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Nitrite Impurities Are Responsible for the Reaction Observed between Vitamin B_{12} and Nitric Oxide in Acidic Aqueous Solution

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The reaction between aquacobalamin, Cbl(H_2O), and NO was studied at low pH. As previously reported, the final product of the reaction is the same as that obtained in the reaction of NO and reduced Cbl(H_2O), viz. Cbl(NO^-). Nevertheless, this reductive nitrosylation is preceded by a faster reaction (accompanied by small absorbance changes) that depends on the HNO_2 concentration but not on the NO concentration. Kinetic and UV–vis spectroscopic data show that Cbl(NO_2^-) is generated during this reaction. Spectroscopic data show that the dimethylbenzimidazole group trans to the NO_2^- ligand is protonated and partially dechelated at pH 1, by which a reaction with NO is induced. DFT calculations were performed to compare the ability of NO and NO_2^- to bind to cobalamin and their influence on the stability of the dimethylbenzimidazole group. The reductive nitrosylation reaction shows a quadratic dependence on the HNO_2 concentration and an inverse dependence on the NO concentration. It also strongly depends on pH and is no longer observed at pH > 4. On the basis of earlier work performed on a series of Co(III) porphyrins, a mechanism is proposed that can quantitatively account for the HNO_2 and NO dependencies. The reductive nitrosylation reaction is practically dominated by a *back reaction*, i.e., the reaction between Cbl(NO^-) and HNO_2, which accounts for the strange NO and HNO_2 concentration dependencies observed.

Introduction

Nitric oxide (NO) plays important roles in mammalian biochemical processes such as cytotoxic response, vasodilatation, neurotransmission, etc.[1] Vitamin B_{12} has been claimed in numerous investigations to interact with NO in vivo and to regulate its biological activity.[2] Earlier work in our laboratory, however, clearly showed that NO does not react with aquacobalamin, Cbl(H_2O), at pH 7 at all and that the observed reactions could be ascribed to the reaction with nitrite impurities always present in aqueous solutions of NO.[3] In later work, it was shown that the reduced form of Cbl indeed reacts very effectively with NO to form a $Co^{II}-NO^{\bullet}$ (or formally $Co^{III}-NO^-$) complex with a binding constant of $7.4 \times 10^8\ M^{-1}$ (25 °C, pH 7.4).[4] These observations were supported by DFT calculations which clearly showed that cob(III)alamin cannot bind NO in aqueous solution but that reduced cob(II)alamin can.[5] More recently, it was reported that Cbl(H_2O) can indeed react with NO but only at low pH.[6] Under these conditions, the final product is the same as the one obtained when reduced Cbl reacts with NO; in other words, a reductive nitrosylation reaction occurs. The

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authors proposed that under the conditions selected, protonation of the dimethylbenzimidazole group in Cbl(H_2O) is favored, which induces the rate-determining dechelation of this group, followed by a fast reaction with NO. For this reason, the reaction seemed to be independent of the NO concentration.

In the meantime, we have studied the interaction of NO with a series of cobalt(III) porphyrins, Co(P), as a function of pH and reported evidence for a reductive nitrosylation reaction that produces $Co^{II}(P)NO^{\bullet}$ (or formally $Co^{III}(P)NO^{-}$).[7] It was found that nitrite plays a major role in this reaction since it can compete very effectively with NO and, at pH > 3, induces an alternative reaction pathway based on the formation of an intermediate [$Co^{III}(P)(NO_2^-)$] complex. The proposed mechanism for these reactions is different from that proposed for the reductive nitrosylation of Fe^{III} porphyrins, where general base catalysis was found.[8]

We have now extended this work and, as announced before,[7] revisited the claimed interaction of NO with Cbl(H_2O) at low pH[6] and report a detailed mechanistic study of this system. We present unequivocal evidence that NO does not interact with Cbl(H_2O) at low pH at all but that the observed reactions are induced by traces of nitrite impurities present in aqueous solutions of NO under the conditions selected. Our mechanistic conclusions are supported by DFT calculations.

Experimental Section

Materials. Fresh samples of hydroxocobalamin acetate were purchased from Aldrich. NO 99.9% from Alpha Caz was purified from higher nitrogen oxides by passage through an Ascarite II (Aldrich) column. $NaNO_2$ was obtained from Merck. The remaining chemicals were of analytical grade and used without further purification.

General Methods. All solutions were prepared using distilled and purified water (Milli-Q system), deoxygenated upon N_2-saturation in Schlenk tubes, handled with gastight syringes and protected from light. Acetate (pH 4−5) and citrate (pH 3−4) buffers were used at a concentration of 0.01 M to control the pH. $HClO_4$ solutions were used at pH lower than 3. The ionic strength was adjusted with $NaClO_4$ to 0.1 M. NO-saturated solutions were prepared by slowly bubbling NO through deoxygenated buffer or acid solutions. The NO concentration of such solutions is already known from our earlier work on the subject, viz. 1.8 mM at 25 °C.[7] The concentration of the NO_2^-/HNO_2 impurities was determined as described before.[7] The NO solutions were diluted to reach the desired NO concentration level. Aquacobalamin, Cbl(H_2O), solutions were subsequently prepared by dissolving hydroxocobalamin in buffer or $HClO_4$ solutions. To avoid the loss of HNO_2 during saturation with N_2 and/or NO at pH lower than 3, the solutions were prepared by dissolving the necessary amount of $NaNO_2$ in water and were subsequently mixed with a double concentrated acid and ionic strength solution. pH measurements were performed with a WTW inoLab level 1 pH meter at room temperature. All the reported experiments were performed at 25.0 ± 0.1 °C.

