Redetermination of the X-ray structure of nitroxylcobalamin: base-on nitroxylcobalamin exhibits a remarkably long Co–N(dimethylbenzimidazole) bond distance†

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The X-ray structures of three new crystals of nitroxylcobalamin (NOCbl) have been determined. Unlike our earlier reported structure in which NOCbl was partially oxidized (L. Hannibal, C. A. Smith, D. W. Jacobsen and N. E. Brasch, *Angew. Chem.*, *Int. Ed.* 2007, **46**, 5140), the O atom of the nitroxyl ligand is located in a single position with a N═O bond distance of 1.12–1.14 Å, consistent with a double bond. The Co–N–O angle is in the 118.9–120.3 Å range. The α-axial Co–N(dimethylbenzimidazole) (Co–NB3) bond distance is a remarkable 2.32–2.35 Å in length, ~0.1 Å longer than that reported for all other cobalamin structures. The change in the Gibbs free energy for the base-on/base-off equilibrium now correlates extremely well with the Co–NB3 bond distance, as observed for other cobalamins.

Introduction

The nitric oxide derivative of vitamin B_{12}, nitroxylcobalamin (NOCbl; also known as nitrosylcobalamin and nitrosocobalamin) is of considerable interest to chemists and biochemists alike. Cobalamins inhibit nitric oxide (NO)-induced physiologies and pathologies (smooth muscle relaxation,[1–3] vasodilation,[4] NO-mediated inhibition of cell proliferation,[5] NO-induced neural tube defects[6]) and both mammalian B_{12}-dependent enzymes are inhibited by NO or NO donors.[7–11] It has been suggested that *in vivo* Cbl directly scavenges NO to form NOCbl,[5,12] and indeed, the reaction between a major intracellular form of Cbl, cob(II)alamin, and NO to form NOCbl approaches the rate of diffusion and is thermodynamically favorable ($K \sim 1 \times 10^8$ M^{-1}, 25 °C).[13,14] The therapeutic potential of Cbls and related cobinamides for treating diseases associated with high NO levels is currently under investigation.[2,6,12,15–17]

Recently we reported the X-ray structure of NOCbl.[18] The NO ligand was bound to the cobalt center through the nitrogen atom in a bent configuration (O–N–Co ranged from 117.4–121.4°), consistent with Co(III)–NO$^-$. During the initial stages of structure refinement, only one position for the NO oxygen was observed in the electron density; however in the latter stages of refinement two smaller difference peaks were also observed near the nitrogen and all three were modeled as alternate conformations of the oxygen atom. Evidence for the presence of a NO$^-$ group as opposed to an oxidized NO_2 came from a comparison of the N–O bond and Co–NB3 bond distances of NOCbl with those of three reported NO_2Cbl complexes. The NO bond lengths for the three orientations were consistent with a N═O double bond rather than the N–O/N═O bonds of nitrite. In addition, the Co–NB3 bond length for NOCbl was 2.13 Å, whereas in the three NO_2Cbl it ranged from 1.99–2.01 Å. NO$^-$ exerts a greater *trans* influence compared with NO_2^-; hence the longer Co–NB3 bond length in NOCbl compared with NO_2Cbl. However, the subsequent realization that the three locations of the O atom in this structure could alternatively be modeled as a mixture of NOCbl and NO_2Cbl motivated us to structurally characterize several new NOCbl crystals. We therefore now wish to report the structures of three new NOCbl crystals from different batches. The new structures show that base-on NOCbl has a remarkably long Co–N(dimethylbenzimidazole) bond length – a bond distance that is significantly longer than that reported for all other base-on cobalamins.

Experimental

Synthesis of NOCbl

Both the synthesis and handling of NOCbl were carried out inside a glove box under an argon atmosphere. NOCbl was synthesized according to a published procedure.[19] Formation of the desired product was checked by UV-vis and ^{1}H NMR spectroscopies.[18]

Crystallization of NOCbl

NOCbl was crystallized by the addition of anaerobic acetone (300 μL) to vials containing concentrated NOCbl solution (50 μL, 2.91 mM) in anaerobic water. The vials were capped (teflon sleeves with screw caps) and a syringe needle inserted through the top of the caps. The vials were kept in the freezer inside the glove box (–30 °C). Small orange crystals of NOCbl grew after 4 weeks in the vials.

