Kinetics and Mechanism of the Reversible Binding of Nitric Oxide to Reduced Cobalamin B_{12r} (Cob(II)alamin)

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Abstract: The reduced form of aquacobalamin binds nitric oxide very effectively to yield a nitrosyl adduct, Cbl(II)−NO. UV−vis, ^{1}H-, ^{31}P-, and ^{15}N NMR data suggest that the reaction product under physiological conditions is a six-coordinate, "base-on" form of the vitamin with a weakly bound α-dimethylbenzimidazole base and a bent nitrosyl coordinated to cobalt at the β-site of the corrin ring. The nitrosyl adduct can formally be described as Cbl(III)−NO^-. The kinetics of the binding and dissociation reactions was investigated by laser flash photolysis and stopped-flow techniques, respectively. The activation parameters, $\Delta H^\ddagger$, $\Delta S^\ddagger$, and $\Delta V^\ddagger$, for the forward and reverse reactions were estimated from the effect of temperature and pressure on the kinetics of these reactions. For the "on" reaction of Cbl(II) with NO, the small positive $\Delta S^\ddagger$ and $\Delta V^\ddagger$ values suggest the operation of a dissociative interchange (I_d) substitution mechanism at the Co(II) center. Detailed laser flash photolysis and ^{17}O NMR studies provide evidence for the formation of water-bound intermediates in the laser flash experiments and strongly support the proposed I_d mechanism. The kinetics of the "off" reaction was studied using an NO-trapping technique. The respective activation parameters are also consistent with a dissociative interchange mechanism.

Introduction

Numerous studies on the structure and reactivity of vitamin B_{12} have resulted in speculations about the mechanisms of its biological functions. One of the recent interests in this area concerns the use of aquacobalamin and its reduced form (vitamin B_{12r}, Cbl(II)) to modify the physiological action of nitric oxide in vivo.[1−3] According to observations reported in the literature, such interactions may play a role in various biological processes involving nitric oxide. The first possible explanation for these experimental observations involved nitrosyl complex formation according to the overall reaction given in 1.

$$\text{Cbl(III)}-\text{H}_2\text{O} + \text{NO} \rightleftarrows \text{Cbl(III)}-\text{NO} + \text{H}_2\text{O} \qquad (1)$$

Detailed spectroscopic and kinetic studies have recently shown that no direct interaction between NO and aquacobalamin occurs and therefore reaction 1 cannot account for the observed physiological effects. However, UV−vis, EPR, and resonance Raman spectroscopic studies indicate that the reduced form of vitamin B_{12} reacts with nitric oxide to form a Cbl(II)−NO complex,[2,3] and the formation constant for this process, $K = (1.0 \pm 0.5) \times 10^8\ \text{M}^{-1}$, has recently been reported.[3] Thus, the effective binding of NO to reduced cobalamin (which is present under physiological conditions[4,5]) may be responsible for inhibition of NO actions in vivo, as well as NO-induced inactivation of selected cobalamin-dependent enzymes.[6] However, to our knowledge, kinetic and mechanistic details of the reaction between reduced cobalamin, Cbl(II), and NO are presently unknown.

In this study we report our findings on the kinetics and mechanism of the reversible binding of NO to the reduced form of cobalamin in aqueous medium. In this context, temperature and hydrostatic pressure effects on the reaction rate were studied by laser flash photolysis and stopped-flow techniques. This enabled a quantitative kinetic and mechanistic description of the reaction system on the basis of rate constants and activation parameters ($\Delta H^\ddagger$, $\Delta S^\ddagger$, and $\Delta V^\ddagger$) for the forward and reverse reactions. We recently reported a similar study on the reversible binding of NO to metmyoglobin.[7] In addition, ^{1}H-, ^{15}N-, and ^{31}P NMR experiments were performed to resolve the nature of the reaction product. Since nitrite ions are usually present as impurity in aqueous solutions of NO,[1b,8] their reactivity toward reduced cobalamin was also studied, and the results are compared with that obtained for NO.

Experimental Section

Materials. Hydroxocobalamin hydrochloride and methylcobalamin were obtained from Sigma. Other chemicals used throughout this study were of analytical reagent grade. All solutions were prepared from

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deionized (Millipore) or triple distilled water. Reagent solutions were prepared and handled in gastight glassware due to the high oxygen-sensitivity of cob(II)alamin, nitrosylcob(II)alamin and NO. Oxygen-free argon and nitrogen were used to deoxygenate reagent solutions. Stock solutions of nitric oxide were prepared by saturation of deoxygenated buffer with NO gas (Linde 93) with the use of vacuum line techniques. The NO gas was purified from higher nitrogen oxides by passing through concentrated KOH solutions or an Ascarite II column. Solutions of $[Fe^{II}(edta)H_2O]^{2-}$ were prepared in water and adjusted with NaOH to the desired pH under nitrogen atmosphere. Acetic acid/sodium acetate buffer (0.2 M) and Tris buffer (0.1 M, Sigma, pH adjusted with $HClO_4$) were used to control the pH where required.

Preparation of Cbl(II) and Cbl(II)−NO Solutions. Reduced vitamin B_{12} was prepared via chemical reduction of aquacobalamin under nitrogen atmosphere. Three different reducing agents (ascorbic acid, sodium borohydride, and sodium formate) were used in the study, the selected reducing agent depending on the type of experiment performed. To avoid the presence of reductant in studies on the redox reaction between cob(II)alamin and NO_2^-, zinc amalgam was used to generate Cbl(II). The identity and concentration of the reduced cob(II)alamin were determined by UV−vis spectroscopy. In most of the experiments performed, the nitrosylcob(II)alamin complex was prepared by addition of gaseous NO to reduced cobalamin under nitrogen atmosphere. Other procedures were used in the case of the laser flash photolysis and ^{15}N NMR studies, as will be described in detail below.

Kinetic Measurements. Laser Flash Photolysis. Kinetic data and activation parameters for the binding of NO to cob(II)alamin were studied by nanosecond laser flash photolysis using a LKS 60 Applied Photophysics spectrometer. In ambient-pressure experiments the deoxygenated solution of methylcobalamin was mixed in an appropriate volume ratio with the NO-saturated solution, transferred to a gastight quartz cuvette and photolyzed (for 1 min) with the full spectrum of a high-pressure xenon lamp. The sample was allowed an additional 10−15 min to equilibrate in a temperature-controlled cuvette holder. A pill-box optical cell combined with a high-pressure cell and equipment[9] was used for experiments at pressures up to 160 MPa.

Flash photolysis of Cbl(II)−NO was initiated with a laser beam generated from the second harmonic of a Nd:YAG laser (Surelite I-10, Continuum; $\lambda_{exc} = 532$ nm, pulse duration 7 ns, pulse energy 245 mJ). Spectral changes were monitored at 313 nm using a 100 W xenon arc lamp, monochromator, and photomultiplier tube (PMT-1P22). The absorbance reading was balanced to zero before the flash. Data were recorded on a digital storage oscilloscope, DSO HP 54522A, and transferred to the computer for subsequent analysis.

Stopped-Flow Kinetics. Samples of Cbl(II)−NO for studies on the “off” reaction were prepared by bubbling NO through the chemically reduced cob(II)alamin. The excess NO was removed from the solution with the use of nitrogen.

The kinetics of NO release from nitrosylated cob(II)alamin was studied on a thermostated (±0.1 °C) stopped-flow spectrometer (SX-17MV, Applied Photophysics) by rapid mixing of a Cbl(II)−NO solution (4×10^{-4} M) with an excess of $[Fe(edta)H_2O]^{2-}$ (0.2 M). The reaction was monitored at 520 nm (pH 5.0 and 7.4) or 471 nm (pH 3.6) (the measured rate constants at a given pH were independent of the observation wavelength). High-pressure stopped-flow experiments were performed at pressures up to 130 MPa on a custom-built instrument described previously.[9] Kinetic traces were recorded on an IBM-compatible computer and analysed with the OLIS KINFIT (Bogart, GA, 1989) set of programs.

Kinetics of the reaction of Cbl(II) with NO_2^- was followed at pH 4.0 in the thermostated (±0.1 °C) stopped-flow unit described above. Additional experiments at pH 5.5 and 7.4 were performed in the thermostated (±0.1 °C) cell compartment of a Shimadzu UV-2001 spectrophotometer. The absorbance change at 455 nm was monitored to determine the observed reaction rates.

Acid Dissociation Constant of Nitrosylcob(II)alamin. The pK_a of nitrosylcob(II)alamin was determined by spectrophotometric titration of a nitrosylcobalamin solution (prepared in 0.1 M $HClO_4$) with deoxygenated NaOH under nitrogen atmosphere. The pK_a value was determined at 25 °C for two different concentrations of nitrosylcob(II)alamin (1×10^{-4} and 2×10^{-4} M). Spectrophotometric titration of reduced cobalamin under similar experimental conditions ($[Cbl(II)] = 1 \times 10^{-4}$ M, 25 °C, $I = 0.1$ M) resulted in $pK_a = 2.9 \pm 0.1$, a value in good agreement with literature data.[10]

NMR Measurements. Samples for ^1H- and ^{31}P NMR were prepared by chemical reduction of aquacobalamin in deoxygenated D_2O followed by addition of gaseous NO. The apparent pH (pH_{app} = pH meter reading = pD − 0.4) was adjusted to the desired value by additions of DCl or NaOD. Samples were transferred under nitrogen atmosphere to oxygen-tight NMR tubes. Spectra were recorded on a Bruker Avance DPX300 NB spectrometer operating at 300.13 and 121.5 MHz for ^1H NMR and ^{31}P NMR, respectively. Chemical shifts were referenced internally to TSP (trimethylsilyl propionate, ^1H NMR) and TMP (trimethyl phosphate, ^{31}P NMR).

^{15}N-enriched nitrosylcobalamin was prepared in D_2O by mixing aquacobalamin (2×10^{-2} M) with 99% enriched nitrite in a ca. 1:5 molar ratio and subsequent anaerobic reduction of the $Cbl(III)-^{15}NO_2$ complex with ascorbic acid. The solution pH was adjusted to the desired value as described above. Formation of the nitrosyl complex was confirmed by the UV−vis spectrum of a diluted sample. ^{15}N NMR spectra were recorded on a Bruker Avance DRX400 WB spectrometer equipped with a superconducting BC-94/89 magnet system, at 40.56 MHz. Chemical shifts were referenced externally to neat nitromethane.