Thermodynamic Experiments. Reaction of Cbl(H_2O) with HNO_2/NO_2^-. Aliquots of a $NaNO_2$ solution were added to 20 mL of a solution 0.1 mM Cbl(H_2O), pH 1−5. The spectral changes were monitored on a Varian Cary 5G spectrophotometer. At pH 1.0, the equilibrium constant was obtained from a nonlinear analysis of absorbance changes at 250, 318, 352, 370, 460, 505, and 543 nm using the Origin 7 program.[9] The equation employed was the same as that used in our earlier work on the Co(III) porphyrins.[7] The spectra obtained at high enough NO_2^- concentration as a function of pH (the spectra did not change appreciably with [NO_2^-]) were used to estimate the equilibrium constant for the protonation of the dimethylbenzimidazole group.

Reductive Nitrosylation. The necessary amount of $NaNO_2$ was weighed inside a 1 cm optical path cuvette (final concentration: 1−5 mM; in this way, the final product was mainly Cbl(NO_2^-), i.e., only small amounts of Cbl(H_2O) and Cbl(NO_2^-)$_2$ were present), which after purging with N_2, was filled with a Cbl(H_2O) solution (0.1 mM) saturated with NO. When the reaction was complete, the absorbances (A) at 534, 517, 470, 448, 352, and 316 nm were analyzed as a function of the [HNO_2], using eq 1. This equation was derived using mass balances for HNO_2, NO, and Cbl(H_2O), the expression of A as a function of the concentrations, and considering that [Cbl(H_2O)] ≪ [HNO_2] and [NO]. A better fit of the data was obtained using a square dependence on [HNO_2] and [NO].

$$K = \frac{\left(1 - \frac{A - A_o}{A_\infty - A_o}\right)[HNO_2]^2}{\frac{A - A_o}{A_\infty - A_o}[NO]^2} \qquad (1)$$

In eq 1, A_o is the absorbance of the Cbl(H_2O) solution saturated with NO at a certain wavelength without addition of HNO_2, A_∞ is the absorbance extrapolated to a very high [HNO_2], and K is defined in eq 10. A_o and [NO] were taken as fixed variables. The reported K value is the average of three independent experiments. A similar procedure involving changing [NO] at [HNO_2] = 0.5 mM was attempted, but the plots of A vs [NO] did not reach a saturation behavior in the [NO] range 0−1.8 mM, with the result that no accurate K values could be obtained in this way. Nevertheless, a better fit of the data could be reached by using a square dependence on [NO].

Kinetic Experiments. Stopped-flow measurements were performed on a SX-18 MV stopped-flow spectrophotometer from Applied Photophysics. The complex solutions (0.02−0.1 mM) were rapidly mixed with an equal volume of a NO (0−1.8 mM) and/or $NaNO_2$ solution (0−4 mM). Some experiments were also performed by mixing a Cbl(H_2O)/NO solution ([NO] = 0.23−1.8 mM, 0.2 M $HClO_4$), with a $NaNO_2$ solution (1.0, 2.6 and 4.0 mM). Kinetic traces were usually monitored at 316, 352, 448, and 534 nm. To study the reaction with HNO_2 in the absence of NO, kinetic traces were monitored at 318, 352, 370, 460, and 543 nm. Traces were fitted to single or double exponential functions with the SX-18 MV program package. Most of the experiments were done in duplicate, the reported rate constants are the averages of at least four kinetic runs at the different wavelengths, and the reported errors are the standard deviation of the data. The slowest reactions ($k_{obs} < 1 \times 10^{-3}$ s^{-1}) were studied on the Varian Cary 5G spectrophotometer. Nonlinear fits of the plots of k_{obs} vs [HNO_2] (eq 9) were performed with the Origin 7 program.[9]

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To obtain complete spectral changes during the reductive nitrosylation reaction (including the first faster reaction step), a 0.10 mM Cbl(H_2O) solution was placed in one of the compartments of a tandem cuvette, with a saturated NO solution in the other compartment. After the solution was thermally equilibrated and mixed, UV−vis spectra were recorded on a Hewlett-Packard 8452A diode-array spectrophotometer with a cycle time of 1.5 s over the first 100 s of the reaction.

Computational Methods. Geometries were fully optimized at the B3LYP[10] level of theory with a 6-31G(d)[11] basis set using the Gaussian 03 program package.[12] All structures were characterized as true minima by calculating their normal vibrations within the harmonic approximation.

Results and Discussion

In a series of preliminary experiments, we followed the reaction of Cbl(H_2O) with NO at low pH with a spectrophotometer. We were able to reproduce in a qualitative way the results reported in the literature.[6] Upon saturation with NO at pH 1.0, the final product is the same as the one obtained through reaction of NO with reduced Cbl(H_2O) at pH < 3, viz. Cbl(NO^-). Nevertheless, the data were not very reproducible and sometimes the kinetic traces showed a more complex behavior than for a clean first-order process.

