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Abstract of the Ph.D. Thesis

Reactivity of vitamin B_{12} and related compounds with small molecules

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1. Introduction

Cobalamin (Figure 1) is one of the most complex vitamins in terms of structure. The description of the crystal structure by Hodgkin et al.[1] was a pioneering work in structural characterization of biomolecules, for which a 1964 Nobel prize was awarded.

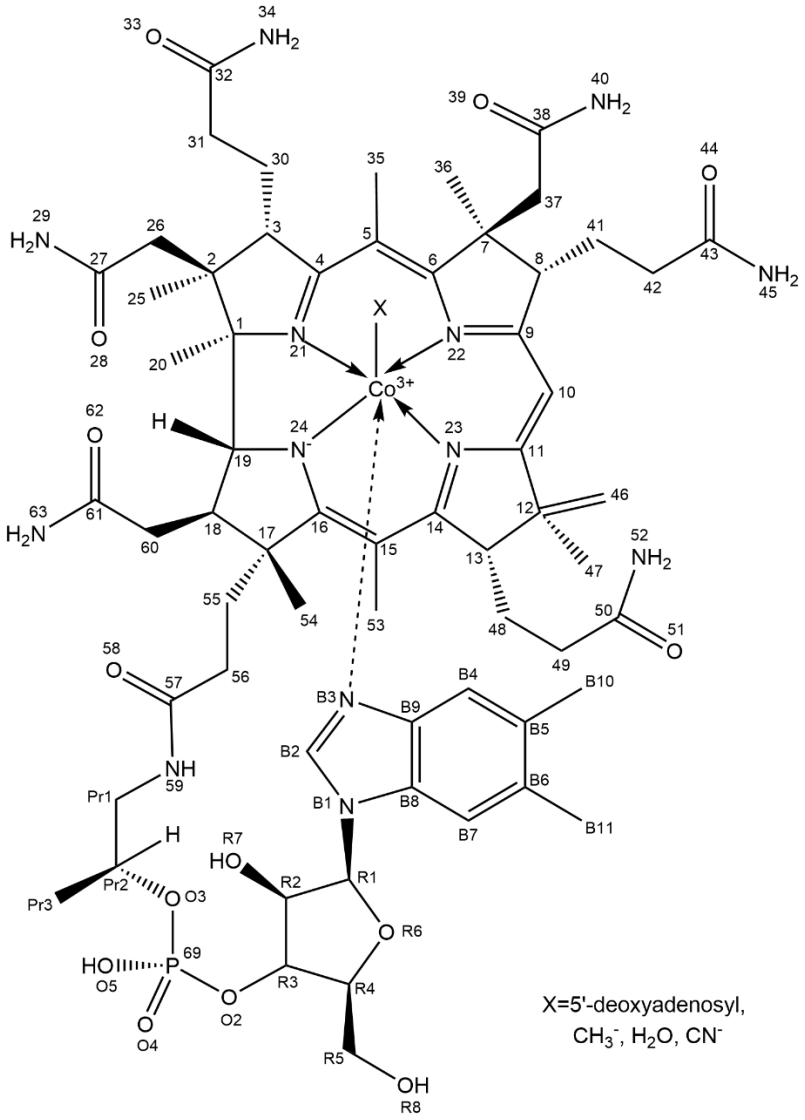

Figure 1. Structure of cobalamin and its most typical exogenous ligands.

The main structural characteristic of cobalamin is the cobalt ion, coordinated axially by the aromatic ring of the 5,6-dimethyl benzimidazole (DMBZ) and equatorially by the corrin

macrocycle.[2] The sixth coordination position of the cobalt is available for the coordination of a variety of groups. The most notable of these is $^{-}CH_3$; methylcobalamin (MeCbl) is a biologically active form of vitamin B_{12}, used as methyl carrier in the process of catalysis of methyltransferases. The transiently-bound methyl group in methylcobalamin facilitates the methylation process in various metabolic pathways including biosynthesis of methionine, methane, acetone and antibiotics such as carbapenems and thiostrepton. AdoCbl, adenosylcobalamin which is also a biologically active forms of vitamin B_{12} has a 5′-deoxyadenosyl group that is bonded to the cobalt from cobalamin through its 5'-carbon. AdoCbl facilitates the conversion of a toxic byproduct, methylmalonyl-CoA to succinyl-CoA through a radical-based isomerization reaction. Thus, both methylcobalamin and adenosylcobalamin contain a covalent cobalt-carbon bond, are rare examples of naturally occurring organometallic compounds. Another vitamin B_{12} form is cyanocobalamin (CNCbl), with a cyanide group as ligand. CNCbl is nonfunctional and has to be converted in vivo to MeCbl or AdoCbl for biological activity. It is usually used to prevent and treat vitamin B_{12} deficiency because it is the most accessible commercial form of cobalamin. In the absence of other ligands, vitamin B_{12} in aqueous solution binds a water molecule forming hydroxocobalamin or aquacobalamin; the latter predominates at physiological pH. Hydroxo/aqua cobalamin has an extremely high affinity for cyanide and forms cyanocobalamin, so it is employed for treating cyanide poisoning.

