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Reactions of Nitric Oxide with Vitamin B_{12} and Its Precursor, Cobinamide[†]

Vijay S. Sharma,*[,‡] Renate B. Pilz,[‡] Gerry R. Boss,[‡] and Douglas Magde*[,§]

Departments of Medicine and Chemistry and Biochemistry, University of California—San Diego, La Jolla, California 92093-0652

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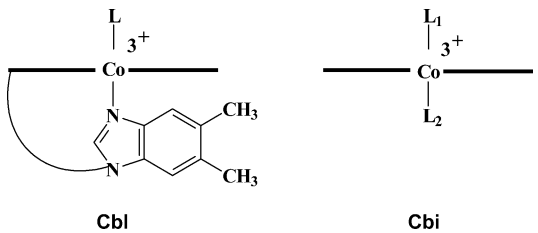
ABSTRACT: Despite early claims that nitric oxide does not react with cobalamin under any circumstances, it is now accepted that NO has a high affinity for cobalamin in the 2+ oxidation state [Cbl(II)]. However, it is still the consensus that NO does not react with Cbl(III). We confirmed that NO coordinates to Cbl(II) at all pH values and that Cbl(III) does not react with NO at neutral pH. At low pH, however, Cbl(III) does react with NO by way of a two-step process that also reduces Cbl(III) to Cbl(II). To account for the pH dependence, and because of its intrinsic interest, we also studied reactions of NO with cobinamide [Cbi] in the 2+ and 3+ oxidation states. Both Cbi(II) and Cbi(III) react readily with NO at all pH values. Again, Cbi(III) is reduced during the process of coordinating NO. Compared to cobalamin, cobinamide lacks the tethered 5,6-dimethylbenzamidazolyl moiety bound to the cobalt ion. It may, therefore, be considered a "base-off" form of Cbl. To explain the reaction of Cbl(III) at low pH, we infer that the base-off form of Cbl(III) exists in trace amounts that are rapidly reduced to Cbl(II), which then binds NO efficiently. Base dissociation, we postulate, is the rate-limiting step. Interestingly, Cbi(II) has 100 times greater affinity for NO than does Cbl(II), proving that there is a strong trans effect due to the tethered base in nitrosyl derivatives of both Cbl(II) and Cbl(III). The affinity of Cbi(II) for NO is so high that it is a very efficient NO trap and, consequently, may have important biomedical uses.

Nitric oxide participates in numerous reactions of biological importance. Most of these reactions are with metal derivatives or free radicals. Reactions of NO with the iron in heme proteins have been studied in considerable detail. Reactions of NO with cobalt in cobalamin (vitamin B_{12}) have not been studied as thoroughly. It has been reported that NO inhibits the vitamin B_{12}-dependent enzyme methionine synthase activity both in vitro and in isolated rat hepatocytes (*1*, *2*). Furthermore, it has been observed that hydroxycobalamin diminishes the biological effect of NO in endotoxin-induced hypotension in rodents (*3*) and in nitrosothiol-induced relaxations of rat aorta and anococcygeus muscle (*4*). By analogy to nitrosyl-heme, it can be postulated that these observations result from the reaction of NO with various oxidation states of cobalt formed in the two major types of vitamin B_{12}-dependent enzymatic reactions (*5*):

methyltransferases	Cbl(III) ⇄ Cbl(I)
mutases	Cbl(III) ⇄ Cbl(II)

In an earlier report (*6*), we showed that the inhibitory effect of NO on the methyltransferase activity of methionine synthase involves its reaction with Cbl(I).[1]

Until a few years ago, there was considerable disagreement whether NO reacted with either Cbl(II) or Cbl(III). In 1969, a leading laboratory summarized the data by saying: "No complex was found between NO and either aquocobalamin or the cobalt(II) complex, vitamin B_{12r}" (*7*). Subsequently, different laboratories (on occasion, even the same laboratory) argued both sides of the issue (*8*−*10*). Recently, however, a definitive study proved conclusively that NO does have high affinity for Cbl(II) (*11*). It still appears to be the consensus that NO does not react with Cbl(III) (*12*). We asked why not, and whether there might be some conditions under which NO does react with Cbl(III), and how does NO react with other, cobalamin-related compounds? Cobalamin has a cobalt atom coordinated to four nitrogens in a corrin ring, which is roughly planar, with a 5,6-dimethylbenzimidazolyl moiety, Bzm, occupying a fifth coordination site and attached by a nucleotide tether to the D ring of the corrin. Cobinamide (Cbi), a late precursor in the biosynthesis of cobalamin by bacteria, lacks the Bzm, and the tether is abbreviated to an aminopropanol side chain of the corrin. Since these are among the larger nonpolymeric molecules found in nature, it is convenient to represent the structures as



Cbl Cbi

The ligands L, L_1, and L_2 may be H_2O, OH^-, or some other ligand, such as NO. In Cbl, the Bzm base can dissociate from the cobalt ion while remaining attached to its tether.

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[‡] Department of Medicine.

[§] Department of Chemistry and Biochemistry.

[1] Abbreviations: Bzm, 5,6-dimethylbenzimidazole; Cbi, cobinamide; Cbl, cobalamin; Cbl′, base-off Cbl; Co A, co-enzyme A; DTT, dithiothreitol; Mb, ferrous myoglobin (horse heart); MbO_2, oxymyoglobin; $metMb^+$, oxidized ferric myoglobin.

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The resulting species is described as "base-off cobalamin" and will be represented as Cbl′. Its properties might be expected to be like those of cobinamide. We found that there is indeed similarity, but with an important difference. In earlier studies, cobinamide and its derivatives have been used to provide useful information regarding the redox chemistry of the corrinoid ring and the role of the Bzm side chain in enzymatic reactions (*13*−*16*). Cobinamide historically has been referred to as Factor B; it is found in the serum of animals and humans. As it appears to be relatively nontoxic (*17*−*21*), it might bear consideration as a pharmaceutical agent.

We present data showing, first, that base-off Cbl′(III) reacts readily with NO, as does Cbi(III), and, second, that Cbi(II) has 100 times greater affinity for NO than does Cbl(II). These observations indicate that there is a strong trans effect in nitrosyl derivatives of both Cbl(II) and Cbl(III), and they suggest that cobinamide could be a much more efficient NO scavenger than cobalamin and might be useful in clinical states of excess NO production, such as septic shock.