Stopped-flow experiments clearly showed that reductive nitrosylation of Cbl(H_2O) is preceded by a faster reaction step, which is accompanied by small absorbance changes and is over within 1 min at room temperature. Figure 1a shows the spectral changes observed with a diode-array spectrophotometer. Figure 1b shows the kinetic traces, from which the formation of an intermediate is clearly seen. The spectrum of the sample after 90 s (see Figure 1c) is very similar to the one observed in the direct reaction between HNO_2 and Cbl(H_2O) at pH 1 in the absence of NO ([HNO_2] = 0.1 mM). Since it is well known that NO_2^- and HNO_2 are common impurities in aqueous NO solutions,[3] we performed a systematic study of the observed reaction steps as a function of the NO and NO_2^- concentrations, as well as of pH.

Reaction with HNO_2/NO_2^-. Cbl(H_2O) reacts with HNO_2 according to eq 2. At pH 1.0 and under an excess of HNO_2, a first-order process is observed and the plot of the observed rate constant (k_{obs}) vs [HNO_2] (Figure 2) shows a linear dependence in the concentration range 0−2 mM, from which the slope (k_2) equals 106 ± 5 M^{-1} s^{-1} and the intercept (k_{-2}) equals (3.3 ± 0.6) × 10^{-2} s^{-1}.

The order of magnitude of k_2, when compared with published data for the reaction with NO_2^- at pH 5,[3] clearly indicates that HNO_2 is the nucleophile that participates in reaction 2, i.e., traces of NO_2^- do not play a significant role under such conditions. At HNO_2 concentrations higher than 5 mM, the plot of Figure 2 shows deviations from linearity

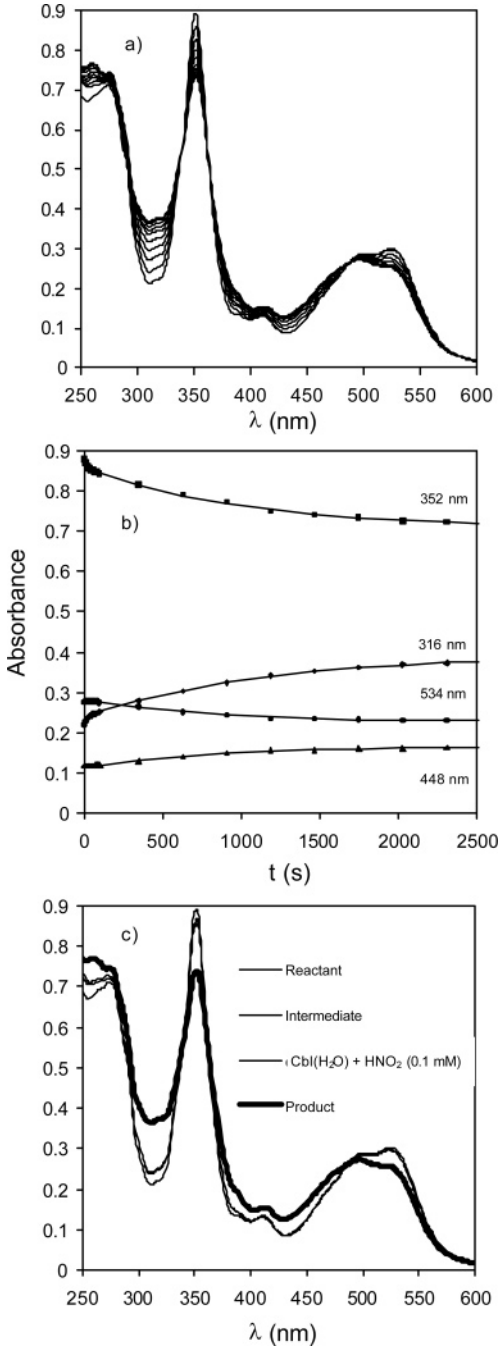

Figure 1. (a) UV−vis spectral changes recorded during the reaction of 5.0 × 10^{-5} M Cbl(H_2O) with 0.90 mM NO: pH = 1.0, I = 0.1 M ($HClO_4$), T = 25.0 °C. (b) Kinetic traces, $k_{1(obs)}$ = 0.048 s^{-1}, $k_{2(obs)}$ = 1.0 × 10^{-3} s^{-1}. (c) Spectra of the reactant, the reaction mixture after 90 s, the product of the reaction, and of a 5.0 × 10^{-5} M Cbl(H_2O) and 10^{-4} M HNO_2 solution.

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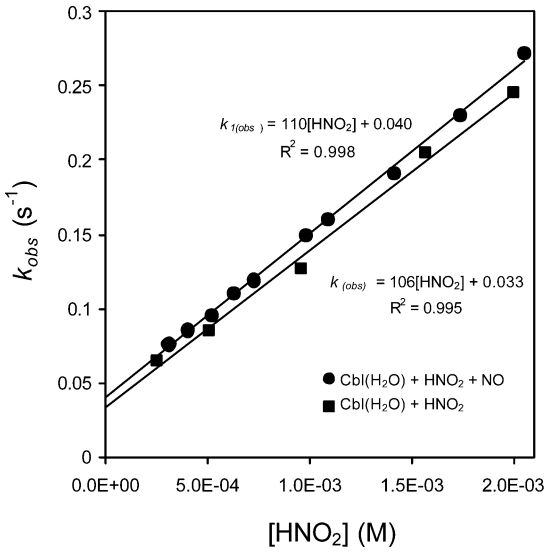