X-ray diffraction studies

Three crystals of NOCbl (crystal 1, 0.15 × 0.05 × 0.05 mm; crystal 2, 0.08 × 0.04 × 0.03 mm; crystal 3, 0.22 × 0.07 × 0.05 mm)

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Table 1 Crystal data and structure refinement parameters for the three NOCbl crystals

Parameter	NOCbl-1	NOCbl-2	NOCbl-3
Wavelength/Å	0.79987	0.85503	0.91840
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Images/oscillation angle	90/1.0	180/1.0	95/1.0
Reflections, collected/unique	29732/15650	39986/10203	19976/9742
Maximum resolution/Å	0.73	0.84	0.89
R_{merge}, R_{sym}	0.0195, 0.0482	0.0451, 0.0471	0.0215, 0.0485
Cell dimensions/Å	$a = 15.96$	$a = 15.94$	$a = 15.93$
	$b = 20.93$	$b = 20.92$	$b = 21.08$
	$c = 24.13$	$c = 24.04$	$c = 24.11$
V/Å	8060.5(7)	8016.5(7)	8096.2(7)
Z	4	4	4
μ/mm^{-1}	0.296	0.298	0.295
F(000)	2912	2912	2912
Empirical formula	$C_{62}H_{89}CoN_{15}O_{15}P\cdot 15H_2O$	$C_{62}H_{89}CoN_{15}O_{15}P\cdot 15H_2O$	$C_{62}H_{89}CoN_{15}O_{15}P\cdot 17H_2O$
FW/g mol^{-1}	1539.72	1565.35	1598.83
Limiting indices	$-16 \le h \le 19$	$-17 \le h \le 17$	$-15 \le h \le 15$
	$-22 \le k \le 27$	$-23 \le k \le 22$	$-19 \le k \le 22$
	$-25 \le l \le 29$	$-26 \le l \le 24$	$-23 \le l \le 26$
Refinement resolution/Å	0.75	0.90	0.89
Data/restraints/parameters	17097/0/997	11465/2/998	10616/2/1021
GOF on F^2	1.035	1.048	0.999
Flack parameter	0.075	0.066	0.091
R factors ($F > 4\sigma(F)$)	$R_1 = 0.0640$	$R_1 = 0.0690$	$R_1 = 0.0556$
	w$R_2 = 0.1856$	w$R_2 = 0.1999$	w$R_2 = 0.1603$
R factor (all data)	$R_1 = 0.0695$	0.0779	0.0610
Largest difference peak and hole/e Å^{-3}	+0.77 and −1.41	+0.69 and −0.65	+0.48 and −0.44

were mounted under paraffin oil in a nylon loop and flash cooled in liquid nitrogen. The diffraction data for the structures were collected at beam lines BL9-2 and BL11-1 at the Stanford Synchrotron Radiation Lightsource (SSRL). Data were collected at 100 K on a MarMosaic 325 CCD detector using X-rays produced either by a 16 pole wiggler insertion device using a liquid nitrogen cooled double Si(111) crystal monochromator (BL9-2) for NOCbl-1 and NOCbl-2 (X-ray energy 15500 eV and 14500 eV respectively), or by a 26 pole wiggler using a side-scattering monochromator (BL11-1) for NOCbl-3 (X-ray energy 13500 eV). In all cases, the data were processed with the program XDS and scaled with the program XSCALE.[20] Bijvoet pairs were not merged and no absorption correction was applied. Data collection statistics are given in Table 1.

The structures were solved by the Patterson method to locate the cobalt and phosphorus atoms, then the lighter atoms located by difference Fourier synthesis, as implemented in the program SHELXS.[21] The structures were refined by full matrix least-squares on F^2 using SHELXL. All non-hydrogen atoms were refined with anisotropic atomic displacement parameters (ADPs), and hydrogen atoms were added in idealized positions and refined in riding positions. A correction for anomalous scattering from cobalt at the appropriate energy was applied during refinement for each crystal. Additional difference electron density peaks were initially modeled as water molecules with site occupancy factors (SOFs) of 1.0, and in later stages of refinement, the occupancies of some of these water molecules were refined. The final crystallographic data collection and refinement are given in Table 1.