The B_{12} 5,6-dimethyl benzimidazole can exist in a coordinated (base-on) or free (base-off) conformation. The base on/off equilibrium plays an important role for the binding and catalytic activity of cobalamin within proteins, while the upper ligand directly affects the chemistry and biology of vitamin B_{12} cofactors. In general, cobalamin exits in solution in its base-on form, meaning that 5,6-dimethyl benzimidazole is coordinated to the corrin ring.[2] However, the Co-N coordination bond between and cobalt and DMBZ is not strong, and the nucleotide base can dissociate depending on pH, temperature and the oxidation state of the cobalt ion.

At pH<2.9 the DMBZ is replaced by a water molecule, cf. Figure 2. The redox potential of Cbl(III) is strongly pH dependent and remains independent only between 2.9 and 7.8. At pH < 2.9 the values are more positive and at pH > 7.8 the values are more negative. While at pH > 7.8 the aqua-ligand is deprotonated to hydroxide, at pH < 2.9 the 5,6-dimethyl benzimidazole attaches the proton and gives Cbl(III) base-off species.[2]

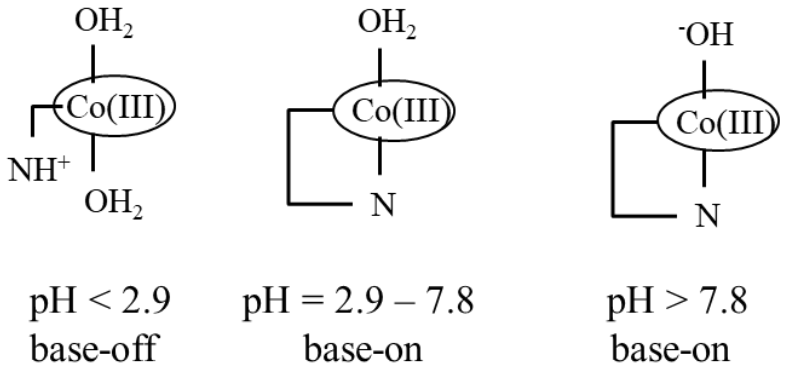

Figure 2. Base-off and base-on species of cobalamin.

Under reducing conditions, the Co(II) cobalamin is formed relatively easily. This form, dubbed B_{12r}, is pentacoordinated and paramagnetic (and hence detectable by electron paramagnetic resonance spectroscopy, EPR). This form is relatively inert and under anaerobic conditions, but oxidizes rapidly in the presence of molecular oxygen. Further reduction of B_{12r} by an additional electron leads to the super-reduced form, Co(I), which is tetracoordinated and dubbed B_{12s}. The Co(I) form is highly nucleophilic and a potent reactive reducing agent. Cob(I)alamin participates in methyl group transfer reactions, the dehalogenation of organic substrates, and the in vivo synthesis of coenzymes forms. The formation of Co(I) form is an important step in several catalytic systems, including cobalamins and their derivatives.[3] A summary of the coordination states described above is shown in Figure 3.

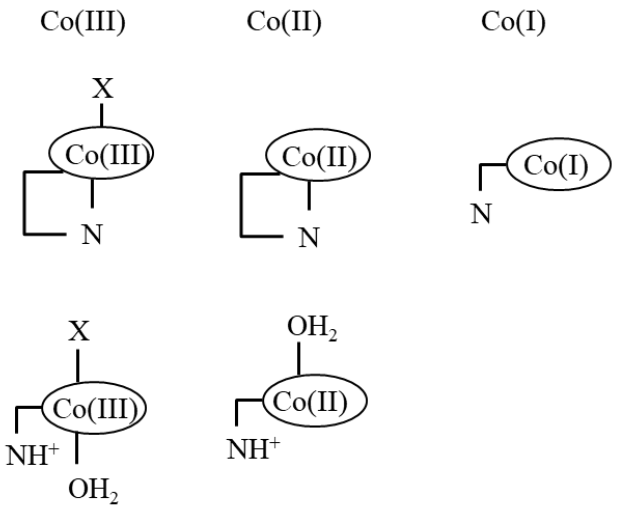

Figure 3. The coordination states of Co(III), Co(II) and Co(I)-cobalamins. [2]

Cobalamin was discovered in 1926, as the anti-pernicious anemia factor (though it was first isolated in 1948). Pernicious anemia is an autoimmune disease that results in a deficient production of intrinsic factor (IF), the protein that facilitates cobalamin absorption. This condition can cause severe vitamin B_{12} deficiency (hypocobalaminemia), even with an adequate dietary intake of the vitamin. Pernicious anemia is characterized by anemia, as well as neurological and digestive dysfunctions.