Figure 2. $[HNO_2]$ dependence of the observed rate constant for the first reaction ($k_{1(obs)}$) in the presence of NO and for the reaction with HNO_2 in the absence of NO (k_{obs}). Experimental conditions: $[NO] = 9.0 \times 10^{-4}$ M, pH = 1.0, I = 0.1 M ($HClO_4$), T = 25.0 °C. $[Cbl(H_2O)] = 0.01-0.05$ mM.

which are ascribed to the coordination of a second HNO_2 molecule.

$$Cbl(H_2O) + HNO_2 \; \Omega \; Cbl(NO_2^-) + H_3O^+ \quad k_2, k_{-2}, K_2 \quad (2)$$

Direct measurements of the apparent equilibrium constant for the coordination of HNO_2 at pH 1.0 afforded a value of $(2.8 \pm 0.8) \times 10^3$ M^{-1}, which is in close agreement with the value of $(3.7 \pm 0.7) \times 10^3$ M^{-1} estimated from the kinetic data, i.e., the ratio of k_2 and k_{-2} from Figure 2. Spectra recorded at $[HNO_2] > 5$ mM show evidence for the coordination of a second HNO_2 molecule.

Similar experiments were done at pH 1−5; the equilibrium constants were analyzed only qualitatively, and the values were found to increase with increasing pH as expected. Comparison of the spectra at sufficiently high NO_2^-/HNO_2 concentrations (independent of $[NO_2^-]$, but at < 5 mM, i.e., $Cbl(NO_2^-)$ is the predominant species) as a function of pH gave evidence for the protonation of the dimethylbenzimidazole group in the $Cbl(NO_2^-)$ complex (vide infra). According to these data, we estimated the pK_a of the protonated form of the $Cbl(NO_2^-)$ complex to be around 1. pK_a values have been reported for a large number of cobalamins and range from 4.0 for Cbl(*n*-heptyl) to −2.5 for $Cbl(H_2O)$.[13] As intermediate cases, there are values of 0.1 for Cbl(CN) and 1.44 for $Cbl(CF_3)$, from which it follows that our assignment for the value of $Cbl(NO_2^-)$ is very reasonable.

First Reaction Step in the Presence of NO: Reaction with HNO_2. Experiments at different NO concentrations (0.23−0.90 mM, $[HNO_2]$ = 0.4 and 1.0 mM, pH = 1.0) showed that the first reaction step does not depend significantly on the NO concentration. Moreover, Figure 2 shows the values for the observed rate constant of the first reaction

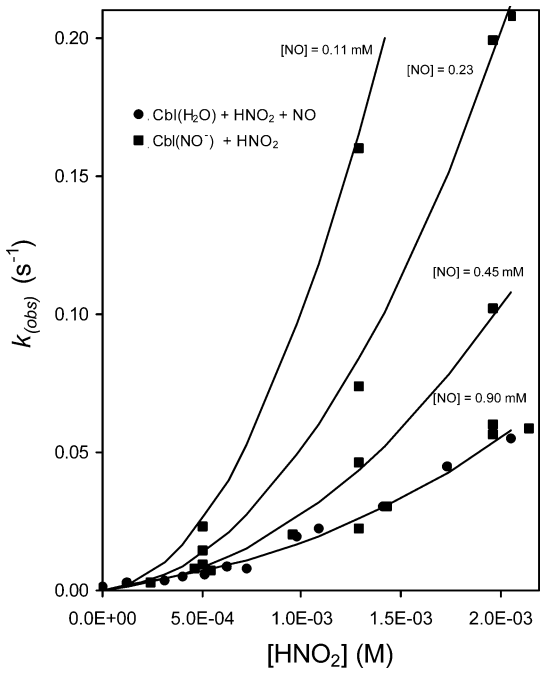

Figure 3. $[HNO_2]$ dependence of the observed rate constant for the second reaction step ($k_{2(obs)}$) and of the reaction between $Cbl(NO^-)$ and HNO_2 (k_{obs}). The solid line corresponds to the simulated data according to eq 9 using K_2 = 2800 M^{-1} (fixed variable), $k_{NO} = 7 \pm 2$ M^{-1} s^{-1} and $K = 0.6 \pm 0.2$. Experimental conditions: pH = 1.0, I = 0.1 M ($HClO_4$), T = 25.0 °C. $[Cbl(H_2O)] = 0.01-0.05$ mM.

($k_{1(obs)}$) as a function of the HNO_2 concentration, from which a slope of 110 ± 2 M^{-1} s^{-1} and an intercept of $(4.0 \pm 0.2) \times 10^{-2}$ s^{-1} were obtained. These values are very similar to the ones observed for the reaction of NO_2^- with $Cbl(H_2O)$ in the absence of NO. The small shift in the intercept can be ascribed to HNO_2 impurities present in the NO solutions, ca. 0.1 mM HNO_2 for a 0.90 mM NO solution.[3,7]

The pH dependence of this reaction shows a clear correlation with the pK_a of HNO_2 of 3.3,[14] since the values of $k_{1(obs)}$ are almost constant at pH < 3 (ca. 0.1 s^{-1}, [NO] = 0.9 mM, $[HNO_2]$ = 0.5 mM) but increase at pH 3−4 to around 0.4 s^{-1} at pH 5.0 (see inset in Figure 4), in agreement with earlier reports on the coordination of NO_2^- to $Cbl(H_2O)$ at this pH.[3]

All the kinetic data, together with the UV−vis characterization of the intermediate, strongly suggest that HNO_2/NO_2^- impurities coordinate to $Cbl(H_2O)$ before any reaction with NO can take place, such that the $Cbl(NO_2^-)$ complex is the one that actually reacts with NO in the subsequent reaction.