EXPERIMENTAL PROCEDURES

Materials. Hydroxocobalamin acetate salt and cobinamide dicyanide were from Sigma. Stock solutions of hydroxocobalamin were prepared in water to produce appropriate UV−visible spectra ($\epsilon_m = 2.62 \times 10^4$ M^{-1} cm^{-1} at 350 nm) (*22*). Diaquocobinamide was prepared according to the method of Hayward et al. (*23*) by photolysis of $Cbi(CN)_2$ acidified to pH 2−4 until no further change in the visible absorption spectrum occurred; the resulting HCN was removed by a stream of argon passed through the solution. The final solutions yielded λ_{max} at 348 nm ($\epsilon_m = 2.8 \times 10^4$ M^{-1} cm^{-1}), similar to Cbl(III) in strong perchloric acid. Pure $Cbi(OH)_2$ has been reported to have an A_{344}/A_{356} absorbance ratio of 1.05−1.11 at pH > 11, and it was noted that impurities give a broad band near 455 nm (*24*). Our diaquocobinamide preparation yielded an $A_{344}/A_{356} = 1.06$ at pH 12, with no additional band at 455 nm.

Cbl(II) is believed to be five-coordinate, with no water coordinated trans to Bzm (*5*). There is no information in this regard for Cbi(II), and what little information exists for Cbi(III) points to complicated equilibria involving both aquo and hydroxo ligands (*25*). For this reason, we will follow the usual practice of not specifying explicitly the axial ligands of Cbi. The pK_a for the reaction $Cbl(III)-H_2O \rightleftarrows Cbl(III)-OH^-$ is ∼7.8, and those for $Cbi(III)-(H_2O)_2$ are ∼6.0 and ∼10.3. The nature of the axial ligands for these species can be inferred from the pH of solutions (*25*).

Nitric oxide gas, 99.9% pure, was from Matheson and was purified further by passing it through a 5 M solution of NaOH. Nitric oxide solutions were prepared by passing NO gas through 0.1 M phosphate buffer, pH 7.4 ([NO] = 1946 μM at 23 °C for water in equilibrium with 1 atm NO gas), with appropriate dilutions made in deoxygenated buffer using gastight syringes.

Cbl(III) was reduced to Cbl(II) using dithiothreitol (DTT, Calbiochem) or sodium borohydride (Fisher), with both reducing agents yielding identical spectra in the 300−700 nm range. Unless otherwise mentioned, spectra were recorded in 0.1 M phosphate buffer at pH 7.4, deoxygenated by bubbling high-purity Ar through the solution. Spectrophotometer cells were capped with double septa in all spectrophotometric experiments.

Stopped-Flow Measurements. Stopped-flow kinetic experiments were performed at 23 °C using a Durrum instrument following protocols described earlier (*26*). To study the kinetics of NO dissociation from Cbl(II)−NO, horse heart oxymyoglobin (MbO_2) was employed as the scavenger. To prepare MbO_2, horse skeletal muscle Mb from Sigma was reduced with dithionite and the dithionite removed on a Sephadex G-25 column. To characterize the kinetics of NO dissociation from Cbi(II)−NO, data were obtained using hemin reduced by borohydride as the scavenger in the presence of 2% cetyltrimethylammonium to keep the heme from polymerizing.

Flash Photolysis. Association rates were measured by flash photolysis using about 8 mJ of green light in an 8-ns pulse from a frequency-doubled Nd:YAG laser (QuantraRay). The probe light was supplied either by a continuous 100-W tungsten−halide bulb or by a 0.5-ms-pulsed xenon flashlamp, depending on the time range to be recorded. Additional instrumental details and procedures have been described previously (*27*). Transients were recorded at single wavelengths; most measurements were made at wavelengths of 446, 470, or 600 nm.

Assessment of Nitrite Contamination. The possibility of nitrite contamination of NO solutions is an issue (*11*, *12*). Nitrite binds to both Cbl(II) and Cbl(III) and might, therefore, affect solution spectra, equilibrium data, or kinetic measurements. It is almost impossible to eliminate completely nitrite from NO solutions. To estimate a conservative upper limit for nitrite possibly present in our experiments, absorbance at 354 nm [λ_{max} for nitrite ion, $\epsilon_m = 22$ M^{-1} cm^{-1} (*28*)] was measured for deoxygenated buffer and deoxygenated buffer with NO added. Manipulations were carried out to duplicate, or exceed, experimental conditions, such as leaving NO solutions in cells for up to 3 h, or adding deoxygenated H_2O two or three times. In no case did the cumulative absorbance change at 354 nm approach 0.001, the limit of measurement. An absorbance change of <0.001 is equivalent to a maximal nitrite contamination of <48 μM, which should be too low to interfere. Direct combination of nitrite is much slower than combination with NO. To verify that, we performed flash photolysis on solutions of Cbl and Cbi in the presence of 2 mM added nitrite and confirmed that NO_2^- reacts quite slowly, as has been found by others (*11*, *12*). Finally, as a check on whether nitrite might have some indirect affect on the associations of NO, we measured NO binding in the presence of known amounts of NO_2^-.

RESULTS

Reaction of Cbl(II) with NO. While our studies were in progress, the results of a comprehensive kinetic study of the reaction of NO with Cbl(II) were published by Wolak et al. (*11*). Given the controversial history surrounding Cbl, and since our procedures differed somewhat, it is worthwhile reporting a brief summary of our findings for comparison. Equally important, these data allow comparison under identical conditions with our new results for Cbl(III), Cbi(III), and Cbi(II).

Titration of Cbl(II) with NO showed that only one NO binds per Cbl(II). Spectra were as reported by Wolak et al.

(*11*). By flash photolysis, we found that, at pH 7.4 in 0.1 M phosphate buffer at 23 °C, the second-order rate constant for NO association to Cbl was $k_{on} = (5 \pm 1) \times 10^8$ M^{-1} s^{-1}. Lowering the pH to 3 did not change the rate constant significantly. At still lower pH, in 2 M $HClO_4$, the spectrum showed formation of base-off Cbl′(II)−NO. The most noticeable feature in the spectrum at low pH is decreased absorption in the range 480−550 nm, a characteristic of the "base-off" nitrosyl derivative of Cbl′(II) (*11*). Flash photolysis experiments yielded an NO combination rate constant at low pH of $(4 \pm 1) \times 10^8$ M^{-1} s^{-1}, which is within error of our value at pH 7.4. Thus, it seems that NO associates with Cbl(II) and Cbl′(II) at almost the same rates. As shown in Table 1, our measured combination rate constants are slightly lower than those reported by Wolak et al. at 25 °C (*11*). In a field with such a controversial history, the slight difference is less important than the general agreement. The two measurements were made at slightly different temperatures, used different buffers, and monitored at different wavelengths. The uncertainties of 20% in Table 1 consider both curve-fitting to any single data set and, more importantly, variations among different samples, which probably stem in part from some scatter in the NO concentrations.