Results and discussion

Multiple batches of new NOCbl crystals were prepared and X-ray diffraction data collected for three new NOCbl crystals (NOCbl-1 to NOCbl-3) from separate batches at the SSRL. The three structures were refined to low crystallographic R-factors using SHELXL.[21] Importantly, the three new structures are consistent with each other, but markedly different from the original NOCbl structure. Table 1 summarizes crystallographic and refinement data for the three new structures. NOCbl·nH_2O crystallizes in the orthorhombic space group $P2_12_12_1$ with one molecule per asymmetric unit. NOCbl-1 was the best resolved of the three structures, with diffraction data to a nominal resolution of 0.75 Å and a final R value of 0.0640. Fig. 1 shows a thermal ellipsoid plot of NOCbl-1. The solvent structure of NOCbl-1 has been modeled as 15 water molecules, all of which are involved in hydrogen-bonding interactions with either oxygen or nitrogen atoms on the cobalamin moiety. Table 2 summarizes bond distances for the first coordination sphere of the Co for the new and original NOCbl structures and for the three reported NO_2Cbl structures. The Co lies essentially on the plane of the four corrin nitrogen atoms (0.036 Å away for NOCbl-1). Importantly, unlike in the previous NOCbl structure in which the O atom of the β-axial ligand was observed in three alternate positions, the O atom of the NO ligand (N70–O71) is located in a single position for NOCbl-1 (Fig. 1) and the other two new NOCbl structures. Although there is some evidence for two additional peaks in the F_o–F_c difference electron density at very low levels (approximately 0.6 e/Å^3) near the NO nitrogen, both peaks are approximately 1.1 Å from the nitrogen and have site occupancy factors (SOFs) estimated at 0.1 (based upon the electron density for the fully occupied NO oxygen atom of 5.5 e/Å^3), implying partially-occupied alternate conformations of the NO oxygen. The Co–N–O angle for the three structures ranges from 119.4 to 120.3° (Table 2), providing support for the oxidation state of the Co center being Co(III), with the bent orientation being a result of the lone pair on the N atom of NO^-.[18] The Co–N–O bond is linear for nitrosyl (NO^+)

Table 2 Co coordination sphere, the N–O bond distances (Å) and Co–N–O bond angles for the new and original NOCbl structures and for NO_2Cbl

	NOCbl-1[a]	NOCbl-2[a]	NOCbl-3[a]	NOCbl (old)[b]	$NO_2Cbl.2LiCl$[c]	$NO_2Cbl.NaCl$[c]	NO_2Cbl[d]
Co–NO_n (Å) ($n = 1,2$)	1.907(2)	1.909(8)	1.940(8)	1.927(6)	1.942(6)	1.912(5)	1.941(5)
N–O (Å)	1.141(4)	1.12(1)	1.14(1)	1.18(1)[e] 1.20(2) 1.14(1)	1.217(9) 1.324(15)[f]	1.221(8)	1.217
Co–NB3 (Å)	2.351(2)	2.353(7)	2.324(6)	2.126(5)	1.992(6)	2.014(5)	2.008(4)
Co–N21 (Å)	1.877(2)	1.867(6)	1.867(7)	1.858(4)	1.873(5)	1.868(5)	1.888(4)
Co–N22 (Å)	1.917(2)	1.911(6)	1.904(6)	1.908(5)	1.920(4)	1.902(5)	1.917(4)
Co–N23 (Å)	1.916(2)	1.911(6)	1.899(6)	1.915(4)	1.919(5)	1.900(5)	1.922(4)
Co–N24 (Å)	1.898(2)	1.877(6)	1.897(6)	1.887(5)	1.894(5)	1.870(5)	1.906(4)
Co–N–O	119.4(2)	118.9(8)	120.2(8)	117.4 121.3 121.4	121.7, 115.4 121.3,[f] 125.6	124.4 120.1	121.2 117.1 129.1[f] 119.5[f]

[a] This work. [b] Reference 18 [c] Reference 29. [d] Reference 30. [e] Three N–O bond lengths were reported. [f] Two orientations of the NO_2 group were observed.