Second Reaction Step in the Presence of NO: Reductive Nitrosylation. The second reaction step corresponds to reductive nitrosylation and correlates with that previously found for the reaction of $Cbl(H_2O)$ with NO at low pH.[6] On keeping the HNO_2 concentration constant at fixed levels (0.4 and 1.0 mM at pH 1.0), we surprisingly found that the rate constant ($k_{2(obs)}$) increases as the NO concentration decreases (see Figure 3). The yield of the $Cbl(NO^-)$ complex decreases dramatically on decreasing the NO concentration, which made it impossible to obtain quantitative information. This

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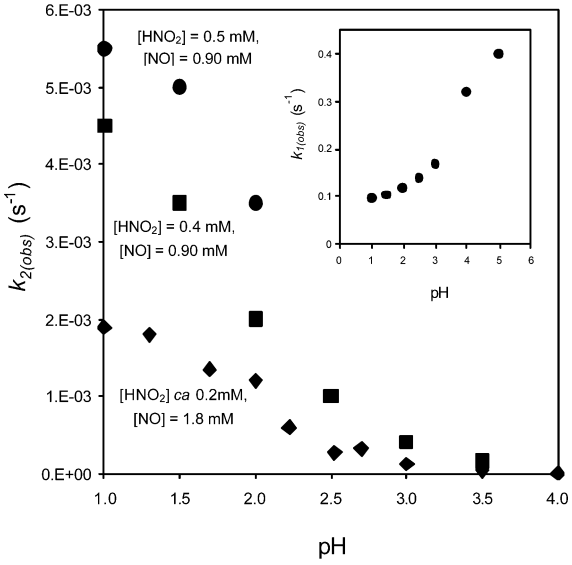

Figure 4. Plot of $k_{2(obs)}$ vs pH at different [HNO_2] and [NO]. Inset: Plot of $k_{1(obs)}$ vs pH ([HNO_2] = 0.5 mM, [NO] = 0.9 mM). Experimental conditions: T = 25.0 °C, I = 0.1 M, [Cbl(H_2O)] = 0.01−0.05 mM.

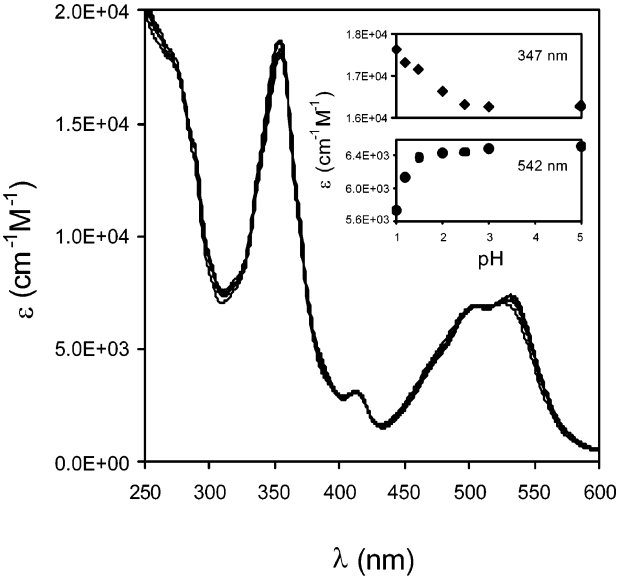

Figure 5. Spectra of the Cbl(NO_2^-) complex at pH 1−5. Inset: Molar absorbances at selected wavelengths. Experimental conditions: T = 25.0 °C, I = 0.1 M, [Cbl(H_2O)] = 0.1 mM.

reaction strongly depends on the HNO_2 concentration. Figure 3 shows the plot of the observed rate constant for the second reaction ($k_{2(obs)}$) as a function of the HNO_2 concentration. A nonlinear, quadratic dependence is observed. Although the $k_{2(obs)}$ values increase with increasing HNO_2 concentration, the yield of the reductive nitrosylation product decreases on increasing the HNO_2 concentration. At [HNO_2] > 2 mM, the second reaction step is no longer observable ([NO] = 0.90 mM, pH 1.0).

This reaction step is also very pH dependent. The values of $k_{2(obs)}$ and the yield of the final product increase on decreasing the pH (Figure 4). The reaction is not observed anymore at pH > 4. These findings are also in agreement with the previous report.[6] The pH dependence correlates with the spectral changes of the Cbl(NO_2^-) complex as a function of pH (see Figure 5). We interpret these changes in terms of the protonation of the dimethylbenzimidazole group in the Cbl(NO_2^-) complex. We propose that the active species toward NO is protonated Cbl(NO_2^-) and not protonated Cbl(H_2O), as was previously proposed,[6] since the pK_a of protonated Cbl(H_2O) is around −2.5 such that the concentration of protonated Cbl(H_2O) must be extremely low at pH 1.[13]

The HNO_2 concentration dependence of $k_{2(obs)}$ and the effect of the HNO_2 concentration on the yield of the reductive nitrosylation reaction suggest that a second HNO_2 molecule may be competing with NO or that HNO_2 can react with the product to regenerate either Cbl(H_2O) or Cbl(NO_2^-), thus induces a *back reaction*. We have no evidence for the coordination of the second HNO_2 molecule at concentrations lower than 5 mM, so the most likely possibility is that HNO_2 oxidizes the reductive nitrosylation product back to Cbl(H_2O) or Cbl(NO_2^-). For this reason, we also studied the reaction between Cbl(NO^-) and an excess of HNO_2 at pH 1.0.