Water Exchange at the Co(II) Center in “Base-Off” Cbl(II). “Base-off” cob(II)alamin was prepared via chemical reduction of aquacobalamin (2×10^{-2} M) with sodium formate (0.2 M) in 0.1 M $HClO_4$ under strictly oxygen-free conditions. Ten percent of the total sample volume of ^{17}O-enriched water (normalized 19.2% ^{17}O H_2O, D-Chem Ltd.) was added to the sample. The solution containing the same components as the NMR sample, except for the Cbl(II), was used as a reference solution. Transfer of the sample into the gastight NMR tubes was performed in a glovebox under nitrogen atmosphere.

Variable-temperature/pressure FT ^{17}O NMR spectra were recorded at a frequency of 54.24 MHz on a Bruker Advance DRX 400WB spectrometer. The temperature dependence of ^{17}O line broadening was determined in the temperature range 278−353 K. A homemade high-pressure probe[11] was used for the variable-pressure experiments performed at the selected temperature (283 K) and at pressures 1, 30, 60, 90, 130, and 150 MPa. The sample was placed in a standard 5 mm NMR tube cut to a length of 45 mm. The pressure was transmitted to the sample by a movable macor piston, and the temperature was controlled as described elsewhere.[11]

Results and Discussion

Spectroscopic Studies. When gaseous nitric oxide is added to a solution of cob(II)alamin, rapid changes occur in the characteristic UV−vis spectrum (Figure 1), indicating the formation of nitrosylcob(II)alamin[1−3] according to reaction 2.

$$\mathrm{Cbl(II)} + \mathrm{NO} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{Cbl(II)-NO} \tag{2}$$

The same final spectrum can also be obtained by anaerobic photolysis of methylcobalamin, adenosylcobalamin, or aquacobalaminin in the presence of nitric oxide,[2,3] or via chemical reduction of $Cbl(III)-NO_2$ under oxygen-free conditions.

The spectral changes observed for reaction 2 depended on the selected pH. At pH 1, five isosbestic points characteristic for the reaction of “base-off” cob(II)alamin with NO (Scheme 1a) were observed at 260, 336, 397, 495, and 535 nm. At pH 4, seven isosbestic points occurred at 292, 332, 390, 423, 460, 485, and 530 nm, featuring the reaction of the deprotonated,

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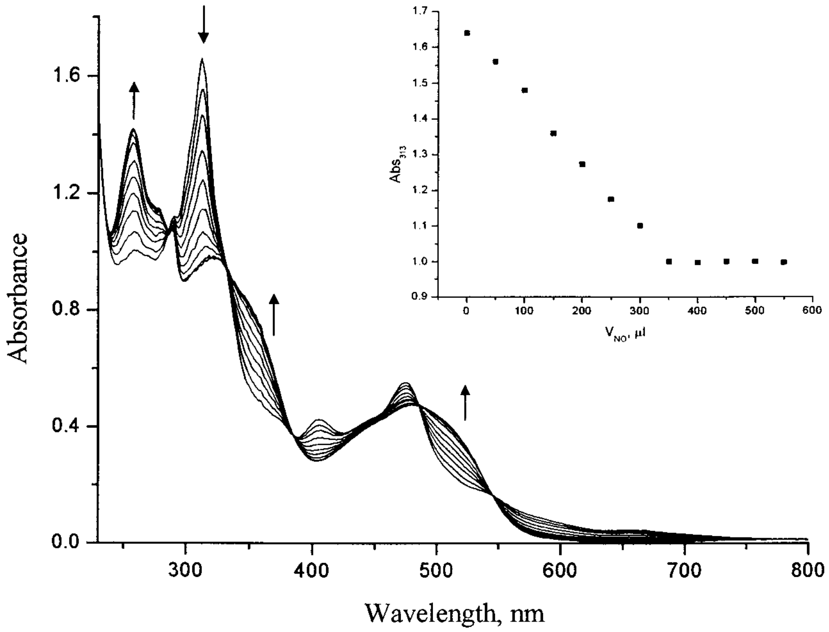
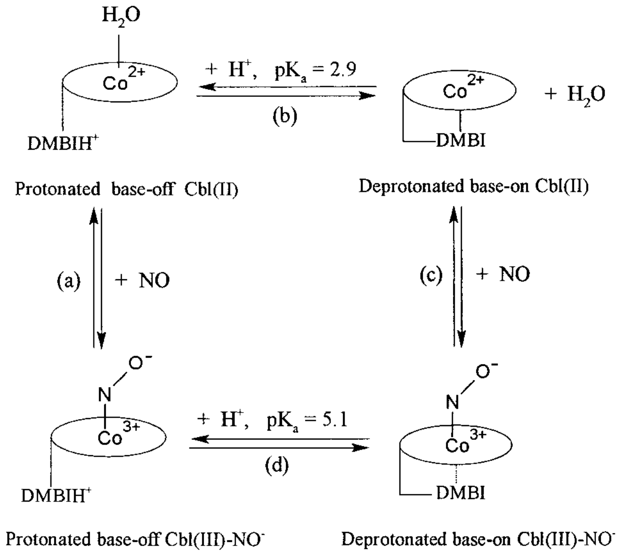


Figure 1. Spectral changes recorded for the titration of a Cbl(II) solution (7.3×10^{-5} M) with an aqueous NO saturated solution (2×10^{-3} M) at pH 7.4 (0.1 M Tris-$HClO_4$). Inset: Plot of absorbance at 313 nm as a function of added volume of NO saturated solution.

Scheme 1



“base-on” vitamin (present at pH > 3, Scheme 1c) with NO. The reaction product formed at pH 4 has essentially the same UV−vis spectrum as that formed at pH 1 (i.e., protonated nitrosylcob(II)alamin). However, small but significant changes in this spectrum occur in the pH range 4−6 (Figure 2). Accordingly, the position of isosbestic points observed in the reaction of “base-on” Cbl(II) with NO at pH 7.4 is shifted to slightly different wavelengths (i.e., to 285, 330, 382, 483, and 545 nm).

The character and magnitude of the pH-induced changes in the UV−vis spectrum of the reaction product suggest dechelation of the weakly coordinated nitrogen base[12] at pH < 6 with formation of protonated, “base-off” nitrosylcob(II)alamin (Scheme 1d). Spectrophotometric titrations of nitrosylcob(II)alamin resulted in a pK_a value of 5.1 ± 0.1 (25 °C, I = 0.1 M).

The pK_a values characterizing the “base-on” − “base-off” equilibrium are one of the most characteristic chemical properties of cobalamins and usually fall in the range from approximately 4.0 to −2.4.[13] It has been stated in the literature that the difference between the pK_a of a given cobalamin derivative and that of the free nucleotide base (i.e., 1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole, pK_a = 5.56 at 25 °C) is a critical parameter that reflects the strength of interaction between the metal center and the nitrogen atom of α-DMBI ligand.[13,14] In the case of Cbl(II)−NO, this difference is unusually small compared to that with most cobalamin derivatives, indicating a weak Co−N(DMBI) bond. It follows that coordination of NO significantly weakens the interaction between α-DMBI and the cobalt atom. Such conclusion is in line with literature data on strong-field Co(II) nitrosyls (representing the $\{M\text{-}NO\}^8$ type species), which are typically square-pyramidal, five-coordinate complexes due to a strong *trans*-labilizing effect of the NO ligand.[15,16] However, the occurrence of pH-dependent spectral changes in the visible spectrum of Cbl(II)−NO and a noticeable (although small) difference in the pK_a value of nitrosylcobalamin and that of the free nucleotide base,[14,17] suggest that the deprotonated DMBI nitrogen donor in Cbl(II)−NO does interact with the cobalt center.

This conclusion is further supported by a 1-D ^1H NMR measurements. As can be seen from the data in Table 1, the ^1H chemical shifts for the benzimidazole B2H, B4H, and B7H protons in deprotonated nitrosylcob(II)alamin at pH_{app} = 8.8 are quite similar to those observed in “base-on” cobalamins,

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[12] (a) Formation of protonated “base-off” species at pH < 6 is clearly evidenced by spectral changes in the range 250−290 nm (characteristic for protonation of 1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole[13]) and additional changes in the range 430−550 nm.[12b,c] The pH-dependent spectral changes were reversible and thus do not result from contamination of the sample with oxygen. (b) Trommel, J. S.; Warncke, K.; Marzilli, L. G. *J. Am. Chem. Soc.* **2001**, 123, 3358. (c) Sirovatka, J. M.; Finke, R. G. *J. Am. Chem. Soc.* **1997**, *119*, 3057.

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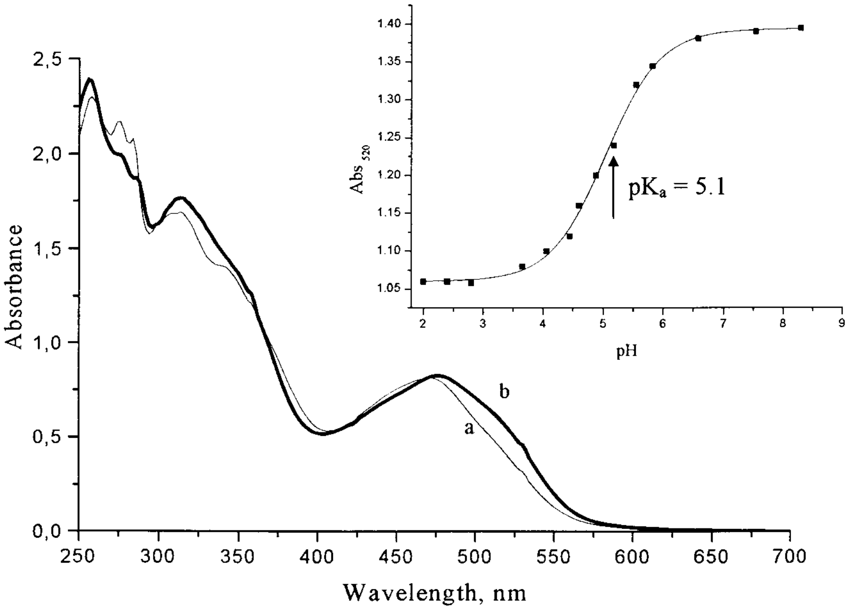

Figure 2. UV−vis spectra of nitrosylcob(II)alamin (1×10^{-4} M) (a) at pH 3.0, λ_{max} (±2 nm): 258, 276, 285, 316, 346 (shoulder) and 472 nm; (b) at pH 7.4, λ_{max}: 256, 277 (shoulder), 320 and 476 nm. Inset: Plot of absorbance at 520 nm versus pH for nitrosylcob(II)alamin. Experimental conditions: [Cbl(II)−NO] = 2×10^{-4} M, 25 °C, $I = 0.1$ M ($NaClO_4$).