Humans have only two cobalamin-dependent enzymes: cytoplasmic methionine synthase and mitochondrial methylmalonyl-CoA mutase. Methionine synthase facilitates the transfer of a methyl group from N^5-methyltetrahydrofolate to homocysteine, generating tetrahydrofolate and methionine. Cobalamin acts as an intermediate carrier of the methyl group and cycles between methylcobalamin and cob(I)alamin. Methylmalonyl-CoA mutase uses the 5′-deoxyadenosylcobalamin form of the cofactor to catalyze the 1,2 rearrangement of methylmalonyl-CoA into succinyl-CoA.

Small-molecule exogenous ligands of cobalamin coordination chemistry have been characterized, some of these bear physiological relevance. Thus, nitric oxide (NO) is a gas formed signaling molecule and it occurs both outside and inside the body.[4] It can have multiple biochemical effects including reactions with metalloenzymes.[5] It is produced by the human body and it is one of the most important molecules for blood vessel health, because it increases blood flow and lowers blood pressure.[6,7] Nitroxyl (HNO) has a unique chemical and biological reactivity[8,9] by comparison with NO, for example it reacts directly with thiols. Like NO, it is a also a vasorelaxant. Nitrite (NO_2^-) is a monovalent inorganic anion and can change into nitric oxide[10] in the body. It has a high affinity for cobalamin. SCN^- (thiocyanate) is a significant biological anion.[11] In humans, it is present in millimolar concentrations in fluids produced by mucous membranes, and to a much lesser extent in the blood. CobIII)alamin reactions with sulfite and thiosulfate have also been studied, as well as Cbl(III) to Cbl(II) reduction by dithionite.

The present thesis aims to investigate the chemical reactivity of vitamin B_{12} towards oxidizing small molecules, including hydrogen peroxide, hypochlorite, chlorite, and m-chloroperoxybenzoic acid. These compounds play roles as intermediates in the homeostatic processes of living organisms. Investigating and pointing out new mechanisms for the reactivity of vitamin B_{12} with these small molecules, which have not been previously studied in this context,

could have significant implications for developing treatments for metabolic deficiencies or diseases, and any conditions related to the metabolism of this vitamin. The oxidizing agents taken into consideration as cobalamin ligands were chosen because of their biological relevance, and can reach relatively high concentrations in certain specialized cellular compartments – and also because their chemistry with related metallamacrocyles (such as hemes) is well known.

2. Interplay and physiological relevance of oxidants and antioxidants on the quintessential metallamacrocyclic moiety: the heme in hemoglobin[1]

Antioxidants, and ascorbate in particular, have long been a subject of ambitious (and at times unsupported) proposed medical treatments.[12] More recently, clinical trials were proposed for intravenous injections of ascorbate, against SARS-CoV-2 / COVID-19.[13]

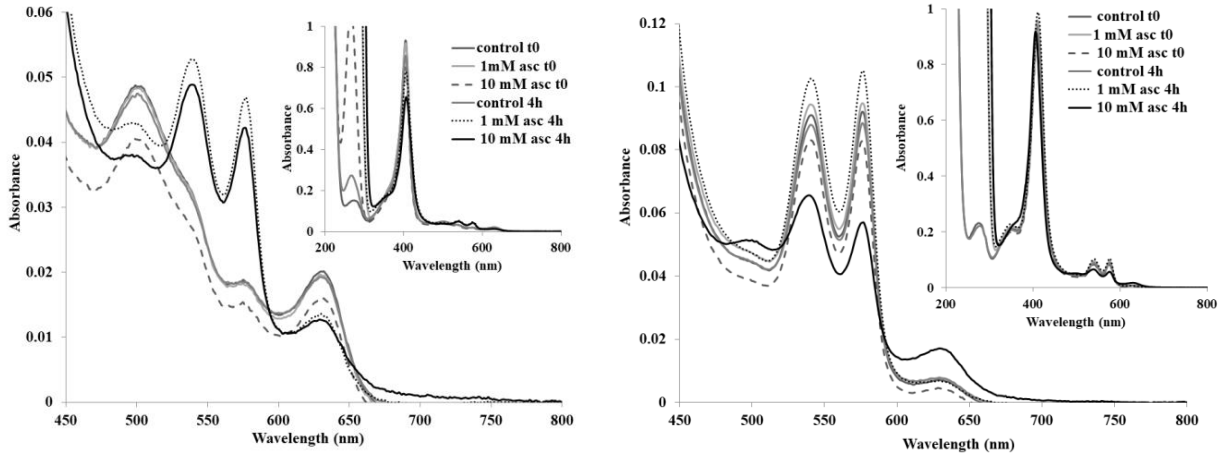

Figure 4. UV-vis spectra of hemoglobin (upper panel: ferric; lower panel: ferrous oxy) with varying concentrations of ascorbate. Conditions: 5 μM met and 7 μM oxy bovine hemoglobin, room temperature, 50 mM phosphate pH 7.