Back Reaction between Cbl(NO^-) and HNO_2. Under an excess of HNO_2, a clean first-order process is observed for the back reaction. The k_{obs} values are not significantly different from the $k_{2(obs)}$ values obtained at the same NO and HNO_2 concentrations (see Figure 3). The HNO_2 concentration dependence of this reaction also shows a quadratic relationship. This type of experiment has the advantage that good spectral changes are observed, especially at high HNO_2 concentrations, where reductive nitrosylation shows very small absorbance changes.

We performed experiments at different NO concentrations, keeping the HNO_2 concentration constant (0.5, 1.3, and 2.0 mM). We observed that k_{obs} increases with decreasing NO concentration in the same way as the reductive nitrosylation reaction does (see Figure 3). For instance, at [HNO_2] = 2.0 mM and [NO] = 0.90 mM, $k_{2(obs)}$ (reductive nitrosylation) and k_{obs} (*back reaction*) have the values $(5.5 \pm 0.7) \times 10^{-2}$ s^{-1} and $(5.8 \pm 0.2) \times 10^{-2}$ s^{-1}, respectively. Reducing [NO] to 0.45, 0.23, or 0.11 mM, increases k_{obs} to $(1.02 \pm 0.07) \times 10^{-1}$, $(2.05 \pm 0.05) \times 10^{-1}$, and $(4.2 \pm 0.5) \times 10^{-1}$ s^{-1}, respectively.

The final product of this back reaction must be Cbl(NO_2^-) in equilibrium with Cbl(H_2O) since the overall reaction is completely reversible and no subsequent reactions were observed. This type of reaction was also observed for the [Co(TPPS)(NO^-)]$^{4-}$ complex (TPPS = tetra-sulfonatophenylporphyrin).[7] In this case, a quadratic [HNO_2] concentration dependence was also observed. Nevertheless, the reaction between NO and the [Co(TPPS)(H_2O)$_2$]$^{3-}$ complex is not influenced by the *back reaction*. We believe that [Co(TPPS)(H_2O)(NO_2^-)]$^{4-}$ is the product in the latter case, as in the case of Cbl(H_2O). It follows that Cbl(H_2O) does not react with NO even at low pH; it is the Cbl(NO_2^-) complex that reacts with NO! This seems to be rather different in the case of the [Co(TPPS)(H_2O)$_2$]$^{3-}$ complex.[7]

Theoretical Calculations. Our earlier DFT calculations[5] showed that Cbl(H_2O) cannot bind NO in aqueous solution, and the results of the present study suggest that Cbl(H_2O) can bind nitrite at low pH, which then in turn results in