Table 1. Comparison of ^1H NMR Chemical Shifts[a] of the Dimethylbenzimidazole Protons in Nitrosylcob(II)alamin with the Corresponding Signals in Selected Cobalamins and Free α-Ribazole-3′-phosphate

	nitrosylcob(II)alamin[b]		adenosylcobalamin[c]		$(CN)_2Cbl$[d]	α-alkylCbl[c]	α-ribasole-3‘-phosphate[c]	
proton assignment	DMBI[e] ($pH_{app} = 8.8$)	DMBIH$^+$[e] ($pH_{app} = 3.1$)	DMBI base-on	DMBIH$^+$ base-off	DMBI base-off	DMBI base-off	DMBI	DMBIH$^+$
B2	7.40	9.15	6.95	9.16	8.35	8.35	8.41	9.13
B4	6.76	7.45	6.24	7.44	7.46	7.37	7.46	7.41
B7	7.20	7.53	7.16	7.55	7.36	7.31	7.35	7.18

[a] Shifts relative to trimethylsilyl propionate, except α-ribazole phosphate (shifts relative to DSS). [b] This work. [c] Alelyunas, Y. W.; Fleming, P. E.; Finke, R. G.; Pagano, T. G.; Marzilli, L. G. *J. Am. Chem. Soc.* **1991**, *113*, 3781 and references therein. [d] Brown, K. H.; Brooks, H. B.; Gupta, B. D.; Victor, M.; Marques, H. M.; Scooby, D. C.; Goux, W. J.; Timkovich, R. *J. Am. Chem. Soc.* **1991**, *30*, 3430. [e] DMBI and DMBIH$^+$ designate deprotonated (DMBI) and protonated (DMBIH$^+$) form of cobalamin, respectively.

but differ significantly from those typical for “base-off”, DMBI-deprotonated cobalamin derivatives. With decreasing pD, the protonation-sensitive B2H and B4H signals progressively moved downfield[18] and were observed at acidic pD as sharp singlets at positions typical for “base-off”, protonated cobalamins (Table 1).

To further investigate the strength of the interaction between the cobalt ion and the DMBI ligand, ^{31}P NMR spectroscopy was employed.[19] The ^{31}P chemical shift for protonated, “base-off” Cbl(II)−NO ($pH_{app} < 4$) was observed in our studies at −4.30 ppm, a value in good agreement with the literature data for other “base-off” cobalamins.[19e] Its position changed slightly with increasing pD and a final value −4.23 ppm was observed for the deprotonated cobalamin ($pH_{app} > 7$). This observation further suggests interaction between deprotonated DMBI and the cobalt center in Cbl(II)−NO. This interaction, however, must be weak, judging from the similarity in the ^{31}P shifts for the protonated and deprotonated forms of the complex.

To obtain direct information on the coordination mode of NO in Cbl(II)−NO, ^{15}N NMR spectroscopy (which is known to be a sensitive technique to probe the nature of the nitrosyl ligand in solution[16]) was employed. The labeled Cbl(II)−^{15}NO complex exhibits a resonance at ca. 785 ppm (see inset in Figure S1, Supporting Information) at $pH_{app} = 7.6$, that is, for “base-on” nitrosylcob(II)alamin. This value falls into the range of ^{15}N shifts observed for porphyrin and Schiff base Co(II) nitrosyls with strongly bent NO, which are usually viewed as diamagnetic Co(III)−NO$^-$ complexes[16] (Table 2). The position of the ^{15}N resonance moved upfield with decreasing pH to 745 ppm (as observed at $pH_{app} = 3.5$), such that protonation of nitrosylcobalamin resulted in a ca. 40 ppm upfield shift of the signal. Deshielding of the NO nitrogen atom in deprotonated Cbl(II)−NO (compared to the protonated form) results from stronger bending of the M−N−O group in a “base-on” complex, that is, a larger shift of Co d-electron density onto the NO ligand.[17]

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(18) (a) The pK_a value for nitrosylcobalamin obtained by following the chemical shift of the benzimidazole B2 hydrogen as a function of pH_{app}, pK_a(apparent) = 5.1 ± 0.2 (measured in D_2O, 22 °C, [Cbl(III)−NO$^-$] = 1×10^{-2} M). This value is in very good agreement with the pK_a value determined by spectrophotometric titration of nitrosylcobalamin. (b) Experimental and literature data demonstrating that the apparent pK_a determined in D_2O equals the pK_a in H_2O are given in: Alelyunas, Y. W.; Fleming, P. E.; Finke, R. G.; Pagano, T. G.; Marzilli, L. G. *J. Am. Chem. Soc.* **1991**, *113*, 3781 and references therein.

(19) It has been shown that the ^{31}P chemical shift of the phosphodiester in the cobalamin “*f*” side chain is almost identical in all “base-off” cobalamins but varies in a linear manner with the strength of the Co−N_{DMBI} bond in “base-on” cobalamins. Although the variation of the observed chemical shifts across the series of “base-on” cobalamins is small (from −3.66 observed for Cbl(III)H_2O to −4.39 ppm observed for $CH_3(CH_2)_2$-Cbl, chemical shifts referenced to TMP), ^{31}P NMR provides a reliable method to estimate the strength of the Co−N_{DMBI} bond in many cobalamin derivatives.[19b−d] (b) Calafat, A.; Marzilli, L. *J. Am. Chem. Soc.* **1993**, *115*, 9182. (c) Brown, K. L.; Evans, D. R.; Zubkowski, J. D.; Valente, E. J. *Inorg. Chem.* **1996**, *35*, 415. (d) Brown, K. L. *Inorg. Chem.* **1986**, *25*, 3111. (e) Brown, K. L.; Hakimi, J. M.; Jacobsen, D. W. *J. Am. Chem. Soc.* **1984**, *106*, 7894.

Table 2. ^{15}N NMR and Structural Data on Square-Pyramidal Co^{II} Complexes with a Bent Nitrosyl Ligand

complex	M−N−O angle	M−NO bond length Å	$\sigma(^{15}N)^a$ ppm
$Co^{II}(TPP)(^{15}NO)^b$	127°	1.833	771
$Co^{II}(OEP)(^{15}NO)^{b,c}$	123°	1.844	760
$Co^{II}(acacen)(^{15}NO)^d$	122°	1.821	714
$Co^{II}(benacen)(^{15}NO)^d$	123°	1.831	723
$Co^{II}(salen)(^{15}NO)^d$	127°	1.807	725
$Co^{II}(corrin)(^{15}NO)^e$			785

[a] Nitrogen shifts relative to neat liquid nitromethane. [b] Reference 16c. [c] Reference 15. [d] Measured in deuterated organic solvents (ref 16a). [e] This work, pH = 7.6.

The well-resolved ^{15}N NMR spectra and the well-defined ^{1}H NMR spectrum of nitrosylated cob(II)alamin (Figure S1 in Supporting Information) indicate that the complex is diamagnetic. This results from the combination of the unpaired electron of the paramagnetic d^7 Co(II) center with the odd electron residing on the π^* orbital of nitrosyl, with subsequent formation of the Co−NO bond. This process can be looked upon as a formal oxidation of Co(II) to Co(III) with formation of a $(L)Co^{III}-NO^-$ species (in analogy to other strong-field five-coordinate $(L)Co^{III}-NO^-$ and $(L)Ir^{III}-NO^-$ complexes). A similar conclusion was recently reached on the basis of resonance Raman data.[3] For this reason the reaction product Cbl(II)−NO will in the remainder of the text be denoted as $Cbl(III)-NO^-$.

To our knowledge, crystallographic data on nitrosylcob(II)alamin are not yet available, and therefore the Co−DMBI bond distance is not known. However, it can be estimated by comparison of structural data on reduced cobalamin[20,21] and typical $Co(III)-NO^-$ complexes.[15] Structural studies on porphyrin and Shiff base Co(II) nitrosyls revealed a 0.18 Å displacement of the metal above the mean plane of the equatorial ligand.[15] In “base-on” cob(II)alamin, however, the relatively large cobalt atom is displaced approximately 0.12 Å below the corrin ring toward the DMBI base.[21] Thus, coordination of nitric oxide to “base-on“ cobalamin presumably results in the 0.3 Å upward movement of the cobalt atom.[22] Consequently, a similar lengthening of the Co−DMBI bond can be expected. Taking into account the data on the Co−DMBI bond distance in “base-on”cob(II)alamin reported in the literature (1.99−2.25 Å),[20,21] the $Co-N_{DMBI}$ bond distance in nitrosylcob(II)alamin should fall into the range 2.29−2.55 Å.

