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Electrochemical and Spectral Studies of the Reactions of Aquocobalamin with Nitric Oxide and Nitrite Ion

Donghong Zheng, Lei Yan, and Ronald L. Birke*

Department of Chemistry and Center for Analysis of Structures and Interfaces, The City College of New York and The Graduate School and University Center of The City University of New York, New York, New York 10031

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Electrochemistry and Raman spectroscopy have shown that aquocob(III)alamin (Cbl(III)) can be reduced by nitric oxide (NO) to form Cbl(II) on an electrode surface. The Cbl(II) formed in this way can bind NO to form nitrosylcobalamin, Cbl(II)–NO, which is reduced to form Cbl(I) at about −1.0 V vs a KCl saturated Ag/AgCl reference electrode. In addition, nitrite was found to bind both Cbl(III) and Cbl(II) and a binding constant of 3.5×10^2 M^{-1} was measured for $(NO_2–Cbl(II))^{1-}$. UV–vis spectrophotometry and mass spectroscopy were used to show that Cbl(I) reduces NO to form Cbl(II)–NO and N_2O and N_2, and this reaction is involved in the cyclic voltammetry of cobalamin in the presence of excess NO where a catalytic reduction of NO occurs involving the cycling of Cbl(II)–NO/Cbl(I). This redox couple is also involved in the electrochemical catalytic reduction of nitrite. These results can be used to explain a number of physiological effects involving NO interaction in biological systems with added cobalamin or with cobalamin in the methionine synthase enzyme.

Introduction

It is well-known that nitric oxide (NO), the intercellular signaling agent for control of mammalian blood pressure, originally identified as the " endothelium derived relaxing factor" (EDRF), is also involved in the cytotoxic immune response to pathogen invasion and is a neurotransmission regulator in the central nervous system. A number of disease states involving NO imbalances have been proposed, and this speculation has stimulated extensive research into the chemistry, biology, and pharmacology of NO and the compounds which directly react with it. For example, the metal porphyrins and related heme proteins are probably the most investigated class of compounds which react with NO. Another class of biologically significant metal macrocyclic compounds which react with nitric oxide are the cobalamins, Cbl, which serve as cofactors in B_{12} dependent enzymatic processes. These are cobalt-containing corrin macrocyclic ring compounds which are similar to cobalt porphyrins in terms of both structure and chemical properties.

Although the interactions of metal porphyrins with NO have been extensively studied, there have been relatively few reports on the interaction of cobalamin with NO. Nevertheless, physiological evidence has already indicated the biological significance of the interaction. For instance, a number of physiological studies show that added cobalamin can reverse the effects of NO in animal studies of blood flow,[1,2] in muscle relaxation,[3−5] and in the cellular growth response to NO stimulation.[6] In other reports, both NO and N_2O were found to be inhibitors in vitro of methionine synthase, which is a cobalamin-dependent methyl transferase enzyme for conversion of homocysteine, Hcy, to methionine.[7−9] This reaction is one of the enzymatic pathways for metabolic regulation of homocysteine, and its inhibition may lead to increased total plasma Hcy, hyperhomocysteinemia, which

* To whom correspondence should be addressed. E-mail: birke@sci.ccny.cuny.edu.

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many epidemiological studies indicate is a risk factor for cardiovascular disease.[10] A very recent report shows that NO inhibits methionine synthase in mammalian cells in vivo at physiological concentrations, and the implications of these results are that NO may act as a physiological regulator of methionine synthase to control metabolic carbon addition processes in the folate pathway.[11]

Although earlier studies failed to provide convincing evidence for the existence of stable Cbl(III)−NO or Cbl(II)−NO complexes,[2,12−16] recently we utilized UV−vis and Raman spectroscopy methods to show that Cbl(II) does bind NO with a very high binding constant and that a Co−NO bond forms as indicated by a Raman vibrational isotopic effect.[17] The Raman data[17] suggested a bent Co−N═O bond, and additional evidence for this structure has been obtained by NMR studies.[18] Furthermore, kinetic and mechanistic experiments have elucidated the reversible binding nature of the reaction of NO with Cbl(II).[18] These results[17,18] are important for explaining some of the physiological effects caused by cobalamin and NO interactions in biological processes, but a comprehensive understanding should involve the possible chemical reactions of NO with all three oxidation states of cobalamin. In addition, some of the contradictions in the earlier studies are because of the ease of oxidation of NO to nitrite in an aqueous solution.[16] Indeed, recently it has again been proposed that NO binds to Cbl(III) based on the reduced rate of dithiothreitol reduction of NO-exposed Cbl(III) solutions.[11] On the other hand, this result could be accounted for by Cbl(III) binding with nitrite ion which can be formed by oxidation of NO by trace oxygen. At this time, there is still no proof of Co(III)−NO bond formation in cob(III)alamin in aqueous solution. Thus it is also necessary to investigate both the interactions of NO and nitrite with cobalamin. In this paper, we report results for the interactions of all three oxidation states of aquocobalamin with both NO and nitrite based on UV−vis spectrophotometry, Raman spectroscopy, and electrochemical studies.

There have been some previous cyclic voltammetry studies of similar water soluble cobalt macrocyclic complexes in the presence of NO, such as cobalt tetrakis(*N*-methyl-2-pyridyl)porphine, Co(2-TMPyP),[19] and cyanocobalamin, vitamin B_{12}.[20] Both the cobalt porphyrin and cyanocobalamin were found to support the catalytic reduction of NO (nitrite in acidic medium) upon reduction of cobalt to the cobalt(I) species, and we find that the same is true for aquocobalamin. On the other hand, our results show differences in the redox and ligand binding properties of aquocobalamin toward NO compared to cyanocobalamin where the cyanide ligand is strongly bound to Co(III) of cobalamin and to Co(2-TMPyP) where aquo species seem to bind the axial positions in all three cobalt redox states.