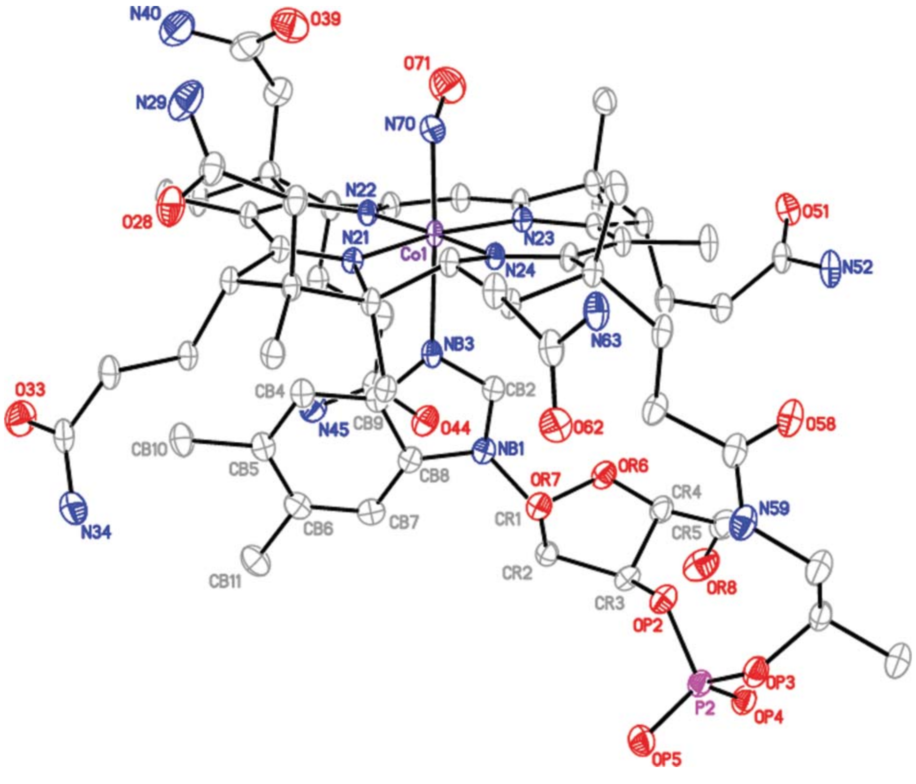


Fig. 1 Thermal ellipsoid plot (30%) of NOCbl-1.

complexes.[22] Whereas the N70–O71 bond length ranged from 1.14 to 1.20 Å in the original structure, the N70–O71 bond length is between 1.12 and 1.14 Å for the three new structures, consistent with an N═O bond. In the NO_2Cbl structures the N70–O71 bond lengths are much longer (1.22–1.32 Å), consistent with a delocalized O═N–O bond. Another notable difference between the earlier NOCbl structure and the three new structures is the Co–NB3 bond distance, which ranges from 2.32 to 2.35 Å. This is ~0.2 Å longer than the Co–NB3 bond distance in the original NOCbl structure (2.13 Å), and ~0.3 Å longer than those found in the three NO_2Cbl structures (1.99–2.01 Å). The superimposition of the original structure (white) and the NOCbl-1 structure (green and yellow) is shown in Fig. 2. The difference in the position of the 5,6-dimethylbenzimidazole (DMB) base is clearly observable. There are no significant differences in the Co–N(equatorial) (Co–N21 to Co–N24) or the Co–N(O) bond distances in the new structures compared with the original structure (Table 2).