Kinetic Studies

Laser Flash Photolysis. Irradiation of the $Cbl(III)-NO^-$ complex with a laser beam (λ = 532 nm) in aqueous solution produces a significant increase in absorbance at 313 nm, indicating the formation of Co(II) species and free NO. The transient spectrum decayed exponentially (in the presence of an excess NO) to the original spectrum of nitrosylcob(II)alamin. The photochemical reaction induced by the laser flash can therefore be expressed as given in reaction 3.

$$\mathrm{Cbl(III)-NO^-} \xrightarrow{h\nu} \mathrm{Cbl(II)} + \mathrm{NO} \xrightarrow{k_{on}} \mathrm{Cbl(III)-NO^-} \qquad (3)$$

To determine the exact nature of the photogenerated Co(II) species, a thorough study of the transient spectra recorded immediately after the flash (usually within 10 μs) was performed. Four examples selected from a series of experiments at pH’s ranging from 1.0 to 7.4 are presented in Figure 3 along with the corresponding spectral changes occurring in the thermal reaction between Cbl(II) and NO. The electronic spectrum in the selected wavelength range is sensitive to changes in the nature of the axial ligands coordinated to the metal center. A comparison of the transient and thermal spectral changes at pH 1 in the wavelength range 380−560 nm clearly shows that in both cases the reaction with NO involves the same process, that is, substitution of a water molecule in the “base-off” form of the complex. In addition, the rapid spectral changes observed in the laser flash experiment at this pH were followed by an approximately 20 times slower process (dotted lines in the spectrum presented in Figure 3a). The nature of this process was not fully investigated (our main interest being the reaction occurring under physiological conditions, i.e., at pH 7.4), but on the basis of the experimental observations it may be ascribed to the photoinduced formation of α-nitrosylcobalamin, in which the nitrosyl ligand is coordinated at the α site of the corrin ring.[23]

The spectral changes observed for the reaction of Cbl(II) with NO at pH 4.0 and 5.0 (Figure 3, b and c) differ in the case of the thermal and photoinduced reactions. In particular, the transient spectra recorded at pH 4.0 (during flash photolysis of protonated nitrosylcobalamin) are significantly different from that obtained in the thermal reaction of “base-on” Cbl(II) with NO at the same pH. The character of the photoinduced spectral changes and location of isosbestic points is, however, very similar to those observed at pH 1. On increasing the pH further, the transient spectra recorded after the flash change progressively from that characteristic for the nitrosylation of the “base-off”, water-coordinated complex to that observed in the reaction of the “base-on” species with NO (see thermal and photoinduced spectral changes at pH 7.4 in Figure 3d). Thus, the electronic spectra of the reactive intermediates formed at the lowest and highest pH are practically identical with those of “base-off” and “base-on” cob(II)alamin, respectively, whereas the character of the photoinduced spectral changes in the pH range 4−6 vary as a result of changes in the molar ratio of the “base-on”/”base-off” intermediates produced from the corresponding forms of nitrosylcobalamin at different pH.

It follows from the above observations that although the formation of the “base-off” cob(II)alamin is thermodynamically unfavorable at pH > 3 (i.e., this form of Cbl(II) does not participate in the thermal reaction at pH above 4), the “base-

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(20) (a) Sagi, I.; Wirt, M. D.; Chen, E.; Frisbie, S.; Chance, M. R. *J. Am. Chem. Soc.* **1990**, *112*, 8639. (b) Giorgetti, M.; Ascone, I.; Berrettoni, M.; Conti, P.; Zamponi, S.; Marassi, R. *J. Biol. Inorg. Chem.* **2000**, *5*, 156.

(21) Kräutler, B.; Keller, W.; Kratky, Ch. *J. Am. Chem. Soc.* **1989**, *111*, 8936.

(22) (a) Upward movement of the Co atom (and, consequently, the change from downward to upward folding of the corrin ring) is expected to be a fast process due to the high flexibility of the corrin ring.[22b] Formation of the nitrosyl complex results in the formal oxidation of Co(II) to Co(III) (i.e., shrinkage of the metal center) and further promotes metal movement through the corrin ring. (b) Brown, K. L.; Zou, X.; Marques, H. M. *J. Mol. Struct. (THEOCHEM)* **1998**, *453*, 209.

(23) (a) Support for such an explanation comes from the following observations: (i) the slower process was observed at pH < 4, i.e., where the α-DMBI ligand is protonated and does not coordinate to cobalt at the α-site of the corrin ring;[23b] (ii) substitution of NO at the more crowded α-site of the corrin ring may be slower than the formation of the β-diastereoisomer, exhibit pseudo-first-order kinetics in the presence of excess NO and depend on the free NO concentration; all of these points are in line with our experimental observations; (iii) the spectral changes for the slower reaction are similar to those observed in the fast process (i.e., formation of the β-nitrosylcobalamin) but shifted to longer wavelengths; thus, the electronic spectrum of the compound formed in the slower reaction exhibits a red-shift typically observed for α-diastereoisomers of alkylcobalamins;[23c] (iv) evidence for the formation of small amounts of α- nitrosylcobalamin under acidic conditions (pH < 5) was also provided by ^{1}H- and ^{15}N NMR spectroscopy. (b) Zou, X.; Brown, K. L.; Vaughn, Ch. *Inorg. Chem.* **1992**, *31*, 1552. (c) Zou, X.; Brown, K. L. *J. Am. Chem. Soc.* **1993**, *115*, 6689. (d) Brown, K. L.; Zhao, D.; Cheng, S.; Zou, X. *Inorg. Chem.* **1997**, *36*, 1764.

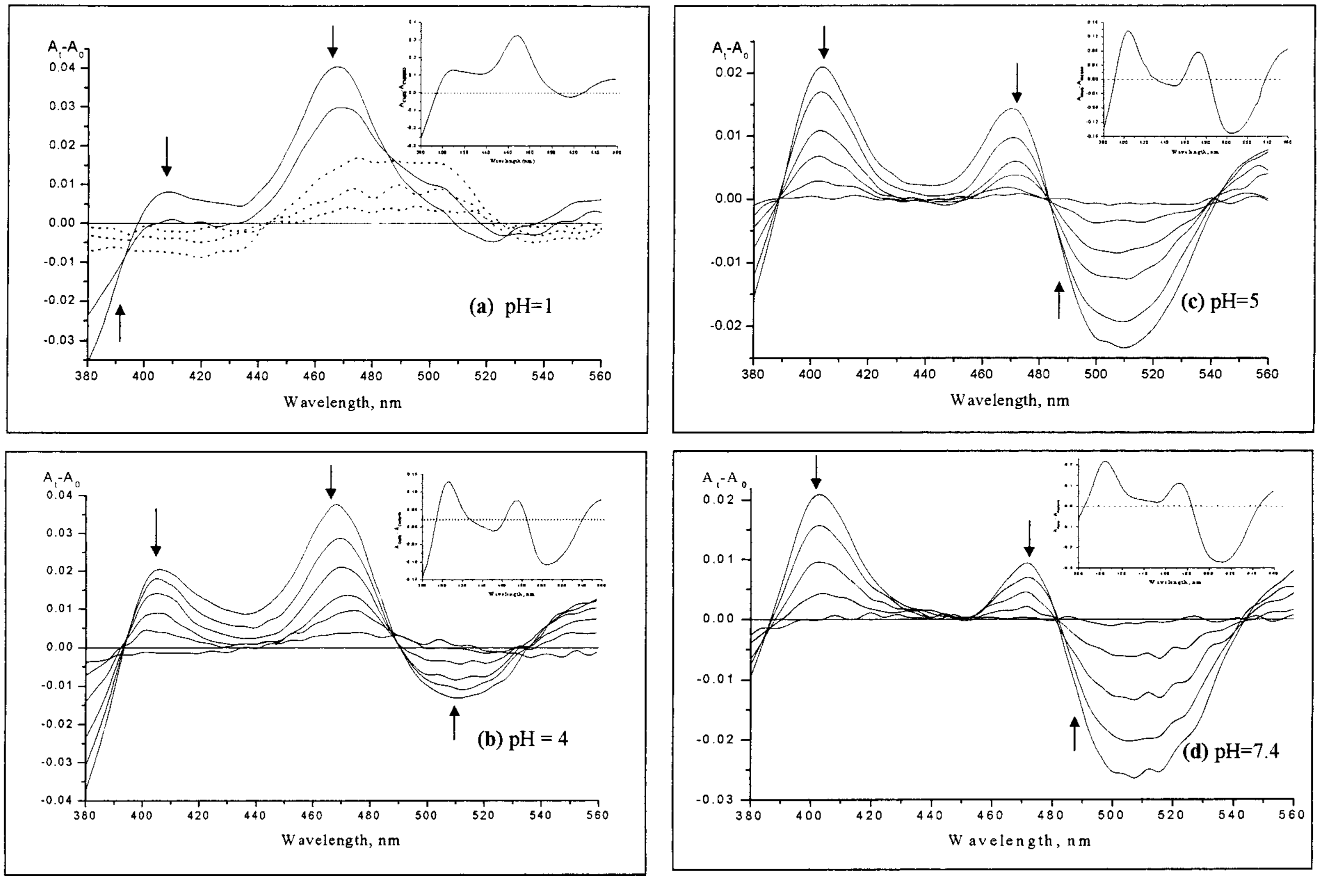
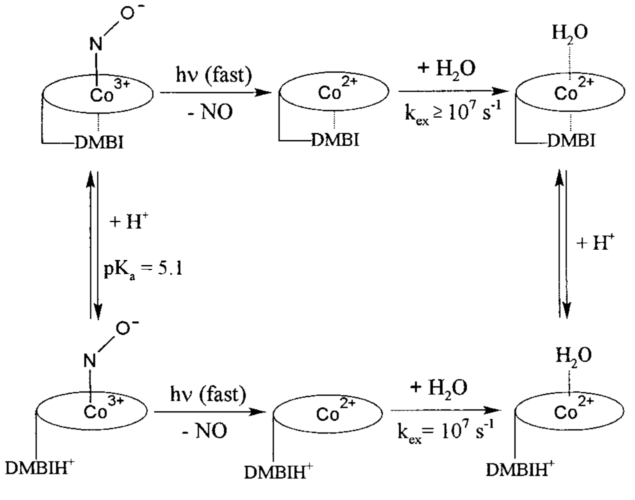


Figure 3. Transient spectra recorded in the laser flash photolysis of nitrosylcob(II)alamin in the pH range 1−7.4. Inset: Corresponding difference spectra obtained in the thermal reaction between Cbl(II) and NO.

Scheme 2



off” species are apparently formed in the laser flash experiments in the pH range 4−6. According to the most self-consistent reaction scheme based on all the experimental evidence outlined above, photodissociation of NO from nitrosylcob(II)alamin leads to the formation of the unstable intermediates presented in Scheme 2. The four-coordinate intermediate formed from “base-off” nitrosylcobalamin (the major photolytic product at pH < 5) rapidly binds a water molecule to produce the “base-off” Cbl(II) species prior to the recombination with NO. This conclusion is supported by ^{17}O NMR measurements on water exchange at the Co(II) center in “base-off” cob(II)alamin (see data reported below), which resulted in $k_{ex} = (3.6 \pm 1.2) \times 10^7$ s^{-1} (298 K, 0.1 M HClO$_4$). Taking into account the high concentration of water compared to that of nitric oxide used in this study (typically $[NO]_{max} = 1.5 \times 10^{-3}$ M), water binding to the “base-off” Co(II) center produced after the flash must occur prior to the reaction with NO (experimental k_{obs} values for the binding of NO did not exceed a value of 1.6×10^6 s^{-1}). Accordingly, photolysis of the “base-on” nitrosylcobalamin (formed as the main reactive intermediate at pH > 5) in aqueous solution results in the formation of the five-coordinate complex with a weakly coordinated α-DMBI group (Scheme 2), which coordinates a water molecule prior to ring-closure (i.e., strengthening of the Co−DMBI bond).