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Experimental Section

Materials. Hydroxocobalamin (vitamin B_{12a}), acetate salt, was purchased from Sigma and used as received. NO (Liquid Carbonic Specialty Gas Corp.) was purchased from T. W. Smith Company. Before being used, NO tank gas was collected with a gas container, then washed with water until colorless, or bubbled through a 1 M NaOH solution twice to purify. The buffer solutions were bought from Spectrum Chemical Mfg. Corp. as standard pH 10.0, and pH 7.0. The total concentrations of the buffer solutions are around 0.05 M. Other pH solutions were adjusted from those above with aqueous solutions of 0.1 M NaOH or 0.1 M HCl. All other reagents were at least analytical reagent grade, and all solutions were prepared with distilled deionized water.

Electrochemical Measurements. All electrochemical measurements were performed in a specially designed five-neck airtight electrochemical cell with O-ring seals. Before each measurement, purified nitrogen gas was bubbled for at least a half-hour to remove oxygen. Then a certain amount of purified NO gas was injected using an airtight syringe. The purification of nitrogen was performed with a Ridox catalyst (Fisher Scientific) which was reactivated before each experiment. After NO solutions (pH 7) were degassed with purified nitrogen, the residual absorbance from 320 to 420 nm was in the background noise, ca. 0.001 absorbance unit, indicating that nitrite was too low to be observed. A three-electrode system was used for measurements with a CH Instruments model 660A electrochemical workstation utilizing a glassy carbon working electrode (3 mm diameter), a Pt wire counter electrode, and a Ag/AgCl reference electrode saturated in KCl. All potentials herein are quoted versus this reference electrode unless otherwise stated. The glassy carbon electrode was polished with 0.3 μm and 0.05 μm alumina on a Buehler felt pad and then cleaned by being sonicated in distilled deionized water. One of the five necks of the electrochemical cell was connected to a mercury manometer for NO determination.

Measurement of NO Concentrations in Solution. According to a method in the literature,[21] the concentration of NO in solution can be calculated from the partial pressure of NO gas and the Bunsen absorption coefficient of NO (4.21×10^{-2} at 1 atm and 293 K). The pressure of the NO gas was measured with a mercury manometer. This method was used for NO concentrations in the 20 μM to 2 mM range since our commercial WPI NO electrode could only be used for NO concentrations lower than 20 μM.

UV−Visible Measurements. Solutions were added to a UV−vis cuvette with a septum cover. Then the solutions were bubbled with purified nitrogen through two needles for at least 1 h followed with NO gas injection with a gastight syringe. All measurements were performed on a Perkin-Elmer UV/vis spectrophotometer (Lambda 18).

GC-Mass Spectrometry. Samples from the headspace of the reaction of Cbl(I) with NO were measured with a Shimadzu GC 17A-MS QP-5000 instrument. A nonpolar column (Econo_Cap EC-5) on the GC did not retain the gaseous products.

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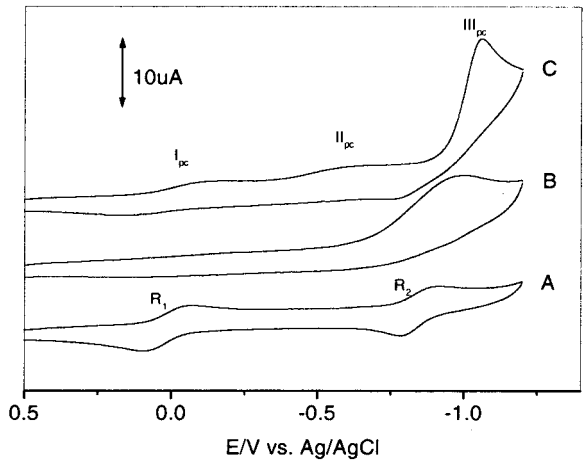

Figure 1. Cyclic voltammograms in pH 7 buffer solution (ca. 0.05 M) at a glassy carbon electrode of (A) 3×10^{-4} M H_2O-Cbl, (B) 1×10^{-3} M NO, and (C) 3×10^{-4} M H_2O-Cbl and 1×10^{-3} M NO. Scan rate: 100 mV/s.

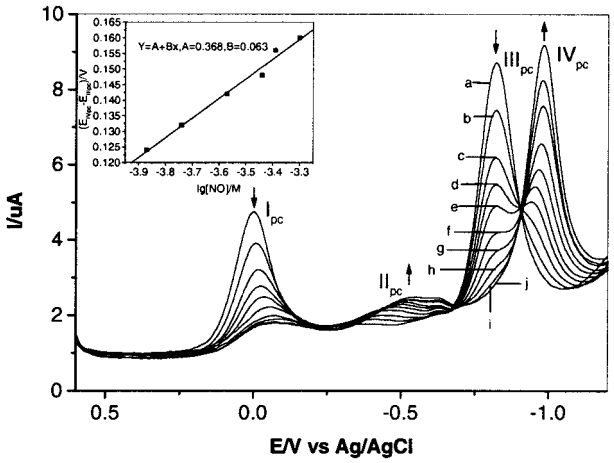

Figure 2. Differential pulse voltammograms of 5×10^{-4} M H_2O-Cbl at a glassy carbon electrode in pH 7 buffer solution as NO is gradually added. The concentration of NO in solution was (a) 0, (b) 0.5, (c) 0.9, (d) 1.4, (e) 1.8, (f) 2.7, (g) 3.6, (h) 4.1, (i) 5.0, (j) 9.1, all $\times 10^{-4}$ M. Arrows show the direction of current change. Pulse magnitude: 50.0 mV. Pulse time: 0.050 s. The inset shows the shift of peak IV$_{pc}$ versus the logarithm of NO concentration, with a slope of 0.063 and an intercept of 0.368.