[1] Published as Lehene, Maria; Fischer-Fodor, Eva; Scurtu, Florina; Hădade, Niculina D.; Gal, Emese; Mot, Augustin C.; Matei, Alina; Silaghi-Dumitrescu, Radu. Excess ascorbate is a chemical stress agent against proteins and cells. Pharmaceuticals, 2020, 13(6), 107.

The spectra of oxy Hb with ascorbate indicate a distinctive effect, cf. Figure 4. Thus at 4 hours the 10 mM sample (but not the 1 mM) has halved its oxy maxima of 540 and 580 nm and has developed a strong 630-nm peak, characteristic of the ferric form. Excess ascorbate is thus an efficient promoter of Hb autooxidation. We have previously shown that at longer incubation times and/or with higher ascorbate concentrations, oxy Hb also develops spectral features of heme degradation, not only oxidation of the iron.[14]

Figure 5 shows SDS-PAGE data on Hb exposed to ascorbate; albumin was in this case also employed, as an alternative example of a blood protein that may be exposed to excess ascorbate. For the Hb-ascorbate sample, a slight decrease in the intensity of the bands due the Hb (monomer, dominant, and dimer, less intense) is seen at 4 hours – together with the appearance of a new band at lower molecular weight. For albumin, lower molecular-weight bands also appear to develop over time. These findings suggest that a minor part of the protein may be degraded at these ascorbate concentrations – and that the process is not unique to hemoglobin. Direct reaction of ascorbate/ascorbyl with molecular oxygen, or of ascorbyl with proteins, may be responsible for initiating such processes.

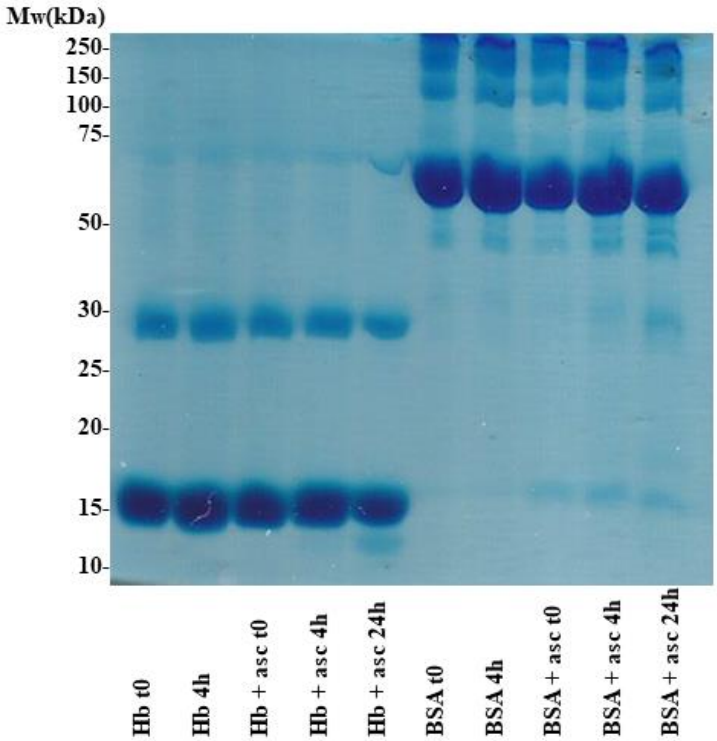

Figure 5. SDS-PAGE (12%) of hemoglobin and albumin exposed to ascorbate. Conditions: 50 µM Hb, 20 µM bovine serum albumin (BSA) were incubated at room temperature for indicated times with or without 10 mM ascorbate, in 50 mM phosphate, pH=7. The samples, from left to right, are: 1. Hb t0 (no incubation), 2. Hb 4h, 3.Hb + ascorbate t0, 4. Hb + ascorbate 4h, 5. Hb + ascorbate 24h, 6.BSA t0, 7.BSA 4h, 8.BSA + ascorbate t0, 9.BSA + ascorbate 4h, 10.BSA + ascorbate 24h.