Interaction of the α-DMBI ligand with cobalt in the five-coordinate intermediate is expected to cause further labilization of the *β*-position (in comparison with that observed in the “base-off” Cbl(II)). Thus, a water molecule present in the proximity of the metal center[24] may bind extremely fast (although weakly) at the *β*-site of the five-coordinate intermediate. The presence of such a labile water molecule would not show up in the electronic spectrum of the “base-on” intermediate but may be anticipated from the experimental evidence presented in the kinetic section of this study.

Kinetics of the “On” Reaction. NO binding to reduced cobalamin was studied under pseudo-first-order conditions with respect to NO. The transient spectra decayed uniformly according to pseudo-first-order kinetics (Figure 4) at each selected pH (i.e., 1.0, 3.6, 5.0 and 7.4). Plots of the observed rate constant versus [NO] were linear under all conditions (see inset in Figure

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(24) (a) In this respect it may be important to point out that structural data on Cbl(II) revealed the presence of two ordered water molecules located in the close proximity of the metal center at the *β*-site of the corrin ligand.[24b] (b) Langan, P.; Lehmann, M.; Wilkinson, C.; Jogl, G.; Kratky, Ch. *Acta Crystallogr.* **1999**, *D55*, 51.

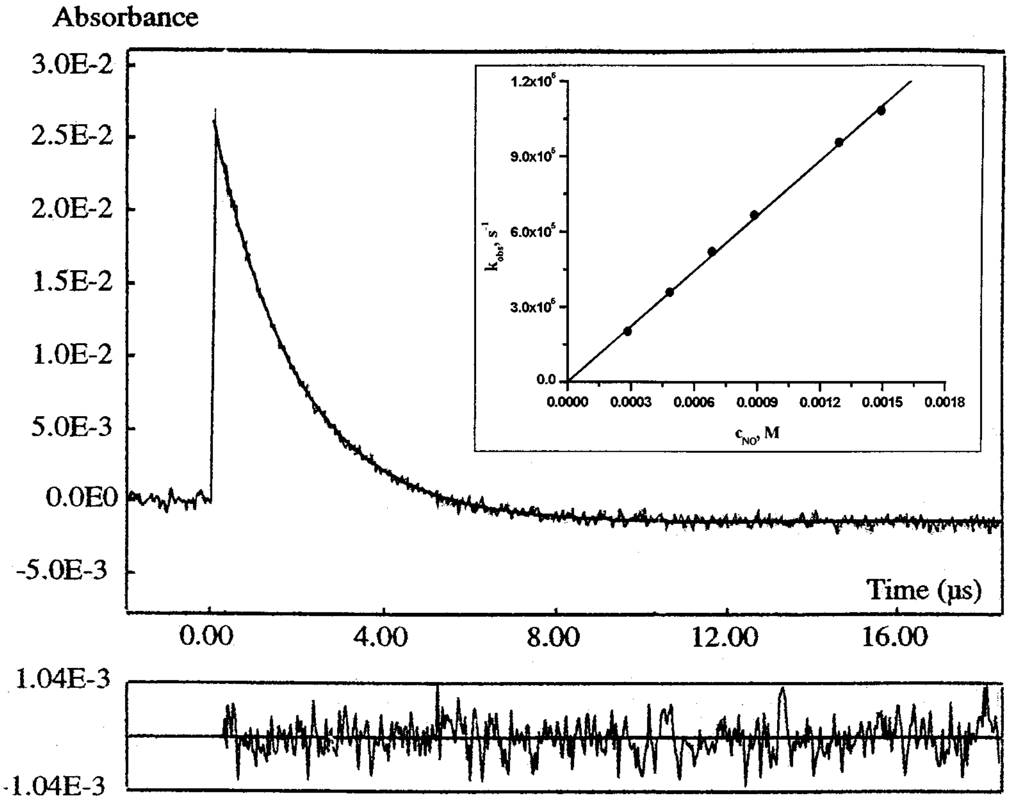

Figure 4. Typical kinetic trace for the formation of the Co−NO bond following the laser flash. (Inset) Plot of k_{obs} vs [NO] at pH 7.4 (0.1 M Tris-HClO$_4$). Experimental conditions: [Cbl(II)−NO$^-$] = 4 × 10^{-5} M, [NO] = 0.3−1.5 × 10^{-3} M.

Table 3. Rate and Activation Parameters for the Binding of NO to Cbl(II)

pH	1.0[a]	3.6	5.0	7.4
$k_{on} \times 10^{-8}$,[b] $M^{-1}\,s^{-1}$	5.0 ± 0.2	5.3 ± 0.2	6.4 ± 0.4	7.4 ± 0.2
$\Delta V_{on}^{\ddagger}$,[b] $cm^3\,mol^{-1}$	+5.3 ± 0.3		+5.2 ± 0.3	+5.4 ± 0.2
$\Delta H_{on}^{\ddagger}$, kJ mol^{-1}			25.6 ± 0.6	24.5 ± 0.7
$\Delta S_{on}^{\ddagger}$, J $K^{-1}\,mol^{-1}$			+9 ± 2	+7 ± 2

[a] Measured in 0.1 M HClO$_4$. [b] Measured at 25 °C, mean value of three determinations.

4). No measurable intercepts were obtained, indicating that the reverse “off” reaction is too slow to be observed in the laser flash experiment. The second-order rate constants (k_{on}) were determined as a function of temperature in the range 5−35 °C at pH 5.0 and 7.4 to construct Eyring plots from which the activation parameters $\Delta H_{on}^{\ddagger}$ and $\Delta S_{on}^{\ddagger}$ were determined. Further information on the reaction mechanism was obtained from the effect of pressure on the observed rate constant, from which the activation volume for the “on” reaction was determined, $\Delta V^{\ddagger} = -RT(\mathrm{d}\ \ln(k_{on})/\mathrm{d}P)_T$. The results of these experiments are reported in Table 3 and in Figure S2 (Supporting Information).

As can be seen from the data summarized in Table 3, reformation of the Co−NO bond in neutral aqueous medium occurs extremely rapidly with $k_{on} = 7.4 \times 10^8\ M^{-1}\,s^{-1}$ (25 °C, pH 7.4). This value compares well with the second-order rate constants reported for the reactions of free radicals with reduced cobalamin and several other Co(II) complexes with macrocyclic ligands.[25,26] Interesting is our observation that the flash photolysis experiments produced practically the same k_{on} value at pH 1.0 and 7.4, although different species exists in solution in the absence of NO under these conditions (see Scheme 1).

The activation parameters determined for the “on” reaction, viz. a small but significant activation enthalpy, a small positive activation entropy, and more diagnostically, a small positive activation volume, cannot be correlated with a simple bond-formation process at the vacant coordination site of “base-on” cob(II)alamin. Such a process would result in a significantly negative volume of activation (due to bond formation and partial oxidation of the Co(II) center), which is apparently not the case. The small positive $\Delta V_{on}^{\ddagger}$ value is, however, very typical for a substitution reaction involving the displacement of a coordinated water molecule at a metal center in terms of an I_d mechanism.[26,27] This suggests that the rate-determining step involves water displacement at the Co(II) center of reduced cobalamin produced during flash photolysis.

At pH 1.0, photolysis of the “base-off” nitrosylcobalamin with a protonated dimethylbenzimidazole group results in the rapid formation of a five-coordinate aqua intermediate. Thus, in this case the binding of NO is expected to be controlled by displacement of a coordinated water molecule. The similarity in the values of k_{on} and the activation volumes at pH 1.0 and 7.4 suggests formation of a similar species during flash photolysis of the nitrosyl complex at pH 7.4, with the difference that the dimethylbenzimidazole group is not protonated under such conditions and interacts with the cobalt(II) center (as mentioned before, stronger labilization of a water molecule in the reactive intermediate produced at high pH compared to that formed at low pH is therefore expected). The striking similarity in the volumes of activation found at pH 1.0 and 7.4 (average values of three series of experiments) provides substantial evidence for the operation of an I_d mechanism in the reaction under study. Additional evidence supporting this conclusion comes from the comparison of activation parameters for the

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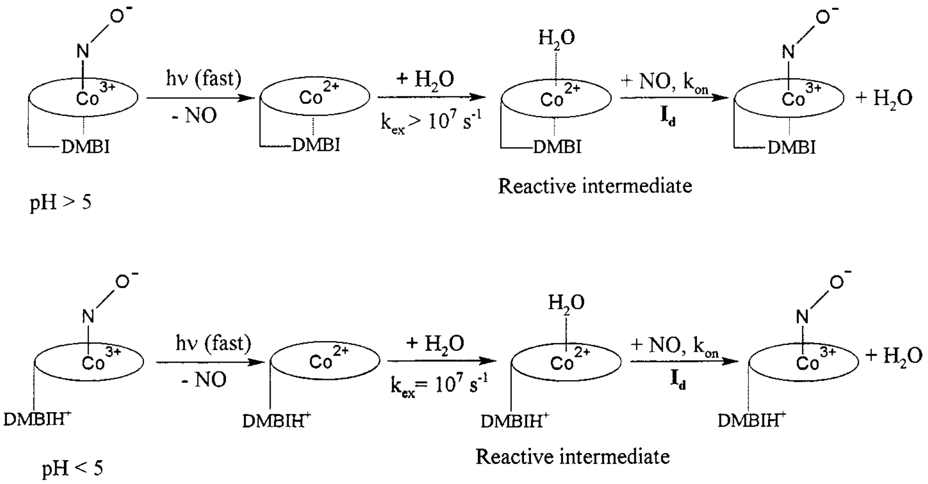
[25] Bakac, A.; Espenson, J. H. *Inorg. Chem.* **1989**, *28*, 4319.

[26] van Eldik, R.; Cohen, H.; Meyerstein, D. *Inorg. Chem.* **1994**, *33*, 1566 and references therein.

[27] Drljaca, A.; Hubbard, C. D.; van Eldik, R.; Asano, T.; Basilevsky, M. V.; le Noble, W. *J. Chem. Rev.* **1998**, *98*, 2167.