Raman Scattering Spectra. The normal Raman scattering, NRS, and surface-enhanced Raman scattering (SERS) spectra were measured on a Spex 1401 scanning double spectrometer (ca. 2 cm^{-1} resolution) with a PMT and photon counting detection. The SERS measurement was made at a 99.999% pure Ag electrode in a three-electrode cell after the surface was roughened by a standard oxidation−reduction cycle. Prepurified nitrogen was bubbled through the cell for at least 1 h, and NO was injected with an airtight syringe.

Results

The Interaction of Cobalamin with NO as Followed by Electrochemistry. Figure 1A shows cyclic voltammograms (CVs) of vitamin B_{12a} in a pH 7 buffer solution on a glassy carbon electrode. At pH 7 the aquocob(III)alamin form, H_2O-Cbl(III), of vitamin B_{12a} predominates over the hydroxocob(III)alamin, HO−Cbl(III), form since its pK_a = 7.8. Two separate pairs of reduction−oxidation peaks are observed for H_2O-Cbl(III) at about 0.0 V (R_1) and −0.85 V (R_2) due to the oxidation state changes of the central cobalt atom of the cobalamin, Co(III)/Co(II) and Co(II)/Co(I),[22] respectively. Figure 1B shows the CV of a 1×10^{-3} M NO solution on a glassy carbon electrode in pH 7 buffer. The reduction of NO occurs at about −0.97 V because of the large overpotential for its direct reduction. On the other hand, Figure 1C shows the CV of the H_2O-Cbl(III) solution when it is in the presence of 1×10^{-3} M NO. By comparing Figure 1C with Figure 1A and Figure 1B, it is obvious that significant changes have occurred on mixing NO with cob(III)alamin. First of all, the reduction peak for the Cbl(III) → Cbl(II) process for H_2O-Cbl has decreased, there being only a small reduction peak current (I_{pc}) left at around 0 V. As the potential scan is made more negative, two new reduction peaks, II_{pc} and III_{pc}, can be found at ca. −0.61 and −1.1 V, respectively. It would appear that some of reduction peak R_1 has shifted to a more negative potential at peak II_{pc} and that peak R_2 has shifted to an enhanced peak III_{pc}, while the reduction peak for NO has disappeared. When the potential is scanned back in the positive direction, only a bump of an oxidation peak is found at about −0.8 V, which appears to be associated with peak III_{pc}, and the return wave of peak R_1 is barely observable; thus the oxidation peaks for Cbl(I) → Cbl(II) and Cbl(II) → Cbl(III) have nearly disappeared. We can obtain a better idea of what has happened by stepwise addition of NO as followed by differential pulse voltammetry (DPV).

Figure 2 shows DPVs of H_2O-Cbl(III) in a pH 7 buffer solution on a glassy carbon electrode as a function of 10 different NO concentrations, a−j. In Figure 2a, the two one-electron-reduction peaks, I_{pc} and III_{pc} at 0.0 and −0.85 V, represent the reduction processes of the Co(III)/Co(II) and Co(II)/Co(I) oxidation states of cobalamin in an aqueous pH 7 buffer solution in the absence of NO. Although they are both one-electron-reduction peaks, the peak currents are different because of the different reversibility of the reduction processes. From curve 2b to curve 2j, NO was gradually added to the solution. It can be seen that the reduction peak I_{pc} gradually decreases and finally almost completely disappears at the highest NO concentration. In addition, the potential of peak I_{pc} gradually shifts to more negative values with the addition of NO. At the same time, the second reduction peak III_{pc} also decreases with the addition of NO, but no potential shift is observed for peak III_{pc}. With the decrease of these two peaks, two new peaks (II_{pc} and IV_{pc}) appear and their peak currents increase with the addition of NO. Note especially that the potentials of the two peaks move more negative with increasing NO concentration. The DPV peak potential for peak IV_{pc} in curve 2j at the highest [NO] is at −0.98 V. There is a potential between peaks III_{pc} and IV_{pc} where the current is constant (similar to an isosbestic point) indicating conversion of one form of Cbl(II) into another form. Similar DPV curves were obtained for the same experiment on a Ag working electrode as well (Supporting Information, Figure S1).

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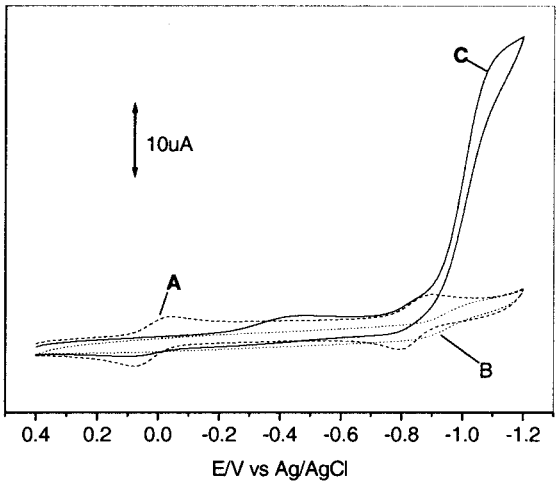

Figure 3. Cyclic voltammograms in pH 7 buffer solution at a glassy carbon electrode of (A) 5×10^{-4} M H_2O−Cbl, (B) 5×10^{-3} M $NaNO_2$, and (C) 5×10^{-4} M HO−Cbl with 5×10^{-3} M $NaNO_2$. Scan rate: 50 mv/s.