The *trans* influence is a key factor in determining the length of axial bonds in cobalamins incorporating non-bulky axial ligands.[23] Thus far the longest Co–NB3 bond distances reported are those for Cbls incorporating the strong σ alkyl donor ligands methyl, 5′-deoxyadenosyl, vinyl and difluoromethyl (2.16–2.19, 2.24, 2.17 and 2.19 Å, respectively [24–27]) at the β-axial site. The Co–NB3 bond lengths for Cbls with typical inorganic ligands such as CN^-, NO_2^-, H_2O and RS^- (RS = γ-GlyCys and N-acetyl-L-cysteine) are significantly shorter at 2.01–2.04, 1.99–2.01, 1.93, 2.05–2.06 Å, respectively.[26,28–33] In pentammine complexes of Co, $[CoX(NH_3)_5]^{n+}$, NO was found to exert an even stronger *trans*

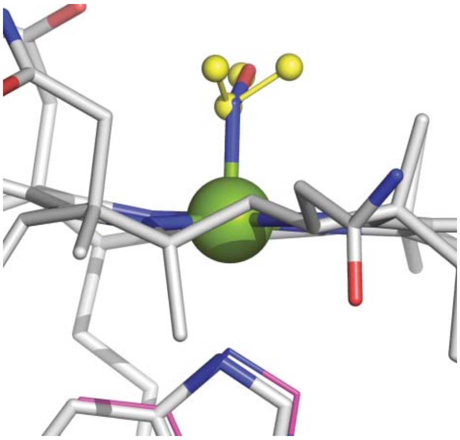
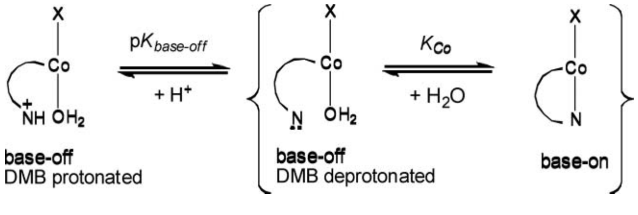


Fig. 2 Superposition of the NOCbl-1 structure (white, thicker bonds) with the original NOCbl structure (thinner bonds, yellow balls for the NO and magenta sticks for the DMB base). The cobalt in NOCbl-1 is shown as a green sphere. The difference in the position of the DMB base is evident.

influence than Me.[31] The longer Co–NB3 for NOCbl can be attributed to the strong σ donor properties of this ligand in addition to NO^- being a moderately good π acceptor ligand.[34] There was no evidence for a deprotonated, base-off NOCbl species in the X-ray diffraction electron density map for NOCbl.

The observation of disorder in the position of the oxygen atom of the NO ligand, the longer N–O bond distances and the much shorter Co–NB3 bond length for the original NOCbl structure compared with the three new NOCbl structures, provides compelling evidence that the crystal used in the original X-ray structural determination of NOCbl was indeed partially oxidized to NO_2Cbl, and therefore represents a mixture of NOCbl and NO_2Cbl structures. It was originally assumed that the disorder for the ligand in the reported structure could be simply represented by three alternate conformations of the NO oxygen with SOFs of 0.5, 0.3 and 0.2. However retrospective analysis of the structure, assuming the presence of NO_2Cbl, gives a model for the disorder consistent with a β-axial NO_2 ligand and two locations for the NO ligand of NOCbl with an average SOF of approximately 0.25 for the NO. Based upon the bond distances in the three new NOCbl structures, and this new analysis of the original SOFs and ADPs, a NO/NO_2 ratio of ~0.25 : 0.75 can be estimated for the original NOCbl crystal. Importantly, full occupancy is observed for all atoms of the DMB; hence NOCbl is base-on and there is no evidence for a base-off component in the structures.