Scheme 3



"on" reaction, $\Delta H_{on}^{\ddagger}$ and $\Delta V_{on}^{\ddagger}$ (Table 3) with that obtained for the water exchange on the "base-off" Cbl(II) aqua complex formed at pH 1.0, which mimics the aqua intermediate produced during flash photolysis (see the following discussion of kinetic results and Table 4).

The kinetic observations are in line with the earlier conclusions on the nature of the reactive intermediate produced in the laser flash experiments and can be rationalized by the reaction sequence proposed in Scheme 3. The unstable intermediate produced during the flash rapidly coordinates a solvent molecule to form the aqua complex, which subsequently undergoes ligand substitution with the NO nucleophile to produce the Cbl(III)−NO⁻ complex. The latter reaction follows a dissociative interchange (I_d) mechanism for which the observed rate constant (k_{on}) and its activation volume are almost identical for pH 1.0 and 7.4.

The operation of a similar dissociative interchange ligand substitution mechanism for the photoinduced reactive intermediates can also account for surprising results obtained in the studies on the recombination of reduced cobalamin and selected macrocyclic Co(II) complexes with organic radicals reported in the literature.[25] In these laser-flash studies the recombination rate constants of the methyl radical with Cbl(II) were identical (within experimental error) in the pH range 1.3−7.0, despite the change in the coordination mode of Cbl(II). It was also noticed that five-coordinate (i.e., with a vacant coordination site) and six-coordinate macrocyclic complexes (with water coordinated as the sixth ligand) reacted with similar rate constants and the recombination rates varied little with the nature of the radical studied. All of these observations can be accounted for in terms of rate-determining water exchange at the Co(II) center in the reactive aqua intermediate produced after the laser flash.

Water Exchange Kinetics. The rate of water exchange on the paramagnetic Cbl(II) aqua complex and the corresponding activation parameters ($\Delta H_{ex}^{\ddagger}$, $\Delta S_{ex}^{\ddagger}$, and $\Delta V_{ex}^{\ddagger}$) were determined at pH 1.0 from the effects of temperature and pressure on the bulk water ^{17}O signal. In this respect, the reduced transverse relaxation times $1/T_{2r}$ were calculated for each temperature and pressure from the difference in line widths observed in the presence and in the absence of the metal complex, ($\Delta\nu_{obs}$ and $\Delta\nu_{solvent}$, respectively).

$$\frac{1}{T_{2r}} = \pi\frac{1}{P_m}(\Delta\nu_{obsd} - \Delta\nu_{solvent}) \qquad (4)$$

In the above equation P_m is the mole fraction of water coordinated to the Co(II) ion.

According to the Swift and Connick equation,[28] the reduced transverse relaxation time $1/T_{2r}$ is related to the exchange rate constant $k_{ex} = 1/\tau_m$ and to the NMR parameters T_{2m} (transverse relaxation time of water in the inner sphere of Co(II) in the absence of chemical exchange), T_{2os} (nuclear relaxation time of water molecules outside the first coordination sphere), and $\Delta\omega_m$ (difference in resonance frequency of water ^{17}O nuclei in the first coordination sphere and in the bulk solvent) as given in eq 5a.

$$\frac{1}{T_{2r}} = \frac{1}{\tau_m}\left\{\frac{T_{2m}^{-2} + (T_{2m}\tau_m)^{-1} + \Delta\omega_m^{2}}{(T_{2m}^{-1} + \tau_m^{-1})^2 + \Delta\omega_m^{2}}\right\} + \frac{1}{T_{2os}} \qquad (5a)$$

In the system studied, the contribution of $1/T_{2m}$ and $1/T_{2os}$ to $1/T_{2r}$ was negligible (as also shown for related systems described in the literature[29]), so that eq 5a can be simplified to eq 5b.

$$\frac{1}{T_{2r}} = \frac{1}{\tau_m}\left\{\frac{\Delta\omega_m^{2}}{\tau_m^{-2} + \Delta\omega_m^{2}}\right\} \qquad (5b)$$

The temperature dependence and pressure dependence of k_{ex} (taken from the transition state theory) are given in eqs 6a and 6b,

$$k_{ex} = (k_B T/h)\exp\{(\Delta S^{\ddagger}/R) - (\Delta H^{\ddagger}/RT) \qquad (6a)$$

$$k_{ex} = k_{ex}^{0}\exp\{(-\Delta V^{\ddagger}/RT)P\} \qquad (6b)$$

where k_{ex}^{0} is the rate constant for solvent exchange at ambient pressure and other symbols have their usual meaning. The NMR and kinetic parameters were calculated by the use of nonlinear least-squares method applied to eq 5b, in which $1/\tau_m$ was replaced by eq 6a and $\Delta\omega_m$ was expressed as a function proportional to A/T.[28−31] In total, four series of measurements were performed for the system under study.[32] Typical temperature dependence of the reduced transverse relaxation rate is presented in Figure S3 (Supporting Information).

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(28) (a) Swift, T. J.; Connick, R. E. *J. Chem. Phys.* **1962**, *37*, 307. (b) Swift, T. J.; Connick, R. E. *J. Chem. Phys.* **1964**, *41*, 2553.

(29) (a) Cossy, C.; Helm, L.; Merbach, A. E. *Inorg. Chem.* **1988**, *27*, 1973. (b) Helm, L.; Lincoln, S. F.; Merbach, A. E.; Zbinden, D. *Inorg. Chem.* **1986**, *25*, 2550.

(30) Bloembergen, N. *J. Chem. Phys.* **1957**, *27*, 595.

(31) Newman, K. E.; Meyer, F. K.; Merbach, A. E. *J. Am. Chem. Soc.* **1979**, *101*, 1470.

Table 4. Rate and Activation Parameters for Water Exchange on “base-off” Cob(II)alamin at pH 1

k_{ex} at 298 K s^{-1}	$\Delta H^{\ddagger}$ kJ mol^{-1} [a]	$\Delta S^{\ddagger}$ J K^{-1} mol^{-1} [a]	$\Delta V^{\ddagger}$ at 283 K cm^3 mol^{-1} [a]	k_{ex} [b] s^{-1}	k_{ex} [c] s^{-1}
$(3.6 \pm 1.2) \times 10^7$	23 ± 3	-24 ± 10	$+3.2 \pm 1.0$	$(2.5 \pm 0.6) \times 10^7$	$(2.2 \pm 0.8) \times 10^7$

[a] The error limit indicates the range of measured values. [b] Rate constant at atmospheric pressure extrapolated from the pressure dependence data measured at 283 K. [c] Rate constant at 283 K calculated from the temperature dependence data.

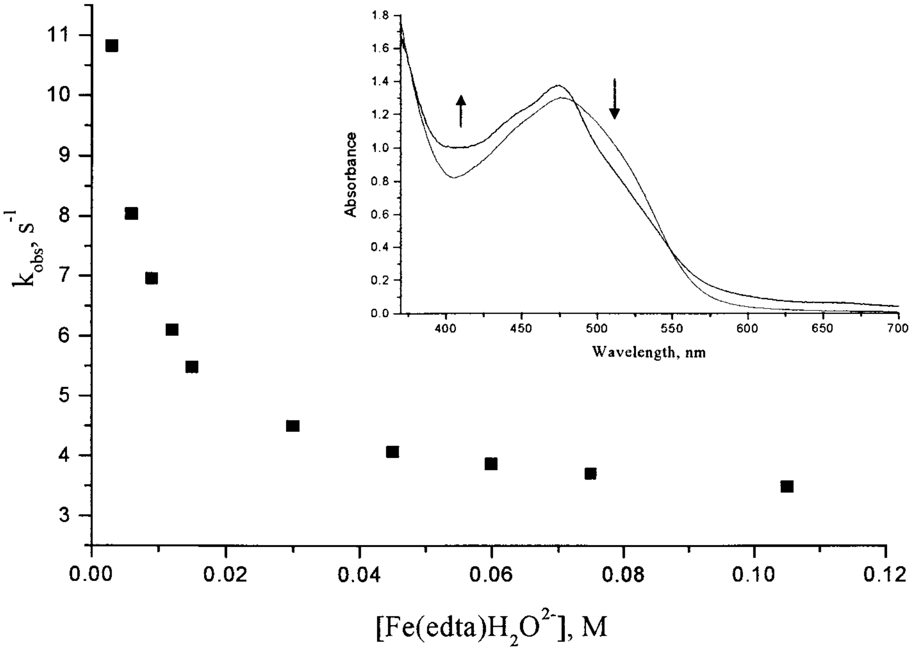
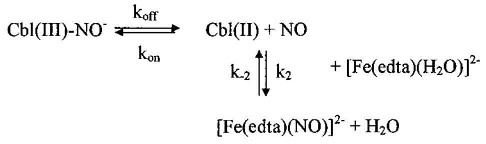


Figure 5. Plot of k_{obs} vs $[\mathrm{Fe(edta)H_2O^{2-}}]$ for the “off” reaction. Experimental conditions: $[\mathrm{Cbl(III)-NO^-}] = 2 \times 10^{-4}$ M, pH = 5.0, 25 °C. Inset UV−vis spectral changes for the reaction of $\mathrm{Cbl(III)-NO^-}$ with $[\mathrm{Fe(edta)H_2O}]^{2-}$ at pH 5.0.

In the case of our system the pressure dependence for k_{ex} was measured at 283, (a temperature close to the maximum in the plot of $\ln(1/T_{2r})$ vs $1/T$, where $\ln(1/T_{2r})$ is dominated by the term $\tau_m(\Delta\omega_m)^2$). The reduced relaxation time (calculated according to eq 4) and the value of $\Delta\omega_m$[33] (which shows a negligible pressure dependence[31]) were substituted into eq 5b to determine $1/\tau_m$ at each pressure. The plot of $\ln(k_{ex})$ versus pressure was linear (as shown in Figure S4, Supporting Information), and the activation volume was calculated directly from the slope ($-\Delta V^{\ddagger}/RT$). The k_{ex}^0 value at ambient pressure extrapolated from this plot was in good agreement with the corresponding k_{ex}^0 value calculated for this temperature (283 K) from the temperature dependence data.

The activation parameters (mean values from four independent experiments) are presented in Table 4. The small positive value found for the volume of activation suggests that water exchange in the “base-off” cob(II)alamin follows a dissociative interchange mechanism.[27,34] A comparison of the rate and activation parameters for the water exchange process with those reported for the binding of NO in Table 2 supports our earlier arguments in favor of a dissociative interchange ligand substitution mechanism involving the displacement of coordinated water by NO on the Co(II) center produced after the flash.