The Interaction of Nitrite with Cobalamin as Followed by Electrochemistry. In Figure 3A and Figure 3B we compare the CVs of separate solutions of cobalamin and sodium nitrite, respectively, in pH 7 buffer on a glassy carbon electrode. It can be seen that, even though nitrite is at 10 times the concentration of cobalamin, its direct reduction on the glassy carbon electrode is a drawn out curve with a much smaller reduction peak current indicating a slow reduction process. However, when the two species are mixed with the same concentrations as they had individually, the current increases dramatically on the second peak, Figure 3C. In fact, both redox peaks of cobalamin have changed in the CV curve. The Cbl(III)/Cbl(II) reduction peak has disappeared, but a new reduction peak appears around −0.35 V. The Cbl(II)/Cbl(I) reduction peak at ca. −0.85 V has also disappeared, but a significantly enhanced current with an s-shaped wave is observed at around −1.0 V. An s-shaped enhanced current wave can be formed by a catalytic process which would, in this case, come from a rapid reaction at the electrode surface of Cbl(I) with nitrite, which is the catalytically reduced species. When the potential is reversed, no oxidation peak can be found for the Cbl(I) → Cbl(II) process since for a catalytic process Cbl(I) is consumed at the surface by reaction with nitrite. In addition a much decreased oxidation peak appears at +0.06 V for the Cbl(II) → Cbl(III) process.

When the solution is made alkaline, pH = 10, the CV of a pure nitrite solution shows no reduction peak (Supporting Information, Figure S2C) and the CV for cob(III)alamin alone shows the usual two reduction peaks. On mixing the two species at pH 10, the only change is that the first reduction peak of cobalamin for the Cbl(III)/Cbl(II) process is shifted more negative to −0.35 V (as shown in Figure S2B), which is identical to what occurs at lower pH values; however, the enhanced current at −1.0 V has disappeared.

Figure 4 shows a DPV curve of H_2O−Cbl(III) at a glassy carbon electrode when $NaNO_2$ is gradually added to a pH 7 buffer solution. It is seen that peaks I and III, which are the reduction processes for Cbl(III) → Cbl(II) and Cbl(II) → Cbl(I), gradually decrease as NO_2^- is added to the solution, and concomitantly peak II at −0.35 V and peak IV at −0.96 V appear and gradually increase with nitrite addition. The new reduction peak IV which grows in at −0.96 V is almost identical to the potential of peak IV_{pc} in Figure 2 (−0.98V) where NO was added to the solution.

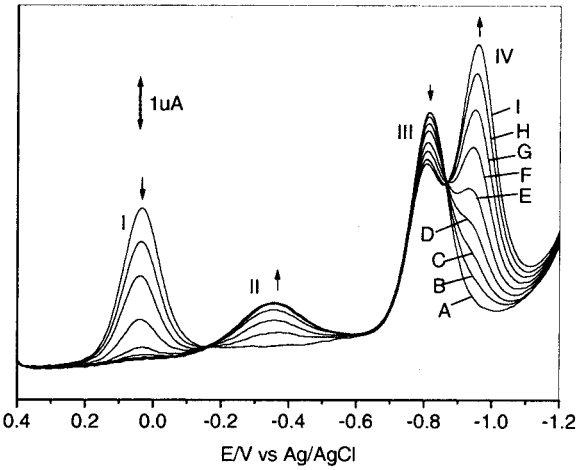

Figure 4. Differential pulse voltammograms of 5×10^{-4} M H_2O−Cbl in pH 7 buffer solution at a glassy carbon electrode as $NaNO_2$ is gradually added. Pulse magnitude: 50.0 mV. Pulse time: 0.050 s. The concentrations of $NaNO_2$ are (A) 0.0, (B) 1.0, (C) 2.0, (D) 3.0, (E) 4.0, (F) 5.0, (G) 6.0, (H) 7.0, (I) 8.0, all $\times 10^{-4}$ M. Arrows show direction of current change.

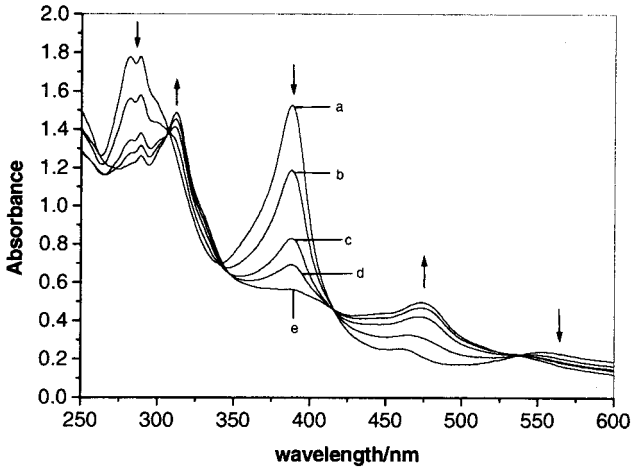

Figure 5. UV−visible spectra at pH 7 of ca. 5 mL of 1×10^{-4} M Cbl(I) as NO is added to solution. From a to e, NO was gradually increased by microliter syringe injection of (a) 0.0, (b) 0.22, (c) 0.45, (d), 0.78, and (e) 0.91, all in micromoles. The actual amount in solution will be less because of equilibration with the headspace above the cell. Arrows show the trend in absorption peaks.

The Interaction of Cob(I)alamin with NO as Followed by UV−Vis Spectrophotometry. In order to observe if NO could react with Cbl(I), we investigated the reaction in a spectrophotometric cell. A Cbl(I) aqueous solution was made by adding excess $NaBH_4$ to a H_2O−Cbl solution. After NO was added, the UV−vis spectrum of Cbl(I), which has absorption peaks at 282, 289, and 388 nm, gradually decreases and peaks at 312 and 474 nm increase, indicating that Cbl(II) had formed (Figure 5). These results agree with a recently reported UV−vis spectrum for this oxidation process.[11]

Discussion

From Figure 2, it is obvious that a strong interaction between cobalamin and NO has occurred when NO is added to the electrochemical cell. The loss in magnitude of the Cbl(III)/Cbl(II) peak I_{pc} can be explained either by the binding of NO to Cbl(III) to form a new compound or by a chemical reaction between Cbl(III) and NO which removes Cbl(III). If Cbl(III) binds NO to form a stable Cbl(III) nitrosyl compound, a new reduction peak of comparable current magnitude to I_{pc} would be expected near the peak I_{pc} for the new complex. However, the absence of such a new reduction peak suggests a mechanism other than the binding of Cbl(III) with NO to form a stable complex. If, on the other hand, the process was simply the electrochemical reduction of Cbl(III) to Cbl(II) followed by a reversible homogeneous chemical reaction for the binding of NO to Cbl(II) to form Cbl(II)−NO, then peak I_{pc} should shift in the positive potential direction, which is also not observed.