3. The adduct of aquacobalamin with hydrogen peroxide [2]

Peroxide reactions with biologically-relevant transition metal centers often follows the paradigm defined by Fenton chemistry, where O-O bond cleavage occurs, generating strong oxidants such as hydroxyl radicals and high-valent metal species, and/or further modifying organic compounds either purposefully (as in peroxidases, cytochromes P450, non-heme iron oxygenases, iron and copper bleomycin, manganese oxygenases, vanadium-containing peroxidases and others) either as a secondary reaction.[15–18]

On the other hand, in some bioinorganic centers stable metal-peroxo complexes are formed – such as the cobalt-peroxo adduct of bleomycin.[19]

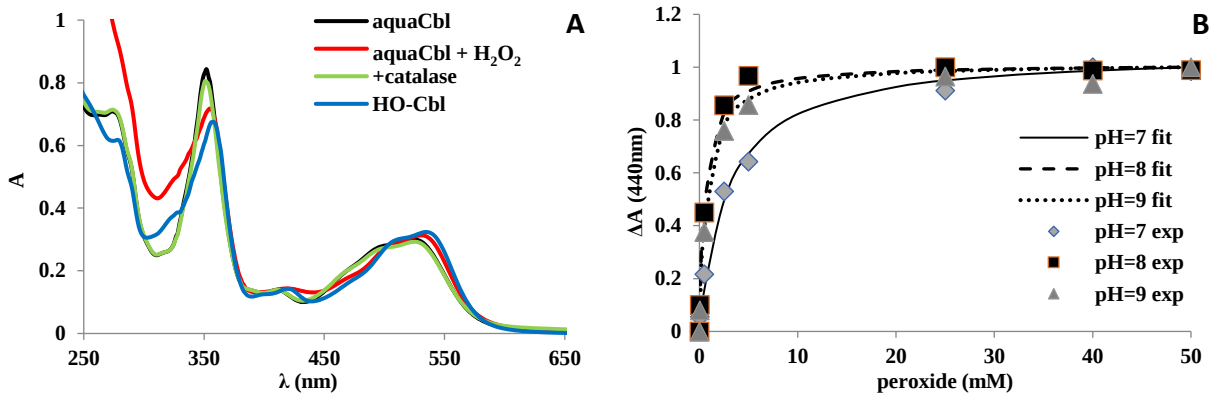

Figure 6. A: the effect of catalase on the UV-vis spectrum of the cobalamin-peroxide complex, at pH 7; shown for comparison is also the spectrum of hydroxo cobalamin (pH 11). **B**: titration curves

[2] Published as Lehene, Maria; Plesa, Diana; Ionescu-Zinca, Stefania; Iancu, Stefania D.; Leopold, Nicolae; Makarov, Sergei V.; Brânzanic, Adrian M.V.; Silaghi-Dumitrescu, Radu. Adduct of aquacobalamin with hydrogen peroxide. Inorganic Chemistry, 2021, 60(17), 12681-12684

for aqua cobalamin reacting with peroxide at pH 6, 7 and 8, monitored at 440 nm. Conditions: 0.05 mM Cbl, 22°C.

Figure 6 and 7 show that the UV-vis spectra of aquaCbl are affected by hydrogen peroxide in a concentration-dependent manner, with an apparent K_d of 0.25 mM at pH 7 (0.56 mM at pH 8, 0.78 mM at pH 9) - somewhat lower than those for nitrite or thiocyanate,[4,20] but much higher than for cyanide.[21,22] The cobalamin+peroxide spectrum is different from that of hydroxo cobalamin and is entirely reverted to aqua cobalamin by catalase, suggesting a reversible Cbl-peroxide.

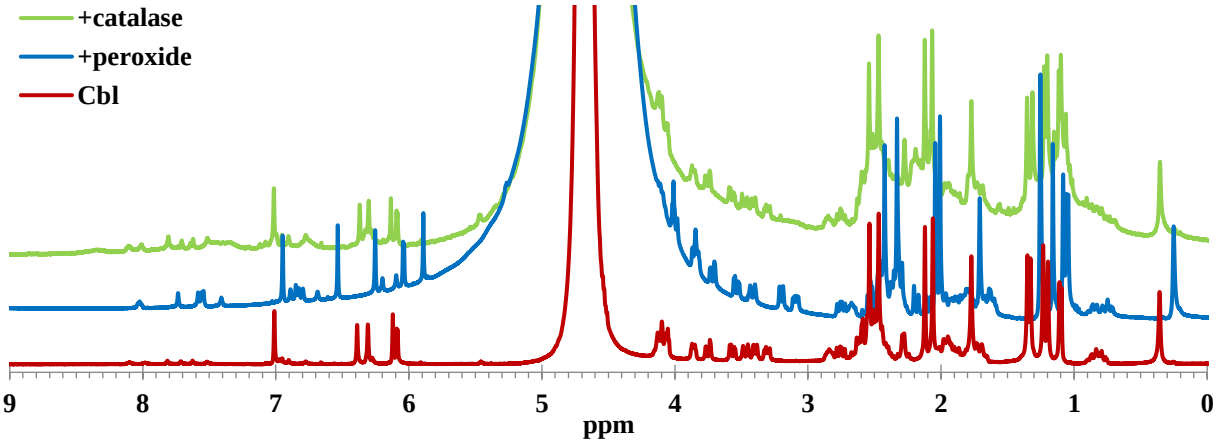

Figure 7. ^{1}H-NMR spectra of Cbl after exposure to peroxide and after subsequent addition of catalase. Conditions: 5 mM Cbl, 50 mM H_2O_2, 200 mM phosphate pH 7, 22°C.