Scheme 4



Kinetics of the “Off” Reaction. As mentioned above, due to the very high k_{obsd} values observed in the laser flash experiments, extrapolation to [NO] = 0 did not give a measurable intercept from which the rate constant for the “off” reaction, k_{off}, could be estimated. It was, however, possible to measure the rate constant for the release of NO directly in a stopped-flow experiment by mixing the $\mathrm{Cbl(III)-NO^-}$ solution with high concentrations of $[\mathrm{Fe^{II}(edta)(H_2O)}]^{2-}$, an efficient scavenger for NO.

The spectral changes observed for the reaction between $\mathrm{Cbl(III)-NO^-}$ and $[\mathrm{Fe^{II}(edta)(H_2O)}]^{2-}$ indicate the formation of cob(II)alamin. The reaction was followed at 520 nm (at pH 5.0 and 7.4) or at 471 nm (at pH 3.6). The observed first-order rate constant decreased to a limiting value with increasing concentration of $[\mathrm{Fe^{II}(edta)(H_2O)}]^{2-}$ (Figure 5). This behavior can be accounted for in terms of the reactions given in Scheme 4.

Application of the steady-state approximation to the NO intermediate allows the observed first-order rate constant to be expressed as in eq 7.

$$k_{obs} = \frac{k_{off}\,k_2[\mathrm{Fe(edta)(H_2O)^{2-}}] + k_{on}k_{-2}[\mathrm{Cbl(II)}]}{k_2[\mathrm{Fe(edta)H_2O^{2-}}] + k_{on}[\mathrm{Cbl(II)}]} \qquad (7)$$

When $[\mathrm{Fe(edta)(H_2O)}]^{2-}$ is in large excess, eq 7 predicts rate

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(32) This was necessary since the line widths obtained for different samples of the complex were not identical. The origin of these differences is not apparent. However, the shape of the curvature presented in Figure S3 (Supporting Information) and the corresponding activation parameters obtained in the separate experiments differed only slightly.

(33) $\Delta\omega_m$ at 283 K was calculated with the use of *A* value determined as a parameter from the temperature-dependence data.

(34) Merbach, A. E.; Helm, L. *Coord. Chem. Rev.* **1999**, *187*, 151.

Table 5. Kinetic Data for the Dissociation of NO from Cbl(III)−NO^-

pH	3.6	5.0	7.4
k_{off},[a] s^{-1}	1.7 ± 0.1	3.3 ± 0.1	5.6 ± 0.2
$\Delta V_{off}^{‡}$,[b] $cm^3\,mol^{-1}$	$+4.1 \pm 0.2$		$+7.9 \pm 0.5$
$\Delta H_{off}^{‡}$, kJ mol^{-1}	72 ± 2		76 ± 1
$\Delta S_{off}^{‡}$, J $mol^{-1}K^{-1}$	$+3 \pm 5$		$+24 \pm 5$

[a] At 25 °C. [b] Mean value of three determinations.

saturation, since under such conditions dissociation of NO (k_{off} step) becomes rate-determining. The decrease in k_{obs} with increasing concentration of $[Fe(edta)(H_2O)]^{2-}$ results from the fact that k_{off} is much smaller than k_{-2}, the rate constant for the release of NO from $[Fe(edta)NO]^{2-}$ ($k_{-2} = 91\ s^{-1}$ at 25 °C, pH = 5.0).[35] Using $[Fe(edta)H_2O]^{2-}$ at the appropriate concentration (i.e., 0.1 M), the temperature and pressure dependences of k_{off} were studied in the ranges 5−35 °C and 0.1−130 MPa, respectively.

It follows from the kinetic data summarized in Table 5 that dissociation of NO from Cbl(III)−NO^- is slow and a high activation barrier must be overcome to break the Co−NO bond. Positive activation volumes obtained for the "off" reaction can be accounted for in terms of a dissociative interchange mechanism in which the breakage of the Co−NO bond is partially accompanied by strengthening of the Co−N(DMBI) bond. A small but significant increase in reaction rate and a more positive activation volume observed at higher pH point to a more dissociative mechanism for the deprotonated form of nitrosylcob(II)alamin. This can be rationalized in terms of a facilitated displacement of the NO ligand due to the presence of a lone pair of electrons on the deprotonated dimethylbenzimidazole base.

An alternative explanation for the more positive activation volume observed at pH 7.4 can be offered. Elongation of the Co−NO bond in the transition state (positive contribution to $\Delta V_{off}^{‡}$) is accompanied by Co−DMBI bond formation (negative contribution to $\Delta V_{off}^{‡}$). At high pH, DMBI is already partially coordinated to the cobalt center, such that the negative contribution to the observed activation volume could be smaller than that observed at lower pH (i.e., in the pH range 3−5), where initially protonated DMBI of "base-off" Cbl(III)−NO^- coordinates to the cobalt ion to form "base-on" Cbl(II).

Overall Reaction Process. In terms of the overall system, the kinetics of the "on" reaction studied by laser flash photolysis differs from that expected for reaction between Cbl(II) and NO at pH > 3. Due to the fact that the intermediates occurring in the laser flash and in the thermal reaction are not the same, the data do not allow construction of energy and volume profiles for the overall reaction 2 at a physiological pH. It is, however, possible to construct a volume profile for the binding of NO to the photogenerated aqua complex at pH 7.4 as shown in Figure 6. The volume profile once again illustrates the I_d character of the reversible displacement reaction.

If the thermal reaction between intact "base-on" cob(II)alamin and NO at pH > 3 is controlled by NO binding at the vacant coordination site, the thermal reaction should occur faster than that observed in the flash photolysis experiments (where the reaction involves substitution of coordinated water on the "base-off" Cbl(II) species generated photochemically). In such cases nitrosylation of Cbl(II) under biological conditions would be close to diffusion-controlled, that is, k'_{on} (NO binding rate constant in the thermal reaction) would be larger than that observed in our laser flash studies. In fact, it has been shown that formation of the Co−NO bond in Co(II) Schiff base and porphyrin nitrosyls in noncoordinating solvents (such as toluene) occurs with second-order rate constants in the range (1.1−1.7) × $10^9\ M^{-1}\ s^{-1}$ and the corresponding activation energies fall in the range 4−12 kJ mol^{-1},[36] that is, the process is almost diffusion-controlled.

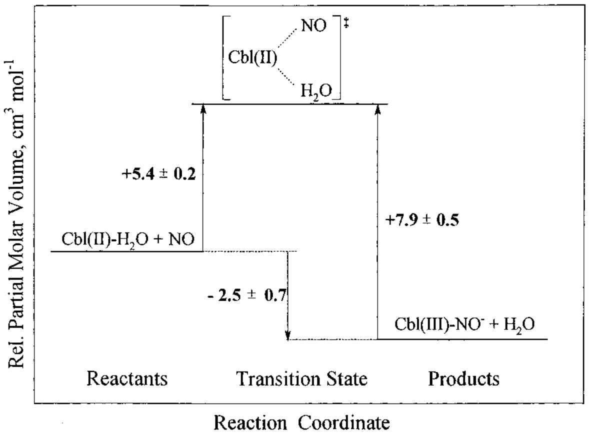

Figure 6. Volume profile for the photoinduced reaction Cbl(II)−H_2O + NO $\rightleftarrows$ Cbl(III)−NO^- + H_2O at pH 7.4.

It proved difficult to verify the actual rate of thermal process. Preliminary experiments showed that the reaction between intact cob(II)alamin and NO is much too fast to be observed on a stopped-flow time scale and it is difficult to conceive another kinetic technique that will allow to follow this reaction. However, the value of the overall equilibrium constant, $K_{NO} = (3.1 \pm 0.6) \times 10^7\ M^{-1}$ (20 °C, pH 7.4, 0.1 M Tris-$HClO_4$) was measured directly through a combination of spectrophotometric and potentiometric techniques.[37] Since the overall equilibrium constant determined in a nonkinetic way equals k'_{on}/k_{off} and the kinetically measured $k_{off} = 3.40 \pm 0.05\ s^{-1}$ (20 °C, pH 7.4), k'_{on} can be estimated as $(1.0 \pm 0.3) \times 10^8\ M^{-1}\ s^{-1}$. Although the k'_{on} value calculated in this way is a bit lower than the value obtained in the laser flash experiments under similar experimental conditions (viz., 6.1 × $10^8\ M^{-1}\ s^{-1}$, 20 °C, pH 7.4, 0.1 M Tris-$HClO_4$), it may be subjected to relatively large errors as a result of uncertainty in the determination of the K_{NO} value.[37] For that reason the difference between k_{on} and k'_{on} does not allow a quantitative comparison of the thermal and photoinduced reaction. However, it may be concluded in a qualitative way that the k'_{on} value is close to that observed in our laser flash experiments. Thus, the kinetics of the thermal reaction is not expected to differ significantly from that measured in our studies.

Reaction between Cbl(II) and NO_2^-. To study the possible influence of nitrite impurities on the investigated system, additional experiments with Cbl(II)/NO_2^- at pH ≥ 4 (i.e., where Cbl(II) is in the α-DMBI-coordinated, "base-on" form[38]) were performed. Addition of nitrite to Cbl(II) solutions under strictly

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(35) Schneppensieper, T.; Wanat, A.; Stochel, G.; Goldstein S.; Meyerstein, D.; van Eldik, R. *Eur. J. Inorg. Chem.* **2001**. In press.

(36) (a) Morlino, E. A.; Walker, L. A.; Sension, R. S.; Rodgers, M. A. *J. Am. Chem. Soc.* **1995**, *117*, 4429. (b) Hoshino, M.; Konishi, R.; Tezuka, N.; Ueno, I.; Seki, H. *J. Phys. Chem.* **1996**, *100*, 13569.