Kinetics of NO Dissociation from Cbl(II)−NO. To study NO dissociation from Cbl(II)−NO and confirm the results of Wolak et al. (*11*) by a different method, we used oxymyoglobin (MbO_2) as an NO scavenger, since it is a more efficient trap than the Fe(II)−EDTA they used and, therefore, need not be added in such high concentrations. On the other hand, it cannot be used at low pH. Nitric oxide reacts irreversibly with MbO_2, with a rate constant of 3.5×10^7 M^{-1} s^{-1} (*29*):

$$MbO_2 + NO \rightarrow metMb^+ + NO_3^- \qquad (1)$$

The product, $metMb^+$ [with ferric Fe(III)], has an absorption band of modest intensity at 630 nm, where MbO_2, Cbl(II), and Cbl(II)−NO all have very low absorption, making it fairly easy to monitor scavenging of dissociated NO according to the mechanism

$$\text{Cbl(II)}{-}\text{NO} \underset{k_{on} = 5 \times 10^8}{\overset{k_{off}}{\rightleftharpoons}} \text{Cbl(II)} + NO \qquad (2)$$

$$NO + MbO_2 \xrightarrow{k_{ox} = 3.5 \times 10^7} metMb^+ + NO_3^- \qquad (3)$$

Making a steady-state approximation gives

$$\frac{d[metMb^+]}{dt} = k_{off}[\text{Cbl(II)} - NO]\frac{k_{ox}[MbO_2]}{k_{on}[\text{Cbl(II)}] + k_{ox}[MbO_2]} \qquad (4)$$

Although NO can, in principle, react further with the product, $metMb^+$, this further reaction is of no consequence for two reasons: first, the rate constant for adding NO to $metMb^+$ is more than 100 times slower than that for adding to MbO_2 (and dissociation is faster), and, second, $metMb^+$ is not present initially and never accumulates to more than a minor extent.

Table 1: Kinetic Parameters[a] for the Reactions of NO with Cbl and Cbi

reaction	pH	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	$t_{1/2}$ (s)	ref
Cbl(II) + NO ⇄ Cbl(II)−NO	7.4	5×10^8	3.0		this work
	7.4	7.4×10^8	5.6		11
	3.6	5.3×10^8	1.7		11
Cbi(II) + NO ⇄ Cbi(II)−NO	7.4	2.4×10^8	0.019		this work
Cbl(III) $\xrightarrow{+NO}$ Cbl(II)−NO	1.05			570	this work
Cbi(III) $\xrightarrow{+NO}$ Cbi(II)−NO	7.1			29	this work

[a] Uncertainties are about 20%.

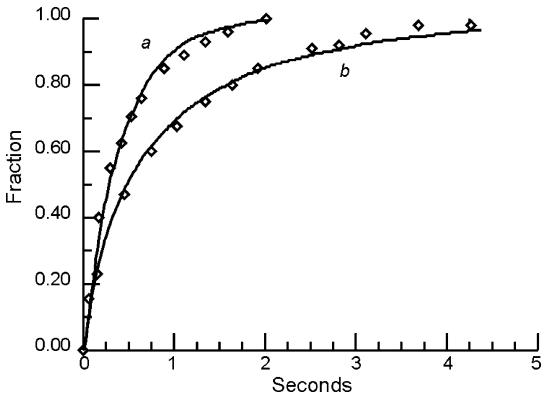


FIGURE 1: Kinetics of NO dissociation from Cbl(II)−NO in 0.1 M phosphate buffer, pH 7.4, at 23 °C; *F* is the fraction of the reaction completed. (a) [Cbl(II)−NO] = 7.7 μM, [MbO_2] = 886 μM. (b) [Cbl(II)−NO] = 33 μM, [MbO_2] = 66 μM. Diamonds, data points at 630 nm; lines calculated according to eq 4.

For 16 μM Cbl(II)−NO, the reaction was studied by stopped-flow mixing using concentrations of MbO_2 from 66 to 886 μM. In Figure 1, we present reaction time courses for the lowest and the highest MbO_2 concentrations. At high MbO_2 concentration, when k_{on}[Cbl(II)] ≪ k_{ox}[MbO_2], reaction 3 is rapid, and the back reaction in eq 2 is negligible. The last factor in eq 4 then simplifies to unity. Observed reaction rates became independent of scavenger concentration above [MbO_2] = 237 μM. Under those conditions, simple curve-fitting gave $k_{off} = 3.0 \pm 0.3$ s^{-1}. An alternative analysis can be made using the literature values for k_{on} and k_{ox} given in the equations above to find the best value of k_{off} from a global fit to all MbO_2 concentrations simultaneously. That gave $k_{off} = 3.7 \pm 0.3$ s^{-1}. Going further, if all three constants k_{off}, k_{on}, and k_{ox} were allowed to vary in the determination of the best global fit to all data at all concentrations, slightly different values were obtained: $k_{off} = 2.7 \pm 0.2$ s^{-1}, $k_{on} = (2 \pm 0.5) \times 10^8$ M^{-1} s^{-1}, and $k_{ox} = (3.8 \pm 1) \times 10^7$ M^{-1} s^{-1}. This independent determination of k_{ox} is in good agreement with the value given by Eich et al. (*29*). Among the different strategies for deducing k_{off}, we consider the value of 3.0 s^{-1} to be the single most reliable, because it was obtained at high MbO_2 concentrations, where the reaction rates become independent of MbO_2 concentrations, and it is in the middle of the results from other strategies. This k_{off} value is slightly lower than the value of $k_{off} = 5.6$ s^{-1} reported by Wolak et al. (*11*). A portion of the difference is due to a 2 °C temperature differential. The value for k_{on} determined from the global fit, which must characterize the thermal combination reaction, is slightly lower than our value of 5×10^8 M^{-1} s^{-1}, determined from flash photolysis. There is