It would appear that another chemical process has occurred between NO and Cbl(III). It has been reported for a number of heme proteins and model complexes that a reductive nitrosylation can occur.[23,24] For example in a surfactant thin-film system in the pH range 6−10, ferric myoglobin, Fe^{III}−Mb, is reduced within minutes (much faster than in bulk aqueous solution) by the overall process[23]

$$Fe^{III}-Mb + 2NO + OH^- \rightleftharpoons NO-Fe^{II}-Mb + HNO_2$$

We propose a similar reduction process for Cbl(III) with NO to explain the observed phenomenon, namely,

$$H_2O-Cbl(III)^+ + NO + 2OH^- \rightleftharpoons Cbl(II) + NO_2^- + 2H_2O \quad (1a)$$

$$Cbl(II) + NO \rightleftharpoons Cbl(II)-NO \quad (1b)$$

$$H_2O-Cbl(III)^+ + NO_2^- \rightleftharpoons NO_2-Cbl(III) + H_2O \quad (1c)$$

with the additional fact that nitrite can bind to Cbl(III), and where we note that above about pH 5, HNO_2, $pK_a = 3.15$, is completely dissociated to nitrite. This set of reactions gives the overall reaction

$$2H_2O-Cbl(III)^+ + 2NO + 2OH^- \rightleftharpoons Cbl(II)-NO + NO_2-Cbl(III) + 3H_2O \quad (1)$$

Reaction 1a accounts for the decreased current on reduction peak I_{pc}, and reaction 1c could account for the new peak II_{pc} which would be from the reduction of the nitritocob(III)alamin, NO_2−Cbl(III), formed in the reaction with NO. The equilibrium constant for reaction 1c, 2.3×10^5 M^{-1}, found in the literature[13] is thermodynamically favorable for binding, and in fact, we have repeated the spectrophotometric competitive binding experiment of NO_2^- with NCS−Cbl(III) and calculate a binding constant for NO_2−Cbl(III), 3.0×10^5 M^{-1}, which is very close to this literature value. We have also shown that reaction 1b is thermodynamically favorable, since we have measured the binding constant of Co(II) with NO to form Cbl(II)−NO and found $K_{1b} = 1.0 \pm 0.5 \times 10^8$ M^{-1} from UV−vis absorbance changes and NO concentrations measured with a WPI commercial NO electrode.[17]

Using redox potentials and binding constants, the overall equilibrium constant of reaction 1 can be calculated. The reduction potential of the NO_2^-/NO couple ($E° = -0.46$ V vs NHE) for basic aqueous solution is more reducing than the reduction potential of the Cbl(III)/Cbl(II) couple ($E° = 0.20$ V vs NHE), and the following complexation steps add additional free energy to drive reaction 1 from left to right. A calculation (footnote 25) shows that the reduction of Cbl(III) by NO is thermodynamically favorable at pH 7 for reaction 1. However, no change can be observed in the normal Raman scattering (NRS) spectrum of a Cbl(III) solution with excitation at 647 nm after the addition of NO. On the other hand, surface-enhanced Raman scattering (SERS) shows a significant change on adding NO with the formation of a characteristic vibration of Co(II)−NO at 505 cm^{-1} (Supporting Information, Figure S3B), indicating that reductive nitrosylation has occurred. The SERS band at 505 cm^{-1} assigned to the Co(II)−NO vibration is slightly shifted down from the 514 cm^{-1} value originally found in our RRS study,[17] most likely due to an electrode surface effect. In that study we showed with resonance Raman scattering (RRS) spectroscopy that, by adding NO to Cbl(II) or by adding NO to Cbl(III) with excitation at 514.5 nm (photolytic reduction), Cbl(II)−NO is formed.[17] Considering that, in the NRS study with 647 nm excitation (avoiding photolysis), the species are in the bulk solution and no changes are observed on NO addition whereas changes are observed in both the electrochemical and SERS studies which take place at an electrode surface, we suggest that the kinetics of the reaction are slow in solution but the electrode surface can catalyze the reduction of Cbl(III) by NO.

Thus the gradual shift of the peak potential on peak I_{pc} in Figure 2 can be understood by this surface chemical reaction. This peak current represents the amount of free Cbl(III) in the diffusion layer which can diffuse toward the electrode and can be reduced to Cbl(II), but after the surface catalytic reaction the diffusion layer would also contain Cbl(II)−NO. Dissociation of this species would yield increased uncom-

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(25) The thermodynamics for the reduction of H_2O−Cbl(III) by NO as a gas can be accounted for by $E° = +0.66$ V for reaction 1a, $K_{1b} = 4.1 \times 10^6$ atm^{-1} for reaction 1b, and $K_{1c} = 3.0 \times 10^5$ M^{-1} for reaction 1c. An equilibrium constant $K_1 = 1.8 \times 10^{23}$ is calculated for reaction 1 at 25 °C using the above values for $E°$, K_{1b}, and K_{1c}. The conditional equilibrium constant, K_c, can be calculated from K_1 for pH = 7.0 and $P_{NO} = 0.025$ atm ([NO] = 1.0×10^{-3} M), namely,

$$K_c = 1.0 \times 10^6 = [OH^-]^2 P_{NO}^2 K_1 = [NO_2-Cbl(III)][Cbl(II)-NO]/[Cbl(III)]^2$$

which shows that reduction of Cbl(III) by NO is thermodynamically favorable under these and similar conditions. The NO_2−Cbl(III) can then be reduced on the electrode giving rise to the irreversible wave at ca. −0.4V vs the Ag/AgCl reference electrode (II_{pc} in Figure 2), and the Cbl(II)−NO liberated by this reduction accounts for the reduction process on peak IV_{pc}.