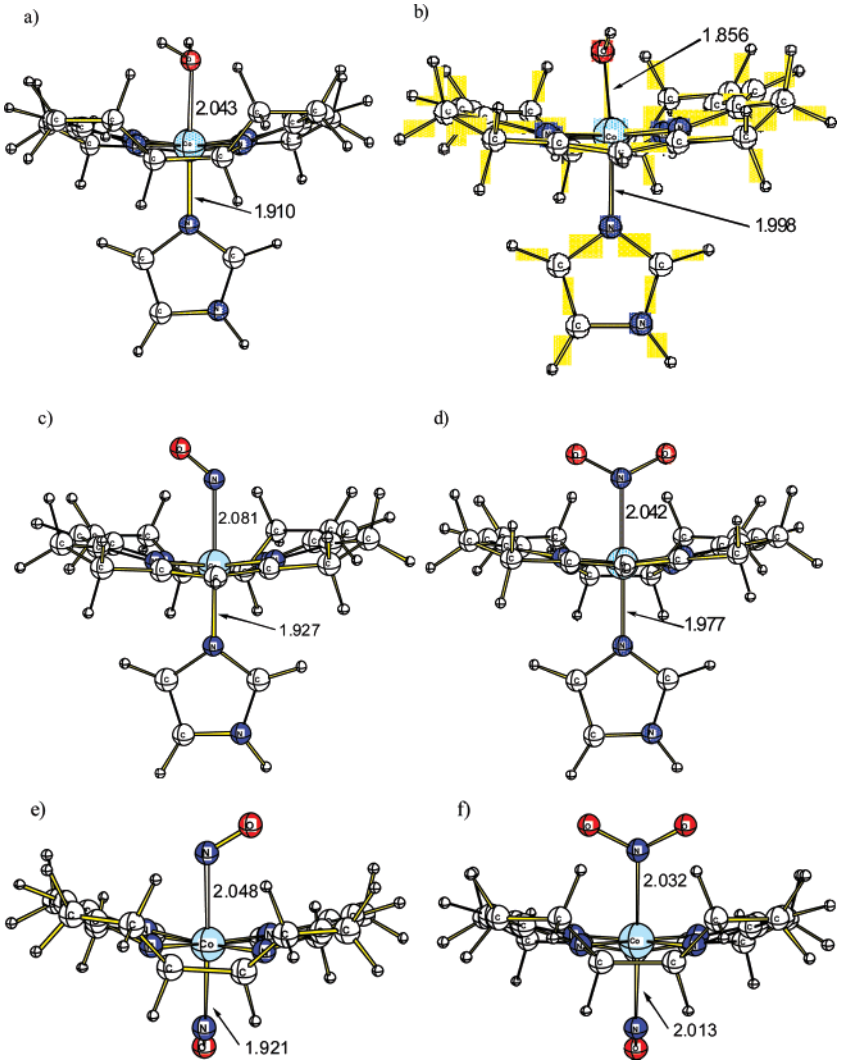

Figure 6. Summary of the optimized B3LYP/6-31G* structures of (a) Cbl(H_2O), (b) Cbl(OH^-), (c) Cbl(NO), (d) Cbl(NO_2^-), (e) Cbl$(NO)_2$, and (f) Cbl(NO_2^-)(NO). All bond lengths are quoted in Å.

protonation and dechelation of the dimethylbenzimidazole group. The structures for the relevant complexes shown in Figure 6 reveal that, on deprotonation of coordinated water in Cbl(H_2O) to form Cbl(OH^-), significant lengthening of the Co−N(Im) bond occurs. A similar situation is seen in the formation of Cbl(NO_2^-) as compared to the formation of Cbl(NO). Also, in the structures of Cbl$(NO)_2$ and Cbl(NO_2^-)(NO) it is clearly seen how nitrite has a trans influence on the coordinated NO. Thus, in the case of Cbl(H_2O), the binding of nitrite causes a significant lengthening of the Co−N(Im) bond, which will enable its protonation and dechelation at low pH, something that is not possible for the Cbl(H_2O) complex. We have shown before that energies calculated for ligand exchange processes strongly depend on the basis set and level of theory employed.[5] Currently, we are performing a comparative study for different DFT functions to estimate exchange energies for Cbl(II) and Cbl(III) systems.

Proposed Mechanism. According to the kinetic data and our earlier work on the reductive nitrosylation of water soluble Co(III) porphyrins,[7] we propose the mechanism outlined in reactions 3−7. Here we consider HNO_2 as the predominant species since most of the work was done in acidic medium (pH 1).

$$\mathrm{Cbl} + \mathrm{HNO_2} \rightleftarrows \mathrm{HCbl(NO_2^-)^*} \quad K_3 \tag{3}$$

$$\mathrm{HCbl(NO_2^-)^* + NO} \rightleftarrows \mathrm{HCbl(NO_2^-)(NO)} \quad K_4,k_4,k_{-4} \tag{4}$$

$$\mathrm{HCbl(NO_2^-)(NO) + H^+} \rightleftarrows \mathrm{HCbl(NO) + HNO_2} \quad K_5,k_5,k_{-5} \tag{5}$$

$$\mathrm{HCbl(NO) + NO} \rightleftarrows \mathrm{HCbl(NO)_2} \quad K_6,k_6,k_{-6} \tag{6}$$

$$\mathrm{HCbl(NO)_2 + H_2O} \rightleftarrows \mathrm{HCbl(NO^-) + HNO_2 + H^+} \quad K_7,k_7,k_{-7} \tag{7}$$

In this reaction sequence, HCbl(NO_2^-)* presents the nitrite complex in which the dimethylbenzimidazole group is protonated and not coordinated to the metal center trans to the NO_2^- group. The dissociation of the dimethylbenzimidazole group following protonation was found to be very fast in related complexes.[13] Moreover, a preliminary value for this reaction in the Cbl(NO_2^-) complex is ca. 0.3 s^{-1}

($[HNO_2] = 4.0$ mM, final pH 1.0), which is much faster than any of the observed reactions. Thus, reductive nitrosylation cannot be controlled by this reaction step, as previously suggested.[6] In the subsequent reaction steps, NO rapidly binds to the vacant coordination site, nitrite is protonated and released as HNO_2, a second NO molecule binds to form $HCbl(NO)_2$, which then induces the reductive nitrosylation process to form $HCbl(NO^-)$. All the cobalamin species in the reaction sequence described above are suggested to contain a dechelated protonated dimethylbenzimidazole group in acidic medium. This also applies to the final product $HCbl(NO^-)$, which is in good agreement with a pK_a value of 5.1 reported for this complex in the literature.[4] On the basis that reaction 4 is a rapid pre-equilibrium, the intermediates $HCbl(NO_2^-)(NO)$, $HCbl(NO)$, and $HCbl(NO)_2$ are in stationary states, and that $k_{-4} \gg k_5$, $k_6 \gg k_{-5}$ and $k_7 \gg k_{-6}$, eq 8 was derived for the formation of $HCbl(NO^-)$.

$$k_{obs} = \frac{K_3[HNO_2]}{1 + K_3[HNO_2]} K_4 k_5 [NO][H^+] + \frac{k_{-5}[H^+][HNO_2]^2}{K_6 K_7 [NO]} \quad (8)$$

For the particular case of pH = 1.0, defining $K = K_4K_5K_6K_7$ and $K_4k_5[H^+] = k_{NO}$, and since $K_3 = K_2$, we obtain eq 9.

$$k_{obs} = \frac{K_2[HNO_2]}{1 + K_2[HNO_2]} k_{NO}[NO] + \frac{k_{NO}[HNO_2]^2}{K[NO]} \quad (9)$$

In fact, K is the equilibrium constant for the overall reaction, which can be defined as in eq 10.