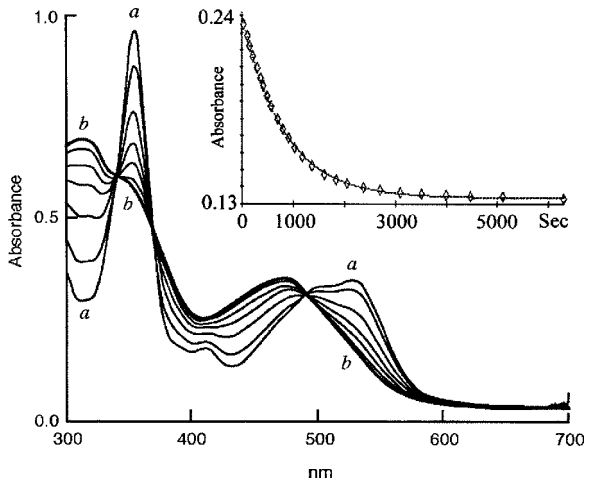


FIGURE 2: Reaction of Cbl(III) with NO as a function of time. Approximately 3 mL of 55 μM deoxygenated aqueous hydroxocobalamin was bubbled with high-purity NO gas for 4 min ([NO] = 1946 μM). Perchloric acid was added to a final concentration of 0.1 M, and spectra were recorded as a function of time until no further change was observed. At the end of the experiment, Ar was bubbled through the solution for 10 min to remove NO before the pH was measured. On other occasions, NO was added after the acid, with the same results obtained. (a) Spectrum immediately after addition of acid. (b) Spectrum when no further changes were observed. (Inset) Time course at 525 nm for the reaction using 28 μM Cbl(III) and 1946 μM NO. Diamonds, data points; line, best single-exponential fit.

always some question whether association after flash photolysis mimics the thermal combination process. Wolak et al. (*11*) noted that they expected the thermal process to be faster and expressed surprise that a calculation combining their measured dissociation rate with an equilibrium constant they measured using a precision NO electrode gave a thermal $k_{on} = 1 \times 10^8$ M^{-1} s^{-1} at 20 °C. This value was 7-fold lower than their value from photolysis. In both their work and ours, the purely thermal rate is slower than the rate from photolysis, but our thermal and photolysis results are closer together and lie between the two numbers reported by Wolak et al. (*11*). Still, the results from both groups show that bond formation after photolysis must be much the same as the true thermal process, as is the case also for NO reactions with heme and heme proteins. We turn now to the three new reactions of NO that we studied.

Reaction of Cbl(III) with NO. Although in past studies small absorbance changes were observed upon mixing Cbl(III) with NO solutions and taken as evidence of Cbl(III)−NO formation (*8*−*10*), more careful, recent studies have revealed that, at pH 7, there is little, if any, reaction between Cbl(III) and NO (*12*). The small absorbance changes seen in the past were due to contamination by nitrite ion, leading to the formation of a Cbl(III)−NO_2 complex.

We found in this study that, at pH 4 or lower, Cbl(III) reacts readily with NO. In Figure 2 are shown changes in UV−visible spectra as a function of time in 0.1 M $HClO_4$, pH 1. Three well-defined isosbestic points are observed, proving that no measurable concentration of intermediate develops. A typical reaction time course is shown in the inset to Figure 2, with the half-life of the reaction being (5.7 ± 1) × 10^2 s ($k_{obs} = 1.2 \times 10^{-3}$ s^{-1}) at pH 1.05 and [NO] = 1946 μM. Experiments were also performed using hydrochloric acid, phosphoric acid, or acetic acid at various pH values. Reaction rates were independent of NO concentration but strongly dependent on pH. For example, in H_3PO_4 at pH 2.7, $k_{obs} = 1.2 \times 10^{-4}$ s^{-1}, while at pH 2.14, $k_{obs} = 5.1 \times 10^{-4}$ s^{-1}. It is not practical to extend this to a full titration curve (which requires pH ≪ −2.5) both because, at such extremely low pH, other sites may be protonated and because, at such high acid concentrations, the anions that are the conjugate bases are likely to bind to cobalt. We discounted the possibility that the pH dependence is due to Cbl(III)−H_2O being more reactive toward NO than Cbl(III)−OH, because the pK_a for the equilibrium Cbl(III)−H_2O ⇄ Cbl(III)−OH^- + H^+ is about 7.8 (30), and NO would react even at pH 7.4 if that were the explanation. Instead, it seems likely that Bzm slowly came off and was protonated, a reaction for which p$K_a \approx -2.5$. We propose that the process by which Cbl(III) reacts with NO below pH 4 involves a rate-limiting step that consists of the formation of four-coordinate Cbl′(III), followed rapidly by reduction to Cbl′(II) and NO ligation:

$$\text{Cbl(III)} \underset{\text{slow}}{\overset{\text{H}^+}{\rightleftharpoons}} \text{Cbl}'\text{(III)} \xrightarrow[\text{fast}]{\text{+NO}} \text{Cbl}'\text{(II)}-\text{NO} \qquad (5)$$

Cobalt(III) frequently exhibits slow ligand dissociation, so much so that it has been used to replace iron in heme in order to slow protein folding in cytochrome *c* (31). Since the BZM is tethered, it cannot escape and will rebind rapidly, unless the proton concentration is high enough for protonation to compete with reattachment. Once Cbl′(III) is formed, reduction and coordination to form Cbl′(II)−NO is fast. At sufficiently low concentrations of NO, a dependence on [NO] would be predicted by our proposal, but we were unable to achieve that condition. The second part of eq 5 involves both a reduction and a ligation. These are separated below in eq 7.

Of critical importance in testing our proposed scheme is identifying spectrum b in Figure 2 with the product in eq 5. This was accomplished by carrying out the same overall steps in a different order. We first reduced Cbl(III) at pH 7.4 using dithiothreitol, then reacted the resulting Cbl(II) with NO, and finally lowered the pH to 1, as shown in eq 6:

$$\text{Cbl(III)} \xrightarrow{\text{+DTT, +NO}} \text{Cbl(II)}-\text{NO} \xrightarrow{\text{+H}^+} \text{Cbl}'\text{(II)}-\text{NO} \qquad (6)$$

Reaction 6 gave the same final spectrum as our proposal in eq 5.

This interpretation of the observations for Cbl(III) reactivity with NO at low pH leads to the prediction that Cbi(III), which does not have the Bzm coordinated to cobalt, should react with NO not only at low pH but even at pH 7.4. Thus, for Cbi(III), the reaction rates should be independent of pH but dependent on NO, with the rate-limiting step being the reduction of Cbi(III) to Cbi(II). Tests of this prediction are described next.