plexed Cbl(II) which would grow as more Cbl(II)−NO is formed. In other words, the potential of peak I_{pc} shifts because of a change in the concentration ratio [Cbl(II)]/[Cbl(III)] in the Nernst expression when reaction takes place because of a surface catalytic process. As noted above, reaction 1a shows that nitrite is formed and it is capable of binding with Cbl(III).[13,16] Thus a fraction of the Cbl(III) could bind with nitrite, reaction 1c, which would give rise to peak II_{pc} observed in both Figures 1 and 2. A similar reduction peak is observed when nitrite is directly added to Cbl(III).

Once Cbl(II) is formed, if NO is available, a subsequent reaction of Cbl(II) with NO will occur according to reaction 1b. This reaction accounts for the current decrease on peak III_{pc} in Figure 2, and it is reasonable to assign peak IV_{pc} to the reduction of the Cbl(II)−NO complex. A similar process occurs in the CV experiment shown in Figure 1. It is found that, with an increase of NO concentration in solution, peak IV_{pc} shifts gradually to more negative potential displaying a 63 mV shift per decade of NO concentration (insert in Figure 2), which is consistent with a Nernst factor of 59 mV for an electrode process involving a 1:1 complex:

$$\text{Cbl(II)}-\text{NO} + e^- \rightleftharpoons \text{Cbl(I)}^- + \text{NO} \qquad (2)$$

From the reduction peak shift on the addition of NO, and NO concentration as calculated from partial pressure measurements, a binding constant $K_{1b} = 2 \times 10^6\ M^{-1}$ for Cbl(II) with NO was calculated from the equation $\Delta E = E_{IIIpc} - E_{IVpc} = 0.059 \log K_{1b} + 0.059 \log [NO]$, see insert in Figure 2. This value, however, is in error since it does not agree with our previously calculated value of $K_{1b} = 1 \times 10^8\ M^{-1}$ for reaction 1b from spectrophotometry in the bulk phase. This discrepancy is clearly accounted for by a more detailed consideration of the electrode process on peak IV_{pc} in the DPV of Cbl in the presence of NO where, in fact, a catalytic electrode process occurs. This catalytic process is clearly observed in Figure 1C where the current magnitude of peak III_{pc} is much larger than even the sum of peak currents around −1.0 V in Figure 1A and Figure 1B. The catalytic process consumes NO in the reaction layer at the electrode surface so that the reaction layer concentration of NO is actually much lower than the bulk concentration. A similar catalytic process was found by studying the effect of nitrite on the cobalamin electrochemistry.

The voltammetry of cob(III)alamin at pH 7 in the presence of nitrite, Figures 3 and 4, indicates that nitrite also reacts with the Cbl(III), Cbl(II), and Cbl(I) species. Peaks I and III are the reduction peaks of Cbl(III)/Cbl(II) and Cbl(II)/Cbl(I) couples. These peaks decrease as peak II and IV grow on gradual addition of nitrite to a Cbl(III) solution (Figure 4), and an enhanced current is found on peak IV. As previously discussed, a complexation reaction between nitrite and Cbl(III) can occur, and we assign the shift of the Cbl(III) reduction peak in the presence of nitrite to this reaction.

Furthermore, Figure 3C clearly shows an enhanced current at about −1.0 V which can be attributed to a catalytic reduction process. The fact that there is no catalytic current at pH 10 shows that this catalytic process is dependent on the availability of hydrogen ions. One effect of pH on the catalytic current would occur if the rate of the oxidation process of Cbl(I) with nitrite directly involves hydrogen ions. Another effect of pH is on the NO_2^-/NO equilibrium since it is well-known that nitrite can disproportionate to NO and nitrate:[19]

$$2NO_{aq} + NO_3^- + 2OH^- \rightleftharpoons 3NO_2^- + H_2O \qquad (3)$$

$$K = 1.1 \times 10^{20}\ M^{-2}$$

Thus low pH values will shift the equilibrium toward the formation of NO. An experiment similar to the one in Figure 3C but with a pH 4 buffer shows an even larger catalytic current than the pH 7 result for the same concentrations. Furthermore, the spectrophotometry results in Figure 5 show that NO can directly oxidize Cbl(I) at pH 7. Therefore, we conclude that both NO and nitrite can be catalytically reduced by cobalamin for a reduction process which involves cycling of its Co(II)/Co(I) oxidation states.

The CV results for Cbl(III) in nitrite solution in pH 7 and pH 10 show the same negative potential shift for the Cbl(III) reduction peak, and we attributed the shift of the peak to the binding of NO_2^-. When an equivalent amount of nitrite has been added to the Cbl(III) solution, peak I is completely gone and peak II at −0.35 V in Figure 4 has reached its maximum current, and we suggest that this peak is due to the reduction of the neutral NO_2−Cbl(III) species. This reduction is irreversible since the DPV peak current of peak II is more than half the magnitude of peak I and the full-width at half-height of peak II is much larger that that of peak I, both relative parameters indicating irreversibility. Note, however, that as the peak current changes, there is no potential shift for peak II. This result can be explained if both Cbl(III) and Cbl(II) can bind nitrite, because if only Cbl(III) binds with NO_2^-, the reduction peak potentials of NO_2−Cbl(III) will shift negative with increasing NO_2^- concentration according to the Nernst equation. Thus the electrochemical process for peak II is

$$NO_2-\text{Cbl(III)} + e^- \rightleftharpoons (NO_2-\text{Cbl(II)})^- \qquad (4)$$