Co–NB3 and the $pK_{base\text{-}off}$ value for the base-on/base-off equilibrium for NOCbl, Scheme 1, are atypical for Cbls with inorganic ligands ($pK_{base\text{-}off}$ = 5.1 for NOCbl *versus* 0.1, ≤ −0.15 and −2.13 for CNCbl, NO_2Cbl and H_2OCbl^+, respectively [13,19,35]). A linear relationship between the apparent Gibbs free energy for coordination of the DMB to cobalt, $-\Delta G_{Co}^\circ$, and the Co–NB3 bond distance, $d_{Co\text{-}NB3}$, has previously been established.[24,36] Using K_{Co} = 1.9,[19] a value of 0.38 kcal mol^{-1} is calculated for NOCbl ($\Delta G_{Co}^\circ = RT\ln K_{Co}$). Fig. 3 gives the plot of $-\Delta G_{Co}^\circ$ *versus* $d_{Co\text{-}NB3}$, incorporating the new Co–NB3 value for NOCbl. The agreement of the NOCbl data with the line of best fit is quite remarkable, and the NOCbl data is similar to that expected for alkylcobalamins. Another established correlation is that between Co–NB3 and the ^{31}P NMR chemical shift of the nucleotide phosphodiester moiety of Cbls.[35,36] The ^{31}P NMR chemical shift reflects changes in the phosphodiester bond angles, which become less strained as the Co–NB3 bond lengthens.[35,37] A plot of ^{31}P NMR chemical shift *versus* Co–NB3 bond distance, $d_{Co\text{-}NB3}$, is given in Fig. 4. Again the correlation points to NOCbl being more similar to an alkylcobalamin rather than other Cbls with inorganic ligands. It has previously been noted that while NOCbl has a single ^{31}P NMR resonance under neutral and acidic conditions as observed for alkylcobalamins, CNCbl and H_2OCbl^+ instead



Scheme 1 Cobalamin forms present in aqueous solution.

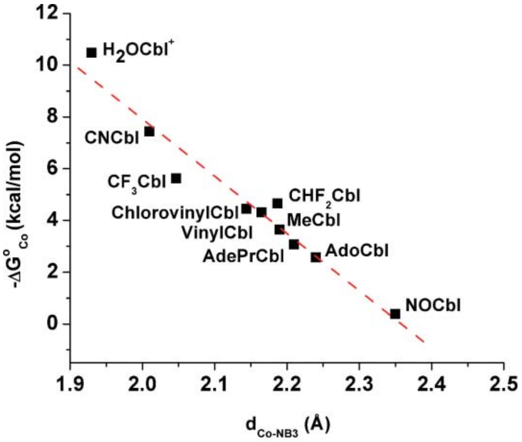


Fig. 3 Plot of $-\Delta G_{Co}^\circ$ *versus* the Co–NB3 bond distance for Cbls. The data has been fitted to a straight line (r^2 = 0.95) with slope = −22.1 ± 1.8 and intercept 52.2 ± 4.0. Co–NB3 bond lengths and $-\Delta G_{Co}^\circ$ are taken from the literature (see references in reference 19).

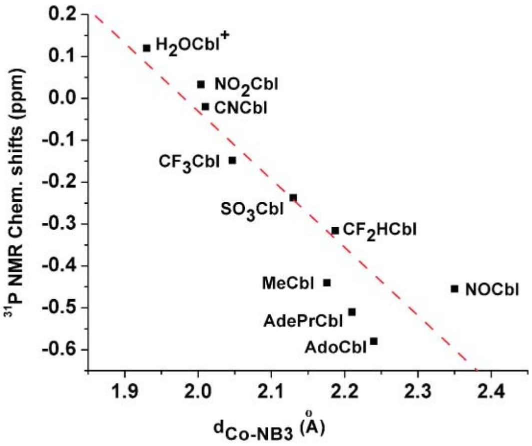


Fig. 4 Plot of ^{31}P NMR chemical shift *versus* the Co–NB3 bond distance for Cbls (in D_2O or H_2O, referenced to 85% H_3PO_4, 25 °C). The data has been fitted to a straight line (r^2 = 0.84) with slope = −1.74 ± 0.27 and intercept 3.46 ± 0.57. Data is taken from the references given in reference 19.

have two distinct peaks corresponding to the base-on and base-off, DMB protonated forms in acidic solution at pH (pD) values close to $pK_{\text{base-off.}}$[19]

To summarize, the structures of three new NOCbl structures have been determined by X-ray diffraction. Significant differences in the NO ligand N–O and Co–NB3 bond distances were observed compared to the original NOCbl crystal. These data provide evidence for substantial oxidation of the NOCbl crystal used in the original NOCbl X-ray structure determination.

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