Reaction of Cbi(III) with NO. We found that, at pH 7.4, Cbi(III) reacts readily with NO, as illustrated in Figure 3. The reaction rates showed first-order dependence on NO concentration. There was only a minor dependence on pH, presumably due to the shift from Cbi(III)−H_2O/OH^- to Cbi(III)−$(H_2O)_2$ at low pH and different reactivities for the two species. The inset to Figure 3 shows a typical reaction time course at pH 7.1, with the half-life of the reaction being

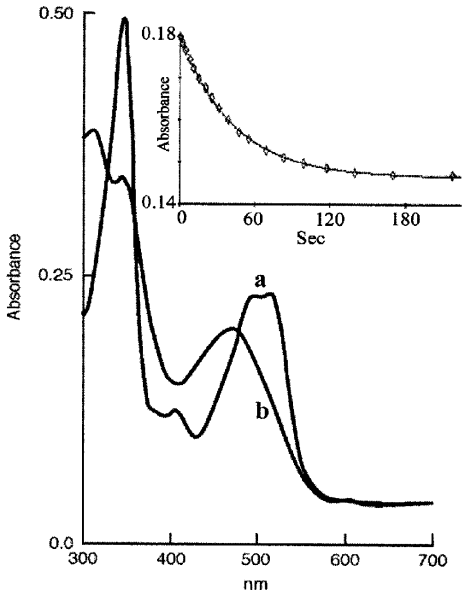


FIGURE 3: Reaction of Cbi(III) with NO. (a) Spectrum obtained after Cbi(III) (21 μM) in 0.1 M phosphate buffer, at pH 7.4, was deoxygenated. (b) Spectrum obtained after reaction with 1946 μM NO. (Inset) Time course at 512 nm for the reaction using 30 μM Cbi(III) and 1946 μM NO. Diamonds, data points; line, best single-exponential fit.

Table 2: Features of the UV−Visible Absorption in Cbl(II) and Cbi(II)

	pH	$\lambda_{max} \pm 2$ (nm)	$\lambda_{shoulder}$ (nm)	$\lambda_{max} \pm 2$ (nm)
Cbi(II)−NO[a]	7.4[b]	314	∼340	466
Cbi(III) + NO[c]	7.4	312	∼344	468
Cbl(II)−NO[a]	7.4	314	∼352	481[d]
Cbl(II)−NO[a]	2.8	314	∼342	470
Cbl(III) + H^+ + NO[c]	∼1	314	∼348	472

[a] From Cbi(III) or Cbl(III) reduced using DTT or sodium borohydride. [b] No change as pH is lowered to 2.8. [c] No added reducing agent, but NO reduces Co(III) to Co(II). [d] Very broad band.

40 ± 10 s. We propose the following for the reaction of Cbi(III) with NO at neutral or lower pH:

$$\text{Cbi(III)} + \text{NO} \rightarrow \{\text{Cbi(III)}-\text{NO} \leftrightarrow \text{Cbi(II)}-\text{NO}^+\} \xrightarrow{-\text{NO}^+,\ +\text{NO}} \text{Cbi(II)}-\text{NO} \quad (7)$$

The final spectrum of the product in eq 7, namely, Cbi(II)−NO at pH 7.4, is identical to that for the product of the reactions discussed above in reactions 5 and 6, confirming that in both reactions 5 and 6 the product is base-off Cbl′(II)−NO, irrespective of whether the reduction process was carried out by NO itself or by DTT introduced before the addition of NO. In all three cases, the final spectrum is that of the five-coordinate nitrosyl−Co(II) complex, as illustrated by the similarity of the sharp spectral features listed in Table 2.

Equation 7 implies that reduction of Cbi(III) [as well as Cbl′(III)] takes place by way of formation of a short-lived Cbi(III)−NO intermediate, followed by reduction of Co(III) to Co(II). Since NO solutions are never entirely free of nitrite ion, another sequence of reactions must be considered:

$$\text{M(III)}^+ + \text{NO}_2^- \rightleftarrows \text{M}-\text{NO}_2 \xrightarrow{+\text{NO}} \text{M}-\text{NO} + \text{NO}_2 \quad (8)$$

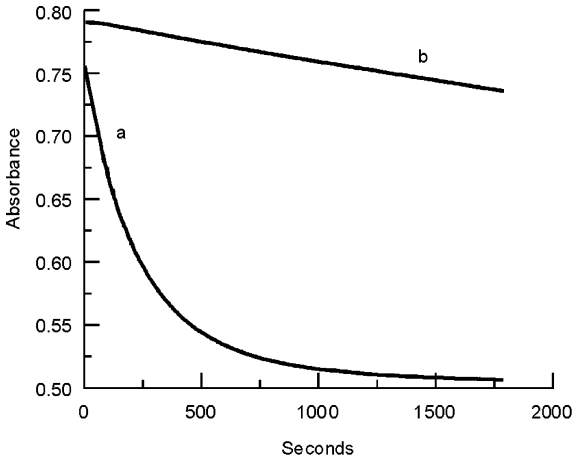


FIGURE 4: Effect of added nitrite ion on the rate of formation of Cbi(II)−NO from Cbi(III), monitored by absorption change at 512 nm. (a) Without nitrite. (b) With 0.043 M nitrite. In both cases, [Cbi] = 90 μM, [NO] = 1946 μM, pH = 7.4.

where M(III) is a transition metal complex. O'Shea et al. (*32*) studied such reactions for chloroiron(III) octaethylporphyrin and potassium (18-crown-6) nitrite in the presence of a wide variety of substrates, including NO. The rate-limiting step was the oxygen transfer. To decide which of the two reactions, 7 or 8, might be valid for our situation, we studied the effect of added NO_2^- on the rates of formation of Cbi(II)−NO from Cbi(III). Reaction 8 demands that the rate for making the nitrosyl derivative must increase with added nitrite, or at least stay the same if our solutions already have so much nitrite that the intermediate M−NO_2 is at a maximum even without added nitrite. The rate can never decrease. On the other hand, if direct reduction by NO is key, then adding nitrite is likely to block the coordination site and eliminate or slow the desired reaction. In Figure 4 are displayed reaction time courses for reaction of NO with Cbi(III), with and without added NO_2^- ion. It is clear that the addition of nitrite dramatically slows the reduction rate. This is not too surprising. O'Shea et al. (*32*) state that the driving force for their reaction resides in the thermodynamic stability of the iron heme−NO adduct. The cobalt corrin−NO adducts are notably less stable; therefore, mechanism 8 should be of less importance than it is for the iron porphyrins. Yet, even if the third-order overall rate constant for nitrosylation by mechanism 8 were as large in the cobalt corrinoids as it is in the iron porphyrins (*30*), the rate would be too slow to account for our measurements, given the upper limits we established for the nitrite concentration.

