Cbl(II). Recently it has been reported that NO can be reduced to N_2O by Ru(II) porphryns (Miranda *et al.*, 1997) and by glutathione (Singh *et al.*, 1996), but this is the first report that Cbl(II) is able to reduce NO.

Why doesn't NO form a complex with Cbl(II), like the NO complex of Co(II)-substituted Hb or Mb? This appears to relate to the reduction potentials of Co-porphyrins and Co-corrins (*i.e.*, Cbl), relative to that of NO. In support of this, we note the following reported reduction potentials (*vs*. normal hydrogen electrode): NO/NO^-, $\epsilon° = 0.39$ V (Stanbury, 1989); Cbl(III)/Cbl(II), $\epsilon° = 0.23$ V (de Tacconi, *et al.*, 1979); Co(III)(2-TMPyP)/Co(II)(2-TMPyP), $\epsilon° = 0.57$ V (2-TMPyP = tetrakis(*N*-methyl-2-pyridyl)porphine; Cheng and Su, 1994). Although pH will affect these potentials (HNO, $pK_a = 4.5$; Cbl(III)H_2O, $pK_a = 7.8$; Co(III)(2-TMPyP)H_2O, $pK_a = 5.5$), as will substitution on the porphyrin (*e.g.,* Co(III)(4-TMPyP)/Co(II)(4-TMPyP), $\epsilon° = 0.41$ V; Chan *et al.*, 1985), these potentials suggest that NO is capable of oxidizing Cbl(II) but not Co(II)-porphyrins, which tend to have higher reduction potentials because of the better π-acceptor ability of more highly conjugated planar macrocyclic ligands (Fujita *et al.*, 1985).

If Cbl(III)NO does not exist, an alternative explanation must be sought for the interference of Cbl(III) with the biological effects of NO. The redox reaction of NO with Cbl(II) is too slow to account for the observed biological effects (Riou *et al.*, 1991; Rajanayagam *et al.*, 1993; Rand and Li, 1993), which are immediate. It is also well documented that NO eliminates the EPR signal of Cbl(III)O_2^- and our data indi-

ate that this reaction, which may result in the formation of peroxynitrite, $OONO^-$, is faster than NO oxidation of Cbl(II). Since $Cbl(III)O_2^-$ is present in Cbl samples under aerobic conditions, it appears quite likely that this species accounts for the biological effects of Cbl(III) (Riou *et al.*, 1991; Rajanayagam *et al.*, 1993; Rand and Li, 1993; Greenberg *et al.*, 1995; Brouwer *et al.*, 1996). Unfortunately, it is not possible to prepare stable pure solutions of $Cbl(III)O_2^-$ (Bayston, *et. al.*, 1969), thus preventing direct dose-response studies of its interaction with NO. Therefore, hemoglobin is very much superior to $Cbl(III)O_2^-$ as a tool for detecting or interfering with the actions of NO in biological systems (Wilcox and Smith, 1995).

One of the early and important discoveries about the biology of NO was that superoxide, under some conditions, leads to rapid inactivation of NO (Gryglewski *et al.*, 1968; Rubanyi and Vanhoutte, 1968a and b), in many cases with formation of $OONO^-$. Just as NO reacts rapidly and preferentially with O_2 liganded to heme iron (Doyle and Hoekstra, 1981; Kruszyna *et al.*, 1993), it appears to react rapidly and irreversibly with superoxide liganded to Cbl(III). Because $Cbl(III)O_2^-$ is rapidly and spontaneously regenerated in aerobic solutions of Cbl(III), this may constitute a cyclic mechanism for the rapid inactivation of NO.

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