The rate of the Cbl-peroxide was examined by stopped-flow UV-vis spectroscopy, as shown in Figure 8. The kinetics may be fit with a simple/single process A→B dependent on peroxide concentration, where species A is identical to the resting spectrum of Cbl while species B features the diagnostic 435 nm characteristic of the Cbl-peroxo adduct.

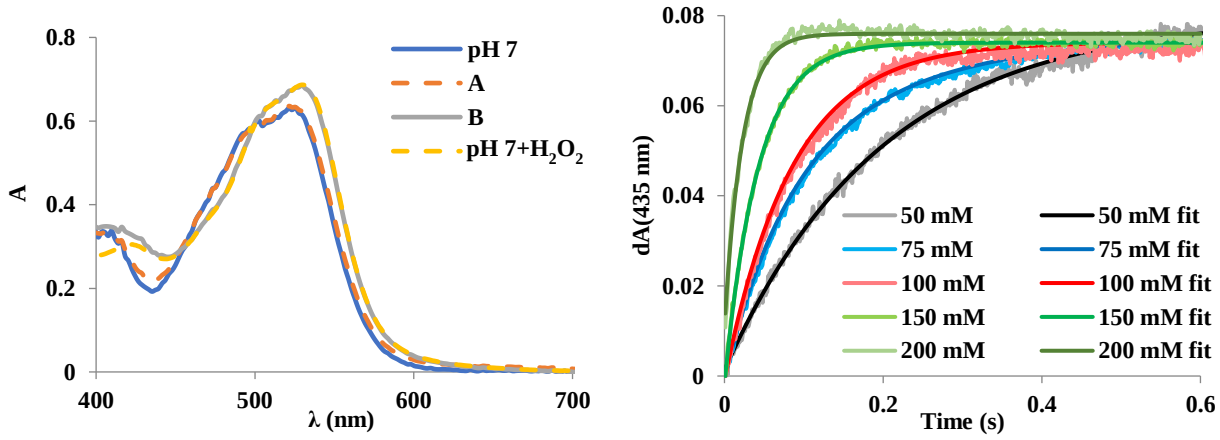

Figure 8. Typical time evolution of absorbance (right) and calculated spectra of species involved (left), for stopped-flow mixing of Cbl with peroxide. Conditions: 0.07 mM Cbl, pH=7, room temperature, peroxide concentrations indicated in the Figure (100 mM H_2O_2 for the spectra in the left panel). Also shown for reference are the spectra of Cbl and of its peroxide adduct obtained in static experiments cf. Figure 6.

4. On the reaction of Co(II) cobalamin with hydrogen peroxide [3]

The reactions of hydrogen peroxide with biologically-relevant transition metal centers typically entail one of two mechanisms: (1) metal-peroxide coordination, or (2) outer-sphere redox reaction. The metal peroxide complexes may either be stable – such as in the case of Co(II) bleomycin or Co(III) cobalamin (Co(III)Cbl),[19,23] or they may undergo O-O bond cleavage such as in the case of Fenton systems, peroxidases, cytochromes P450, iron and copper bleomycin, vanadium-containing peroxidases and others).[15–18]

The outer-sphere reactions of peroxides are typically monoelectronic and, by population of the σ* antibonding orbital of the peroxide bond with electrons received from the metal, lead to O-O bond cleavage and hydroxyl radicals; such processes are typically associated with Fenton chemistry or with unwanted side-reactions of biological metal centers.[24–28]

[3] Published as Plesa, Diana; Lehene, Maria; Silaghi-Dumitrescu, Radu. On the reaction of Co(II) cobalamin with hydrogen peroxide. Reaction Kinetics, Mechanisms and Catalysis, 2023, 136,1791–1799.

Figure 9 shows that reoxidation of B_{12r} with O_2 in the presence of excess ascorbate partially restores an aquaCbl-like spectrum; however, at longer reaction times an increase in the 450 nm region is seen, suggestive of SYC-type species formation (stable yellow corrinoid, with an oxygenated corrinoid system at the periphery). This may be explained by formation of ROS (including peroxide) due to interaction of the excess ascorbate with O_2 and due to the monoelectronic oxidation of Cbl(II) with O_2 in a pseudo ascorbate oxidase reaction.