The inhibition by nitrite suggests that the reduction of Co(III) portrayed in eqs 6 and 7 proceeds by a simple inner-sphere mechanism in which an electron is transferred directly from NO to cobalt. The lifetime of the Co(III)−NO precursor is unknown but must be fairly short, as efforts to detect it have so far remained inconclusive at best.

Another remote possibility to account for the reduction of Cbi(III) or Cbl′(III) would be an outer-sphere reaction. We cannot eliminate this possibility completely, but it seems unlikely. There are two possibilities. An outer-sphere process might be expected to generate a −C−NO derivative somewhere in the corrin ring structure, because NO^+ is a powerful electrophilic entity. This was rejected on two grounds: first, the spectrum for Cbi(II)−NO produced by reduction with

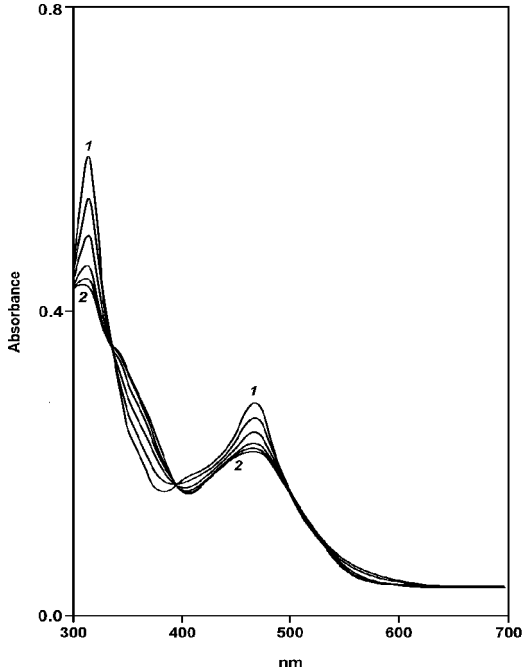


FIGURE 5: Titration of Cbi(II) with NO. Deoxygenated Cbi(III) (36 μM) was first reduced to Cbi(II). Small volumes of 1946 μM solutions of NO were then added. (1) After reduction by DTT; (2) after additions of NO summing to 1 equiv (or more). After 1 equiv of NO was added, no further change was observed.

NO is identical to that resulting from reduction with DTT followed by reaction with NO, a process always accepted to be metal ligation, and, second, the reaction is easily and completely reversible. We proved experimentally that the NO can be removed from Cbi(II)−NO simply by bubbling with a stream of argon, a process too gentle to break any strong −C−NO bond. Alternatively, and despite expectation, an outer-sphere process might simply involve NO donating an electron somewhere in the corrin macrocycle, leaving NO^+ free in solution. This we judge unlikely because the nitrite inhibition experiment suggests that NO must have access to the metal.

Reaction of Cbi(II) with NO. Cbi(II) was produced from Cbi(III) by treatment with an excess of DTT or borohydride. The Cbi(II) reacted readily with NO. Figure 5 shows a titration of Cbi(II) with NO in aqueous solution at pH 7.4. Because no further changes in the spectrum were observed after adding 1 equiv of NO, we infer that only one NO molecule binds per Cbi(II) molecule. The final spectrum was similar to the spectra obtained for Cbi(III) at pH 7.4, reduced by NO instead of with DTT, for Cbl(III) + NO at low pH, or for Cbl(III) reacted with DTT and NO and then exposed to lowered pH to remove Bzm, all described above.

Kinetic studies of NO combination with Cbi(II) at pH 7.4 were carried out by flash photolysis at NO concentrations of 1946, 746, 390, and 186 μM. Figure 6 shows two reaction time courses. Reaction rates were first order in [NO] and yielded a rate constant of $(2.4 \pm 0.3) \times 10^8$ M^{-1} s^{-1}; lowering the pH to 2.2 did not change the rate constant significantly (2.3×10^8 M^{-1} s^{-1}). These reaction rate constants and others are collected together in Table 1.

Cobinamide exists in complicated equilibria of aquo and hydroxo species in aqueous solutions (*24*, *25*), which are likely to show somewhat different kinetic behaviors. Such complications were not evident in the equilibrium studies of Cbi(II); however, the kinetic studies revealed evidence for at least two and probably more reactions, all NO dependent with slightly different rates and spectral evolution. Our work focused on the major and fastest component, since the main point was to gain insight into the behavior of base-off Cbl′. Wolak et al. (*11*) also observed a 20-fold slower, minor component for Cbl′(II)−NO at pH 3.6, and attributed it to photoinduced formation of an α-nitrosyl isomer. It may not be a coincidence that complicated behavior appears when there is more than one free site on Co(II) for reaction with NO.

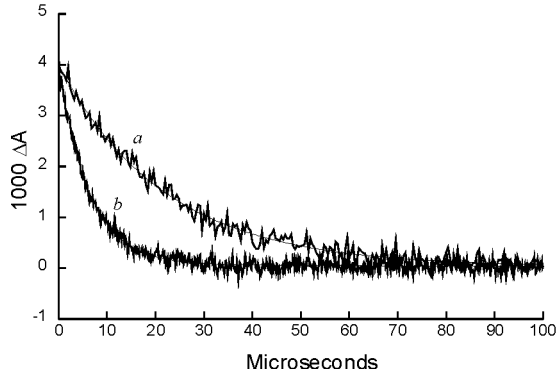


FIGURE 6: Kinetics of Cbi(II) reaction with NO after flash photolysis of Cbi(II)−NO in 0.1 M phosphate buffer, pH 7.4. Absorbance changes, ΔA, were monitored at 446 nm. (a) [NO] = 186 μM; smooth line, exponential decay at 4.4×10^4 s^{-1}. (b) [NO] = 744 μM; smooth line, exponential decay at 1.7×10^5 s^{-1}.

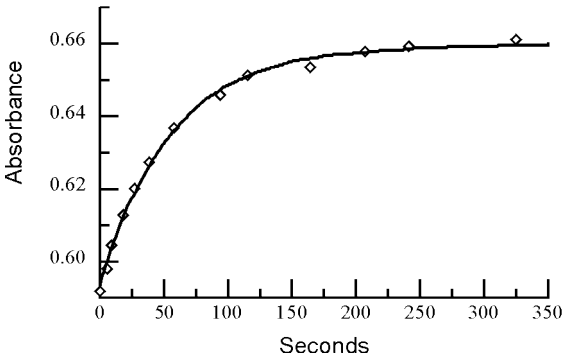


FIGURE 7: Kinetics of NO dissociation from Cbi(II)−NO. The time course of NO dissociation from the Cbi(II)−NO complex (18 μM) in 0.1 M phosphate buffer, pH 7.4, was followed at 469 nm after mixing with 130 μM heme, 2% cetyltrimethylammonium bromide. Diamonds, data points; line, best single-exponential fit.