(37) The equilibrium NO concentrations in solution were measured with the use of an NO electrode (Isolated Nitric Oxide Meter, WPI Instruments, model ISO-NOP200) in solutions of a known Cbl(III)−NO^-/Cbl(II) concentration ratio (determined by UV−vis). The measured equilibrium constant is high, and therefore the equilibrium concentrations of NO in solution were close to detection limits of the electrode, i.e., in the nanomolar concentration range. Although the given K_{NO} is a mean value of several determinations, it may be subjected to experimental uncertainty larger than the calculated standard deviation. However, this value is in reasonable agreement with that reported recently in the literature.[3]

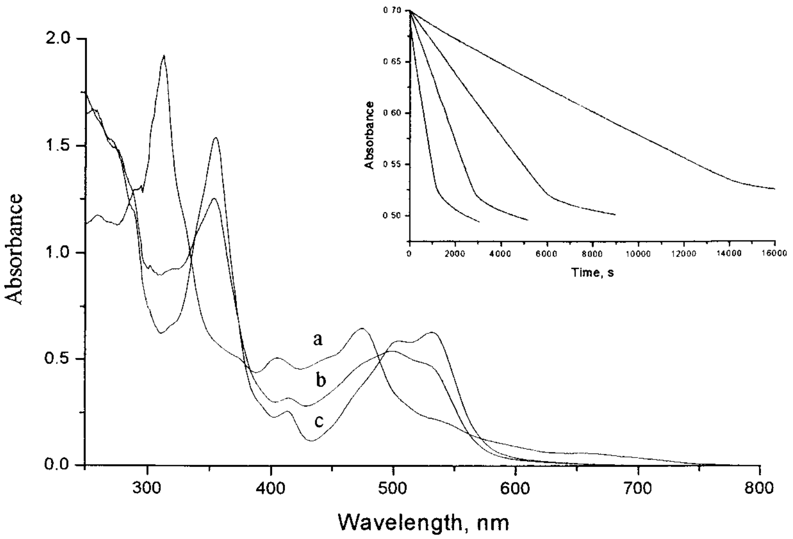


Figure 7. Spectral changes observed for the reaction of Cbl(II) with NO_2^-: (a) [Cbl(II)] = 1 × 10^{-4} M, pH 5.5, (b) after the reaction with NO_2^-, (c) after oxidation of the final reaction mixture with air. (Inset) Typical kinetic traces recorded as a function of nitrite concentration at pH 5.5 (λ_{obs}= 455 nm).

oxygen-free conditions resulted in significant UV−vis spectral changes (Figure 7). These indicate that Cbl(II) is converted to a mixture of Cbl(III)−NO_2^- (formed in the reaction of oxidized cobalamin with excess NO_2^- see reaction 9) and Cbl(III)−NO^- (presented in Figure 7, spectrum b). It follows that nitrite can partially oxidize Cbl(II) to Cbl(III) and simultaneously produce NO, which rapidly reacts with a second Cbl(II) molecule as described above. The very rapid binding of NO to form Cbl(III)−NO^- accounts for the partial (approximately 50%) oxidation of Cbl(II) even in the presence of a large excess of nitrite. The overall reaction (in the presence of an excess of NO_2^-) may therefore be presented by the following reactions:

$$2\text{Cbl(II)} + NO_2^- + 2H^+ \rightarrow \text{Cbl(III)}{-}H_2O + \text{Cbl(III)}{-}NO^- \quad (8)$$

$$\text{Cbl(III)}{-}H_2O + NO_2^- \overset{K}{\rightleftarrows} \text{Cbl(III)}{-}NO_2^- + H_2O \quad (9)$$

The kinetics of reaction 8 was studied under pseudo-first-order conditions with NO_2^- in at least 10-fold excess. The observed reaction rate strongly increased with decreasing pH and increasing total nitrite concentration, which can be accounted for in terms of the increasing HONO concentration. Typical absorbance/time plots recorded at 455 nm[39] are shown in the inset in Figure 7. The observed traces were typical for a pseudo-zero-order reaction and exhibited a sudden inflection point when a 1:1 [Cbl(III)−NO_2^-]:[Cbl(III)−NO^-] product mixture was formed. The observed zero-order rate constant increased linearly with $[HONO]^2$ (as demonstrated by the plot of k_{obs} versus $[HONO]^2$ in Figure 8) and exhibited a substantial dependence on pH. In analogy to the reaction of $[Fe^{II}(edta)(H_2O)]^{2-}$ with nitrite described in the literature,[40] we conclude that the rate-determining step for the pseudo-zero-order path involves formation of NO^+ from two molecules of HONO (presumably via the formation of N_2O_3 which decomposes to NO^+ and NO_2^-), as shown in the second reaction in Scheme 5, and subsequent rapid reaction of NO^+ with Cbl(II).

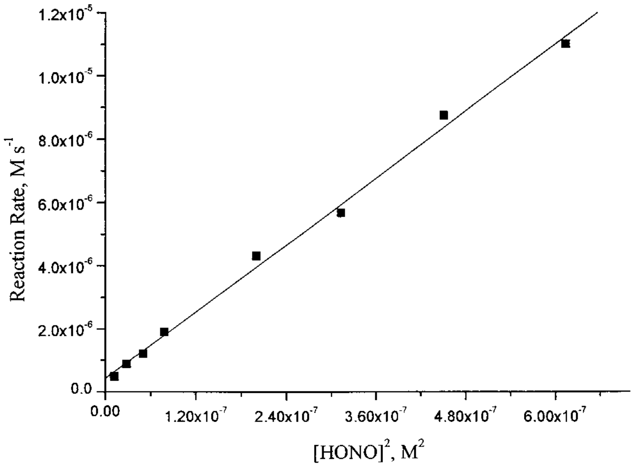
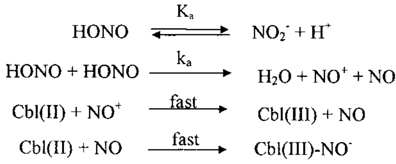


Figure 8. Dependence of the reaction rate on $[HONO]^2$ for the reaction of Cbl(II) with NO_2^-. Experimental conditions: pH = 4.0, [Cbl(II)] = 5 × 10^{-5} M, [NO_2^-] = (1−7) × 10^{-3} M, 25 °C.

Scheme 5



Taking into account that [HONO] = $[NO_2^-]_{total}[H^+]/([H^+] + K_a)$, the pseudo-zero-order rate constant for the mechanism outlined in Scheme 5 can be expressed as in equation 10.

$$\text{Rate} = 2k_a\{[NO_2^-]_{total}[H^+]/([H^+] + K_a)\}^2 \quad (10)$$

The value of $k_a = 18 \pm 1\ M^{-1}\ s^{-1}$ obtained from the slope in Figure 8 is in fair agreement with that reported in the literature for the formation of NO^+ from nitrous acid.[40] Comparison of the standard reduction potentials of HONO and "base-on"

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(38) Redox reactions of Cbl(II) are influenced by the equilibrium between the α-DMBI protonated "base-off" and ring-closed "base-on" forms, but the latter species is considered to be more important in the context of this study.

(39) At this wavelength the extinction coefficients of Cbl(II) and Cbl(III)−NO^- are almost identical, and the decrease in absorbance was only due to oxidation of Cbl(II) to Cbl(III) (i.e., was not affected by the rapid subsequent reaction of Cbl(II) with NO to Cbl(III)−NO^-).

(40) Zhang, V.; Kotowski, M.; van Eldik, R. *Inorg. Chem.* **1988**, *27*, 3279 and references therein.

Cbl(II) (+0.99[40] and +0.20[10] V vs NHE, respectively) suggests that in analogy to the Fe^{II}(edta) system, oxidation of Cbl(II) could also proceed via a pseudo-first-order path. The rate-determining step for this reaction route would involve the direct reduction of HONO by Cbl(II).

$$\mathrm{HONO} + \mathrm{Cbl(II)} + \mathrm{H}^+ \xrightarrow{k_b} \mathrm{Cbl(III)} + \mathrm{NO} + \mathrm{H_2O} \quad (11)$$

$$-\mathrm{d[Cbl(II)]}/\mathrm{d}t = 2k_b\mathrm{[Cbl(II)][HONO]} \quad (12)$$

However, a contribution of the pseudo-first-order pathway was not observed under the experimental conditions employed (i.e., pH 4.0, $[NO_2^-]_{total} = 0.001-0.007$ M, and pH 7.4, $[NO_2^-]_{total} = 0.1-0.5$ M). This indicates that oxidation of Cbl(II) by NO^+ is a very efficient process and the observed spectral changes were therefore controlled by the rate-determining production of NO^+.

Irrespective of the details of the reaction mechanism, the experiments performed clearly show that the reaction of reduced cobalamin with NO_2^- is several orders of magnitude slower than the reaction with NO. At pH above 4.5 and in the presence of relatively low (milimolar) nitrite concentrations, the reaction occurred in minutes to hours. The meaningful but very slow spectral changes at physiological pH (7.4, 0.1 M Tris-$HClO_4$) have only been observed for high (>0.1 M) nitrite concentrations. We therefore conclude that nitrite impurities (although presumably present in the Cbl(II) + NO reaction system) did not affect the kinetics of NO binding and release from reduced cob(II)alamin under the experimental conditions employed in this study.

Conclusions

In summary, the present study provides kinetic and mechanistic information on the reaction of nitric oxide with the reduced form of aquacobalamin (cob(II)alamin). Formation of the Cbl(III)−NO^- adduct in aqueous solution has been shown to occur on the microsecond time scale with a high binding constant ($K_{NO} \approx 3 \times 10^7$ M^{-1}). The reaction product formed at physiological pH has been characterized with the use of several spectroscopic techniques (UV−vis, ^{1}H-, ^{15}N-, and ^{31}P NMR) as a diamagnetic Co(III) complex containing a strongly bent NO^- ligand. The combination of ambient and high-pressure kinetic techniques (i.e., laser flash photolysis, stopped-flow, and ^{17}O NMR) provided key insight into the mechanism of the reaction. In particular, the nature of the reactive aqua intermediate generated in the laser flash could be resolved. Although the nature of this intermediate differ to some extent from that anticipated in the thermal reaction, the kinetics of the thermal and photoinduced nitrosylation of reduced vitamin B_{12} is not expected to differ significantly.

This study provides further chemical evidence that Cbl(II), rather than its oxidized form, efficiently binds NO to form the stable Cbl(III)−NO^- complex. This process may contribute to the observed biological interactions of vitamin B_{12} with NO.

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Supporting Information Available: ^{1}H- and ^{15}N NMR spectra of nitrosylcobalamin (at $pH_{app} = 5.4$ and 7.5, respectively), temperature- and pressure dependence of k_{on} obtained from laser flash photolysis studies, temperature dependence of the reduced relaxation time $1/T_{2r}$, and the corresponding pressure dependence of k_{ex} for ^{17}O water exchange on "base-off" cobalamin (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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