Since the DPV (Figure 4) and CV (Figure 3C) curves show that peak II is not reversible (0.46 V peak separation in the CV), the potential difference between peaks I and II in Figure 4 cannot be used to calculate binding constants for the reactions

$$H_2O-\text{Cbl(III)}^+ + NO_2^- \rightleftharpoons NO_2-\text{Cbl(III)} + H_2O, \quad K_{1c} \qquad (1c)$$

$$\text{Cbl(II)} + NO_2^- \rightleftharpoons (NO_2-\text{Cbl(II)})^-, \quad K_5 \qquad (5)$$

On the other hand, a binding constant for Cbl(II) with nitrite, eq 5, can be estimated from the decrease in peak current on peak III_{pc}, and the calculated concentrations of nitrite based on the amount added. The result of the calculation gives $K_5 = 3.5 \times 10^2\ M^{-1}$. According to eq 1, nitrite is one of the products of the surface catalytic redox reaction of NO with

Cbl(III). In light of the large binding constant for $K_{1c} = 3 \times 10^5$ M^{-1}, Cbl(III) will bind nitrite to form NO_2−Cbl(III), which is reduced around −0.35 V to form the peak II in Figure 2 or Figure 4. However, the DPV peak II in Figure 2 is not as well defined as peak II in Figure 4 and shifts to more negative potentials as NO is added. This difference may be attributed to the formation of nitrite by a surface redox reaction and subsequent additional electrochemical steps which are not found when nitrite is in the bulk solution as it is in the experiment in Figure 4.

As nitrite is added, the Cbl(II) → Cbl(I) reduction peak III decreases in the DPV curves of Figure 4 and a new reduction peak IV grows in at −0.96 V. This value of peak potential is almost identical to the potential of peak IV_{pc} in Figure 2 (−0.98 V), which was attributed to the reduction process of Cbl(II)−NO + e^- → Cbl(I). In the present case, there is also a source of NO which could come from the reaction at the electrode surface of Cbl(I), formed on peak III at −0.82 V, with nitrite which is in the bulk solution according to the reaction

$$2H^+ + NO_2^- + Cbl(I)^- \rightleftharpoons Cbl(II)-NO + H_2O \quad (6)$$

An equilibrium constant for reaction 6 can be calculated from the redox potentials in aqueous acid solution for the NO_2^-/NO couple (1.20 V vs NHE) and the Cbl(II)/Cbl(I) couple (−0.61 V vs NHE), as well as the formation constant for Cbl(II)−NO (1.0×10^8 M^{-1}). At pH = 7, the equilibrium constant $K_6 = 3.8 \times 10^{24}$ M^{-1} is calculated which shows that if the kinetics are fast, nitrite will oxidize Cbl(I) at the electrode surface forming Cbl(II)−NO whose reduction process occurs at ca. −0.96 V. Thus we conclude that the catalytic current which occurs on peak IV in DPV curves (Figures 2 and 4) or around −1.0 V in the CV curves (Figures 1 and 3) comes from the same process which is the cycling of Cbl(II)−NO/Cbl(I) in the presence of either NO or nitrite. In the latter case the catalytically formed Cbl(II)−NO can only be reduced on the more negative reduction peak.

Analysis of Products in the Cbl(I) + NO Reaction. It is clear that cobalamin can catalyze the reduction of NO or nitrite in neutral to acidic solutions on cathodic peaks involving the Cbl(II)−NO/Cbl(I) couple. In order to investigate the reaction products of the Cbl(I) + NO reaction, mass spectrometry was utilized. We formed Cbl(I) as described in the UV−vis experiment in Figure 5 but used isotopic ^{15}NO obtained from $Na^{15}NO_2$,[26] and then sampled the gas headspace above the reaction solution with mass spectroscopy. The mass spectrum showed mass peaks at 30, $^{15}N_2$, and 46, $^{15}N_2O$, indicating the formation of both N_2 and N_2O (Supporting Information, Figure S4). It has been reported[27,28] that Cbl(I) can react with N_2O to form Cbl(II) and N_2. Thus it is quite reasonable that two two-electron reductions, reactions 7 and 8, are involved to form N_2O from NO and N_2 from N_2O by reaction with the supernucleophile Cbl(I):

$$2Cbl(I)^- + 2NO + 2H^+ \rightleftharpoons 2Cbl(II) + N_2O + H_2O \quad (7)$$

$$2Cbl(I)^- + N_2O + 2H^+ \rightleftharpoons 2Cbl(II) + N_2 + H_2O \quad (8)$$

These reactions are most likely occurring in a reaction layer at the electrode surface giving rise to the catalytic process which occurs in the presence of NO on peak IV_{pc} in the DPV curve of Figure 2. Again there is a cycling of the Cbl(II)−NO/Cbl(I) species in the electrochemical reaction layer. A similar catalytic NO reduction process was found on the reduction wave of cyanocobalamin where Cbl(I) is formed;[20] however, in this study a displacement of the 5,6-dimethylbenzimidazole by NO to form a NO−Cbl(III)−CN complex was invoked to explain the mechanism which seems suspect. In the case of the Co(2-TMPyP) porphyrin, a catalytic NO reduction process is found to be catalyzed by both $[Co^I(2\text{-}TMPyP)]^{3+}$ and $[Co^{II}(2\text{-}TMPyP)]^{4+}$ complexes, whereas cobalamin in the Co(II) oxidation state does not seem to catalyze NO reduction since there is no enhancement of the current on NO_2−Cbl(III) reduction waves, e.g., at −0.4 V in Figure 3C.