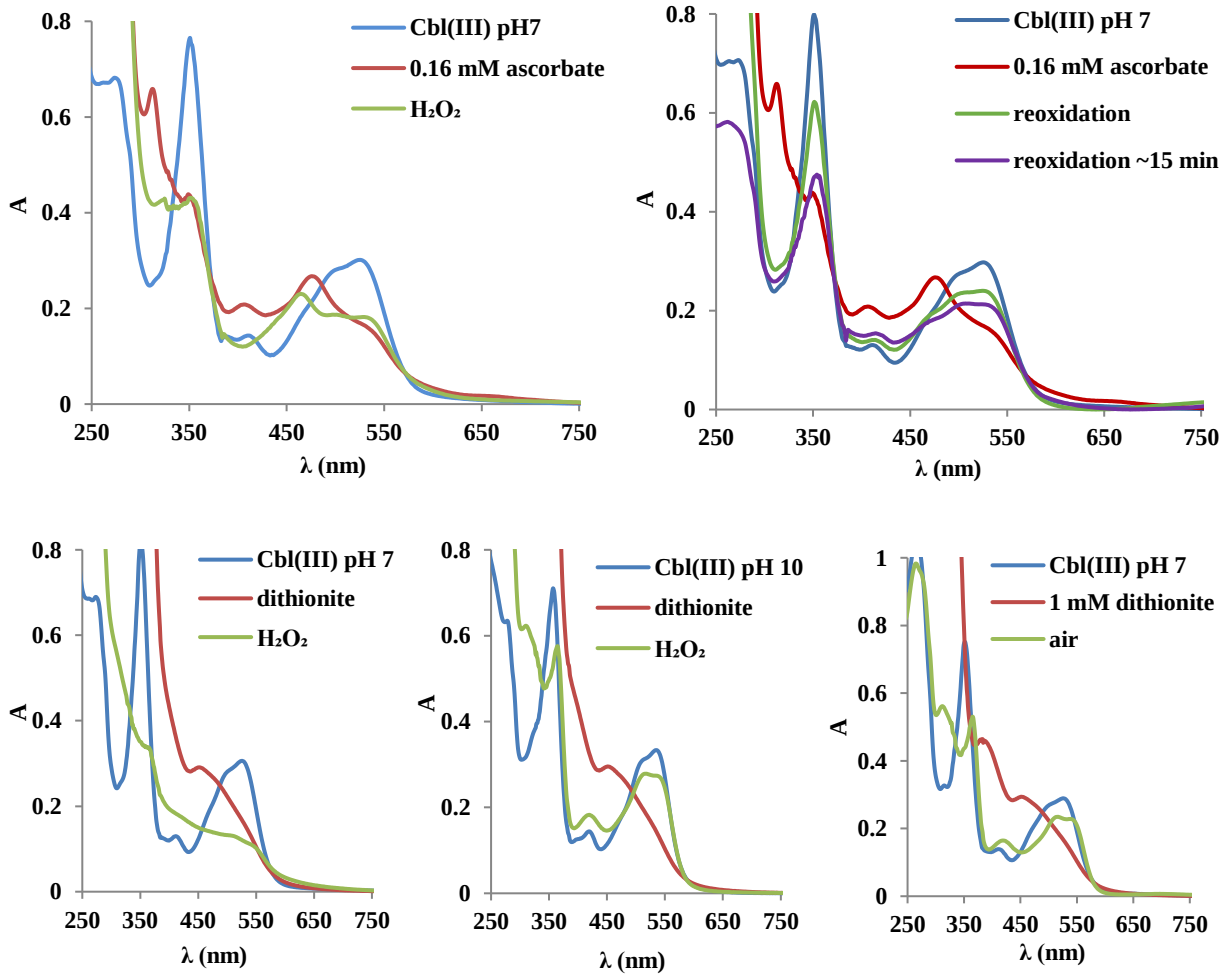

Figure 9. Representative UV-vis spectra illustrating reoxidation of B_{12r} or B_{12rs} with H_2O_2 or O_2. Upper panel: UV-vis spectra of 0.05 mM Co(III) cobalamin with 0.16 mM ascorbate and reoxidation with H_2O_2 and O_2 at pH 7, 22°C. Lower panel: UV-vis spectra of 0.05 mM Co(III) cobalamin with 1 mM sodium dithionite and reoxidation with H_2O_2 at pH 7 and pH 10 and with O_2 at pH 7, 22°C.