Kinetics of NO Dissociation from Cbi(II)−NO. We found by stopped-flow measurements that NO dissociation from cobinamide is much slower than that from cobalamin. Figure 7 shows a typical reaction time course. Measurements over such long times face the difficulty that there is a slow reaction between MbO_2 and DTT, presumably the slow removal of O_2 by DTT to give deoxy Mb. Consequently, instead of MbO_2, we used heme as the NO scavenger. The reactions are

$$\text{Cbi(II)}-\text{NO} \xrightarrow{k_{off}} \text{Cbi(II)} + \text{NO} \qquad (9)$$

$$\text{heme(II)} + \text{NO} \rightarrow \text{heme(II)}-\text{NO} \qquad (10)$$

Reaction 10 is much faster than reaction 9, so the overall reaction is

$$\text{Cbi(II)}-\text{NO} \xrightarrow[\text{heme}]{k_{off}} \text{Cbi(II)} + \text{heme(II)}-\text{NO} \quad (11)$$

The reaction progress was monitored at 469 nm. At that wavelength, the spectral changes due to reduction to Cbi(II), eq 9, and those due to ligation with NO, eq 10, augment each other to maximize the overall absorbance change in eq 11 and facilitate the measurement. In eq 10, the NO association with heme proceeds with a rate constant of about 10^9 M^{-1} s^{-1}, and its dissociation rate constant is only 10^{-5} s^{-1} (*33*, *34*). Thus, even at a 1:1 mole ratio of Cbi(II)−NO: heme, the heme is a very efficient scavenger of NO. We used two concentrations of heme, 130 μM (giving the ratio Cbi:heme = 1:7.3) and 43 μM (giving Cbi:heme = 1:2.4). At both scavenger concentrations, k_{off} was found to be 0.019 ± 0.001 s^{-1}, in 0.1 M phosphate buffer, pH 7.4, at 23 °C. Concentrations of heme higher than 130 μM were precluded by solubility limitations. Observe that NO dissociation from Cbi(II)−NO is about 150 times slower than that from Cbl(II)−NO.

DISCUSSION

We started by asking why NO does not react with Cbl(III), even though it reacts with Cbl(II). Our experiments with Cbl(III) at low pH and with Cbi(III) at the physiological pH of 7.4 answer this question definitively: It is the strong negative trans effect of Bzm coordination that prevents NO from reacting with Cbl(III). As soon as that effect is eliminated by removing Bzm at low pH in Cbl′ or by removing it permanently in Cbi, the reaction occurs. Why, then, does Cbl(II) react with NO near neutral conditions? The pK_a for the dissociation and protonation of Bzm from cobalt is −2.5 in Cbl(III) but +2.5 in Cbl(II). Thus, Cbl(II) reacts near neutral conditions, and Cbl(III) reacts similarly at a pH value 5 units lower. As the binding affinity of Bzm to cobalt decreases from Cbl(III) to Cbl(II), so does the negative trans effect. At the same time, NO reacts more readily.

Our results also show that, in the absence of the Bzm base coordinated to cobalt, NO reacts with Cbl′(III) in a manner generally similar to its reaction with Fe(III)−heme, that is, with one NO reducing the metal followed by coordination of a second NO, as illustrated here:

$$\text{heme(III)} + \text{NO} \rightarrow \text{heme(II)} + \text{NO}^+ \xrightarrow{+\text{NO}} \text{heme(II)}-\text{NO} \quad (12)$$

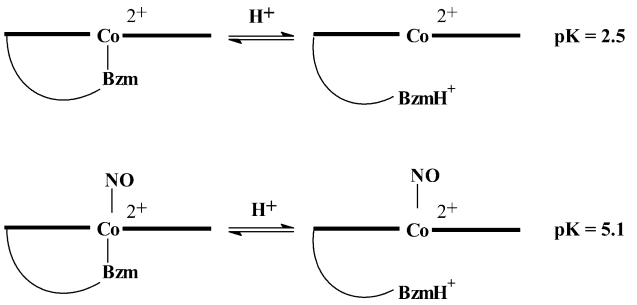
The reason that this reaction does not occur in the corrins in the presence of the axial Bzm is probably related to electronic factors and strain arising from Bzm coordination on the α side of the corrin ring. For small ligands such as NO, steric factors on the β side, i.e., the side on which NO reacts, are probably not significant.

Turning next to the 2+ oxidation state, NO affinity for cobalt in both Cbl(II) and Cbi(II) is much weaker than it is for iron in heme(II). This is mainly due to faster ligand dissociation rates in the corrinoids than in the hemes (*35*):

Heme(II)−NO	$k_{off} = 1 \times 10^{-5}$ s^{-1}	(ref 33)
Base-off Cbl′(II)−NO	$k_{off} = 1.7$ s^{-1} at pH 3.6	(ref 11)
Base-on Cbl(II)−NO	$k_{off} = 5.6$ s^{-1} at pH 7.4	(ref 11)
Base-on Cbl(II)−NO	$k_{off} = 3$ s^{-1} at pH 7.4	(Table 1)
Cbi(II)−NO	$k_{off} = 0.02$ s^{-1} at pH 7.4	(Table 1)

Unlike these large differences in k_{off}, combination rates are quite similar for nitrosyl derivatives of Cbl(II) or Cbi(II) [∼(3−7) × 10^8 M^{-1} s^{-1}, as discussed above] as compared with heme(II) [∼1 × 10^9 M^{-1} s^{-1} (*33*)]. Two conclusions may be drawn: (1) Since the combination rate constants are similar for the nitrosyl derivatives of Cbl(II), Cbi(II), and heme(II), and are fairly close to the diffusion-controlled limit, there seems to be little hindrance to NO bond formation in any of these species due to either electronic or steric factors. (2) Since dissociation rates do differ among these species, it is largely the trans effect of Bzm that causes NO to dissociate 85−280 times faster in Cbl(II)−NO than in Cbi(II)−NO. [This negative trans effect for NO in hemes, heme proteins, and corrinoids is opposite the effect of CO in hemes and heme proteins: for CO, a trans base almost invariably increases affinity of both CO and the base (*35*).] For five- and six-coordinate nitrosyl derivatives of tetraphenylporphinato iron(II), TTP−NO, large differences in NO dissociation rate constants are explained on the basis of displacement of the central iron with respect to the mean plane of the porphyrin ring and steric interactions between the NO and the porphyrin ring (*36*, *37*). The same issues of cobalt displacement and steric repulsions likely account for the difference between the k_{off} values for Cbl(II)−NO and Cbi(II)−NO.