When the catalytic process occurs, the actual concentration of NO in the reaction layer will be lower than that in the bulk solution since it is being consumed. As previously mentioned, this deviation makes the calculated binding constant for Cbl(II)−NO obtained from the electrochemical experiments 2 orders of magnitude lower than the value found from UV−vis experiments where bulk NO concentrations were measured. However, the concentration gradients in the diffusion layer and the reaction layer for NO should vary as the concentration in the bulk solution varies with the addition of NO gas. Thus shifts in potential due to changes in [NO] which affect the mass action quotient of the Nernst equation can still be observed as in peak I_{pc} of Figure 2.

Physiological Consequences of Cobalamin and NO Reactions. The reaction of Cbl(II) with NO leading to the formation of Cbl(II)−NO can be used to explain many of the biological effects observed when cobalamin interacts with NO. First it should be noted that biological systems contain reducing agents which can rapidly form Cbl(II) from aquocob(III)alamin. Addition of aquocob(III)alamin to a biological system involving endogenous NO such as in smooth muscle cells can lead to the sequestering of NO by a reduction process which forms Cbl(II) which then binds NO inhibiting vascular relaxation. In addition, the inhibition of methionine synthase by NO[7−9,11] can be explained by its interaction with both Cbl(II) and Cbl(I).

It is well-known that, in methionine synthase, the strongly nucleophilic Cbl(I) can be methylated by N^5-methyltetrahydrogenfolate (N^5-methylTHF) to form methylcobalamin (Me−Cbl), for the transfer of a methyl group to homocys-

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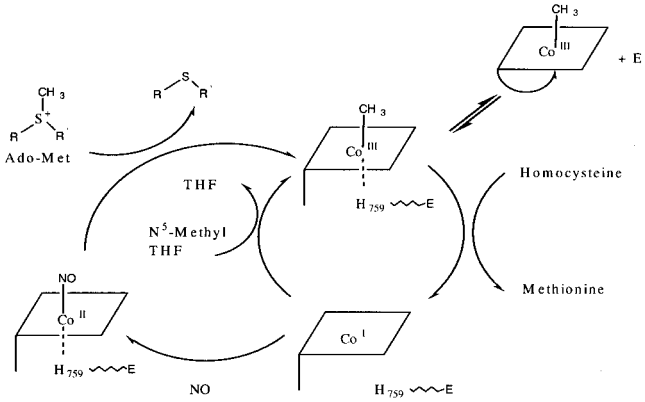
Scheme 1



teine (see Scheme 1).[29,30] However if Cbl(I) is oxidized to Cbl(II), the enzyme will lose activity and a pathway involving *S*-adenosylmethionine (Ado-Met) is necessary to remethylate Cbl(II) to Me−Cbl to recover the enzyme activity. Note that, in the enzyme, histidine-759 forms an axial bond to the cobalt of Me−Cbl in place of the 5,6-dimethybenzimidazole side chain.[30] An inactivation mechanism involving nitrous oxide (N_2O) has been proposed[31,32] where Cbl(I) reduces N_2O to release a potent oxidant, a putative hydroxyl radical or equivalent, that is capable of modifying sites proximal to the cobalamin to inhibit the function of the enzyme. Our results show that Cbl(I) can also reduce NO to N_2O, which could then inhibit methionine synthase by the above mechanism. However, if excess NO is available, its binding to Cbl(II) would also effect the inhibition process. Scheme 1 depicts the scheme proposed for inactivation, where NO causes the inactivation. Our catalytic current results suggest that nitrite could also cause this inhibition.

In this mechanism, NO oxidizes Cbl(I) to Cbl(II), which strongly binds excess NO to form Cbl(II)−NO, which can inhibit the enzyme activity in the methionine synthase mechanism (Scheme 1). An inhibition mechanism involving oxidation of Cbl(I) to Cbl(II) by NO has also recently been proposed;[11] however, NO binding to Cbl(II) was not considered important in this process.[11] According to our electrochemical results, NO binding shifts the reduction potential of Cbl(II)−NO about 0.24 V more negative than that of Cbl(II). This potential could be beyond the reduction ability of the *S*-adenosylmethionine pathway which involves a 1 equiv reduction, further inhibiting the functioning of the enzyme.

Finally, in addition to methyltransferases, there is a class of vitamin B_{12} dependent mutase enzymes, which includes methylmalonyl-CoA mutase found in mammals, that function by the homolytic cleavage of the 5′-deoxyadenosyl-cobalamin to give Cbl(II) and the 5′-deoxyadenosyl radical. Our results would suggest that NO could affect the functioning of these enzymes by forming Cbl(II)−NO, and this would have consequences for human health in the case of the methylmalonyl-CoA mutase enzyme.

Summary

Our results show that although the reduction of Cbl(III) by NO to form Cbl(II) is thermodynamically favorable in solution for pH greater than around 5, it only occurs as a surface-catalyzed process at the electrode surface in our electrochemical experiments. Furthermore, we conclude that this reaction would lead to the formation of Cbl(II)−NO and NO_2−Cbl(III). Reduction of Cbl(II)−NO forms Cbl(I) and NO, and in the presence of excess NO, the reaction of Cbl(I) with NO under our conditions produced N_2O and finally N_2. The electrochemistry also indicates that Cbl(I) can react with nitrite to give Cbl(II)−NO. These reactions yield catalytic waves in voltammetry experiments, and we are in the process of further examining the details of this catalytic electrochemical process in the presence of nitrite to measure rate constants and determine final products on exhaustive bulk electrolysis. The fact that cobalamin species and NO can both be present in the same biological system makes this chemistry relevant to physiological processes. Indeed, the oxidation of Cbl(I) by either NO or NO_2^- to give Cbl(II)−NO provides a mechanism for the inhibition of methionine synthase by NO systems.

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Supporting Information Available: The DPV of Cbl(III) and NO at pH 7 on a Ag electrode (Figure S1) and the CV of Cbl(III) and nitrite at pH 10 on a glassy carbon electrode (Figure S2). SERS of Cbl(III) and NO (Figure S3) and the mass spectrum of the products of the Cbl(I) and NO reaction (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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