$$Cbl(NO_2^-) + 2NO + H_2O \rightleftarrows Cbl(NO^-) + 2HNO_2 \quad K \quad (10)$$

Equation 9 was used to fit the data for $k_{2(obs)}$ for reductive nitrosylation and $k_{(obs)}$ for the reaction between $Cbl(NO^-)$ and HNO_2 ($[NO] = 0.9$ mM). From this analysis, $k_{NO} = 7 \pm 2$ M^{-1} s^{-1} and $K = 0.6 \pm 0.2$ were obtained (see Figure 3). With the same equation and the calculated rate parameters, the curves at different NO concentrations (0.11, 0.23, 0.45, and 0.90 mM) were simulated. The agreement between the simulation and the experimental data can be seen in Figure 3. It can be observed that, at $[HNO_2] < 0.2$ mM, the $k_{2(obs)}$ values are practically independent of [NO], since the two terms in eq 9 compensate each other and apparently cancel out. This may account for the earlier finding that reductive nitrosylation was independent of the NO concentration.[6]

We determined $K = 0.57 \pm 0.15$ from three independent experiments (see Figure 7), which is in very good agreement with the kinetic value determined from Figure 3. The assumption that $K_3 = K_2$ implicates that the fraction of the $Cbl(NO_2^-)$ complex in the protonated form, in which the dimethylbenzimidazole group is dechelated, is close to 1. Probably, a better approximation would be to consider this fraction to be close to 0.5 or 0.7. In the case of the latter approximation, k_{NO} and K would have the values 10 ± 2 M^{-1} s^{-1} and 0.9 ± 0.3, respectively.

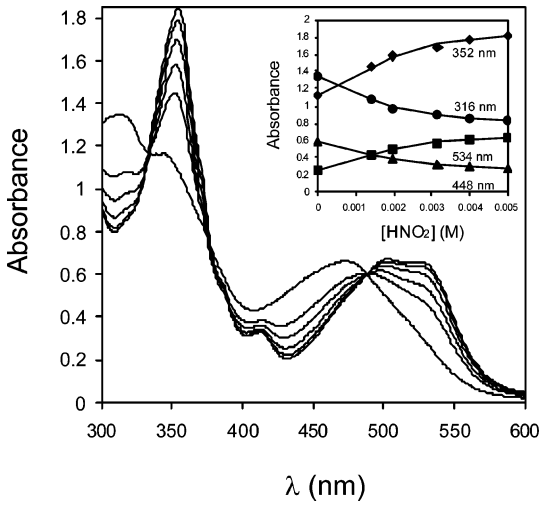

Figure 7. Spectra of a 1.0×10^{-4} M $Cbl(H_2O)$ solution saturated with NO (1.8 mM) at different HNO_2 concentrations. Inset: selected absorbances vs $[HNO_2]$, solid line: fit according to eq 1 using $K = 0.72 \pm 0.06$. Experimental conditions: pH = 1.0, $T = 25.0$ °C, $I = 0.1$ M ($HClO_4$)

In this study, we propose that $k_6 \gg k_{-5}$ in order to account for the observed kinetic data. In contrast, in our previous report on the interaction of Co(III) porphyrins with NO,[6] we proposed the opposite relationship to account for the instability of the Co(P)(NO) complex in the presence of NO_2^- at pH 5. The different pH conditions are suggested to account for this different behavior.

Finally, it can be seen in Figures 4 and 5 that quantitative agreement is not observed. A possible reason could be that in eqs 8 and 9 several terms depend on pH not just as a result of the protonation of the dimethylbenzimidazole group, which could be included in k_{NO}. For instance, the rate of reverse reaction 7 should also increase at low pH, and reaction 5 is also pH dependent as many rate and equilibrium constants. For this reason, we can at present only offer a qualitative description of the reductive nitrosylation process as a function of pH.

Conclusions

We have performed a detailed study of the reaction of aquacobalamin, $Cbl(H_2O)$, with NO as a function of pH, and NO and HNO_2 concentrations. We found that an overall reductive nitrosylation reaction is operative, as reported in the literature.[6] Nevertheless this reaction is preceded by a faster reaction that depends only on the HNO_2 concentration. In this way, we can propose that the reactive species toward NO is not $Cbl(H_2O)$ but the $HCbl(NO_2^-)$ complex in which the dimethylbenzimidazole group is protonated and dechelated. DFT calculations support this suggestion.

We propose a reaction mechanism based on our earlier findings for the reductive nitrosylation of Co(III) porphyrins.[7] On the basis of this mechanism, eq 9 could be derived, which can describe the observed kinetic data quantitatively. The reductive nitrosylation reaction is practically dominated by its *back reaction*, which is the reason for the quadratic $[HNO_2]$ and inverse [NO] dependences shown in Figure 3.

$Co^{III}(P)(NO)$ (P = porphyrin) complexes are very unstable with respect to NO dissociation.[7] The only possible way they

can react with NO is through the reductive nitrosylation process. This is only possible when the ligand trans to coordinated NO is sufficiently labile to allow the reaction with a second NO molecule that induces the reduction process. In the case of Cbl(H_2O), the dimethylbenzimidazole group is located trans to the labile water molecule and is tightly bound even at pH 1. It can therefore not induce the formation of Cbl(NO^-). Once the labile water molecule is displaced by nitrite, the dimethylbenzimidazole group is more easily protonated and able to induce the reductive nitrosylation process.

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