The trans effect should be symmetrical. If Bzm affects the affinity for NO, then the presence of NO should affect the binding of Bzm. That this is the case is clearly seen in the pK_a's reported (*11*) for the following reactions:



In Cbl(III)−H_2O and Cbl(II), introducing one extra electron in the d_{z^2} orbital causes the pK_a of the axial Bzm to change from −2.5 to +2.5, a difference of 5 pH units. Here, in Cbl(II) and Cbl(II)−NO, the difference is somewhat smaller, about 2.6 pH units, but still evident. These variations most likely involve back-bonding and partial delocalization of the unpaired electron on NO to the Co−corrinoid.

Given the utility of the negative trans effect in explaining so much, it may at first be surprising that it seems to be almost absent in one case. The NO affinity constants for Cbl(II)−NO, which were reported by Wolak et al. (*11*),

might have been expected to vary with pH, since Bzm should not be coordinated at low pH. Yet there was only a slight affect. Nitric oxide affinity in Cbl(II)−NO was 1.3×10^8 M^{-1} at pH 7.4 and only slightly larger, 3.1×10^8 M^{-1}, at pH 3.6, where the complex will exist mainly in the base-off form. This is different from other situations discussed earlier for the corrinoids and also quite unlike the situation in nitrosyl hemes. The NO affinity constants for five- and six-coordinate nitrosyl heme derivatives differ significantly: base-off heme(II)−NO has an affinity of about 1×10^{15} M^{-1}, but base−heme(II)−NO has an affinity of only about 8×10^{11} M^{-1} (*35*).

Our data suggest that the lack of any large difference in the NO affinities of base-on and base-off derivatives of Cbl(II)−NO is due to the NO off-rate from the base-off derivative Cbl′-NO (1.7 s^{-1}) remaining close to what it is in Cbl(II)−NO instead of what it should be if the base-off species were truly like Cbi-NO (0.02 s^{-1}). Consistent with this is the observation that the pK_a of 5.1 accompanying axial Bzm dissociation in Cbl(II)−NO is not very different from the pK_a of 5.0 for uncoordinated Bzm in dicyanocobalamin (*38*). Even at pH 7.4, there is only a weak interaction between the central metal ion and Bzm in Cbl(II)−NO; that is, the complex is already somewhat like the base-off form. Even more importantly, the base-off state of Cbl′(II)−NO does not appear to have a protonated $BzmH^+$ that is solvated and located away from the corrin ring. It does release from the Co and allow the NO adduct to form, as we report here; but even when dissociated from the Co atom, the Bzm side chain continues some degree of interaction with the cobalt−corrinoid complex and accelerates NO dissociation rates well above what they are in Cbi(II)−NO. Pratt (*30*) discussed the possibility of interactions in which Bzm is positioned next to the metal ion in Cbl′ by being held in a hydrophobic cleft without direct coordination to the cobalt. In another publication, he suggested that the base interacts with the corrin ring through hydrogen bonding (*39*). Perhaps most convincing were the ^{13}C NMR studies of Brown and Peck-Siler (*40*), which report evidence of base-off cobalamin in which the Bzm remains unprotonated but hydrogen-bonded to a specific side chain of the corrin. Much the same conclusion emerged even earlier from a ^{31}P NMR study (*41*), which also inferred such an interaction and added that it affects puckering in the ring — a process we believe would affect dissociation rates.

Finally, we turn to the implications of NO binding to Cbl for enzymology and physiology. When cobalamin binds to methionine synthase or to methylmalonyl-Co A, the Bzm axial ligand is replaced by a histidine residue of the protein. This may not represent much of a change to the cobalt, as the His-759 N_ϵ–Co bond length is 0.22 nm, close to the distance of 0.219 nm found in methylcobalamin (*42*). Such replacement of the Bzm moiety by an amino acid side chain in the enzyme should be assisted by the trans effect of ligands such as $NO^\bullet$ or $R-CH_2^\bullet$. It should be more likely to take place in six-coordinate alkyl derivatives of the enzyme. The protein structure may assist the process, but by itself, in the absence of the negative trans effect of some axial ligand, the process may be energetically unfavorable.

Previously, we reported (*6*) that methyltransferase activity is inhibited by NO reaction with Cbl(I) and speculated that, in view of a high recombination rate constant for the $RCH_2^\bullet$ radical with Cbl(II) in the intermediate formed in isomerase reactions (*43*, *44*), NO might not be able to interfere in those reactions. Now, however, we must withdraw that conjecture. Kinetic data from the present study and those reported by Wolak et al. (*11*) suggest that the NO association rate constant is, in fact, sufficiently high to compete with $R-CH_2^\bullet$ — particularly under physiological conditions of unusually high NO concentrations, as might occur during septic shock or infection:

$$R-CH_2^\bullet + \text{Cbl(II)} \rightleftarrows R-CH_2-\text{Cbl} \qquad k_{on} \approx 1 \times 10^9\ M^{-1}\ s^{-1}\ \text{(ref 45)}$$

$$NO^\bullet + \text{Cbl(II)} \rightleftarrows NO-\text{Cbl} \qquad k_{on} \approx (5-8) \times 10^8\ M^{-1}\ s^{-1}\ \text{(ref 11)}$$

Finally, we observe that the fact that cobinamide reacts with NO in both the 2+ and 3+ oxidation states at pH near neutral, and has an affinity for NO more than 100 times higher than that of Cbl(II), suggests that cobinamide should be a much more effective NO scavenger than cobalamin, and that cobinamide could potentially be used clinically in pathologic states of high NO production, for example, in the treatment of septic shock.

In conclusion, the kinetic and spectroscopic data provided in this study show that both Cbi(III) and Cbl′(III), in the absence of axial base, behave toward NO in a manner generally similar to their counterpart heme derivatives. They are reduced by NO and then form the nitrosyl complexes. Ligand combination rates and affinities are sufficiently high to make these species potentially very efficient NO traps, as has been shown in rodent models of NO-induced vascular smooth muscle relaxation and hypotension (*3*, *4*). These data re-emphasize the point sometimes missed while explaining the many, complicated roles of NO in biological systems, namely, that NO is trapped very fast by metal derivatives with free ligand binding sites [as well as by the radical $HOO^\bullet$, not discussed herein (*46*)]. Only very rarely can other reactions compete by being very rapid themselves or by occurring in the absence of fast competition.

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