Reoxidation of dithionite-reduced aquaCbl (B_{12rs}, hexacoordinated with an SO_2 ligand trans to the dimethylbenzimidazole, DMBZ) at pH 7 leads to bleaching of the Cbl spectrum in the visible region – likely from a pseudo dithionite peroxidase reaction and from reaction with reactive species inherently present in mixtures of dithionite with oxidizing agents.[29–33] However, at pH 10 a typical Co(III)Cbl spectrum is obtained upon reoxidation of B_{12rs} with H_2O_2. This different reactivity at basic pH may be due to a lower reactivity of dithionite at basic pH (indeed, in this pH range dithionite's reactivity is significantly affected by pH especially via the complex nature of its possible decomposition products such as bisulfite, thiosulfate, sulfur dioxide and others, whereas ascorbate, with a pKa of 4.2 and a single product – dehydroascorbate – exhibits much simpler behavior),[34,35] and also points in general towards a role for protons in SYC formation in the dithionite-peroxide system. This is somewhat at odds a mechanism where hydroperoxide would be the key reagent attacking the corrin ring of Cbl.[36]

5. A complex of cobalamin with an organic peroxide [4]

Peroxide complexes of biological transition metal centres are in some cases reactive intermediates in complex reaction mechanisms Stable peroxo complexes of biological transition metal centres are also known such as with Co(III) cobalamin (Cbl(III)) or Co(II) bleomycin. As we recently demonstrated, cobalamin also forms a stable Co(III) complex with hydrogen peroxide.[19,23] This was the first complex of cobalamin with an oxidizing agent. The present chapter demonstrates that the adduct with hydrogen peroxide is not an exception and that in fact cobalamin also offers coordination possibilities for organic peroxides.

[4] Published as Lehene, Maria; Zagrean-Tuza, Hadade, Niculina; Cezara; Aghion, Andreea; Septelean, Raluca; Iancu, Stefania; Brânzanic Adrian M.V.; Silaghi-Dumitrescu, Radu. A complex of cobalamin with an organic peroxide. New Journal of Chemistry, 2023, 47(39), 18178-18185.

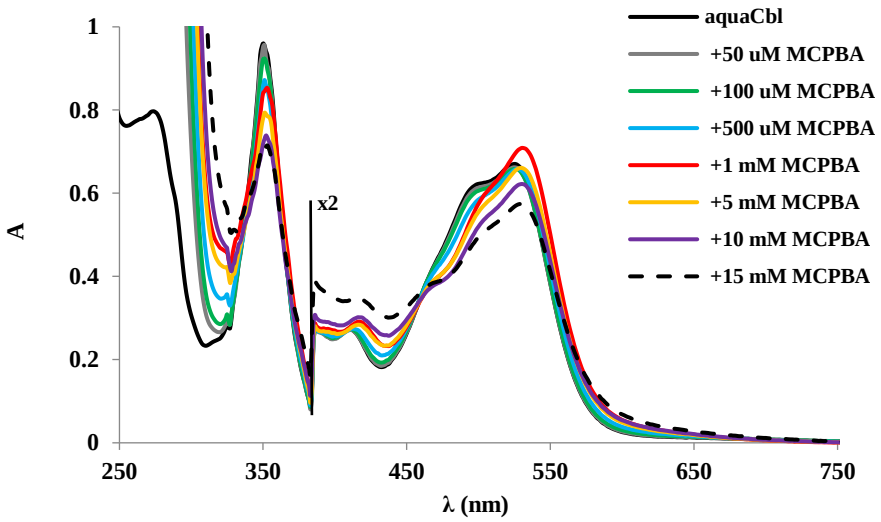

Figure 10. UV-vis spectra of 50 μM aquacobalamin treated with MCPBA.

As shown in Figure 10, upon reaction with MCPBA the UV-vis spectrum of aquacobalamin ($H2OCbl^+$) undergoes bathochromic shifts reminiscent of the hydroperoxocobalamin adduct.[23] Isosbestic points at 461 nm, 340 and 367 nm suggest formation of a single new product, which can in principle be assigned as a peroxoacid(MCPBA)-Cbl(III) complex analogous to the previously-reported cobalamin(III)-hydroperoxo complex. These observations were corroborated with NMR, Raman resonance, mass spectrometry and DFT data.

6. The adducts of cyano- and aquacobalamin with hypochlorite [5]

The biological reactions catalyzed by cobalamin relate to cobalt-carbon bond heterolysis and hemolysis in Cbl(III)-alkyl adducts.[37] The redox chemistry of the cobalt in Cbl(III) is so far known to be constrained mainly to reduction to Co(II) and Co(I).[11,38] Treatment of Cbl(III) with oxidizing agents has been shown to either have no effect (e.g., with hexachloroiridate), or lead to corrin oxidation and bleaching (e.g., with hypochlorite). In the present thesis, however, the reversible formation of relatively stable Co(III)-peroxide adducts was demonstrated.[23] The present

[5] Published as Lehene, Maria; Brânzanic Adrian M.V.; Silaghi-Dumitrescu, Radu. The adducts of cyano- and aquacobalamin with hypochlorite. Journal of Biological Inorganic Chemistry, 2023, 6(28), 583-589.

chapter explores whether this new coordinative chemistry of cobalamin with peroxides can be extended to hypochlorite as an example of a second class of oxidizing agents.

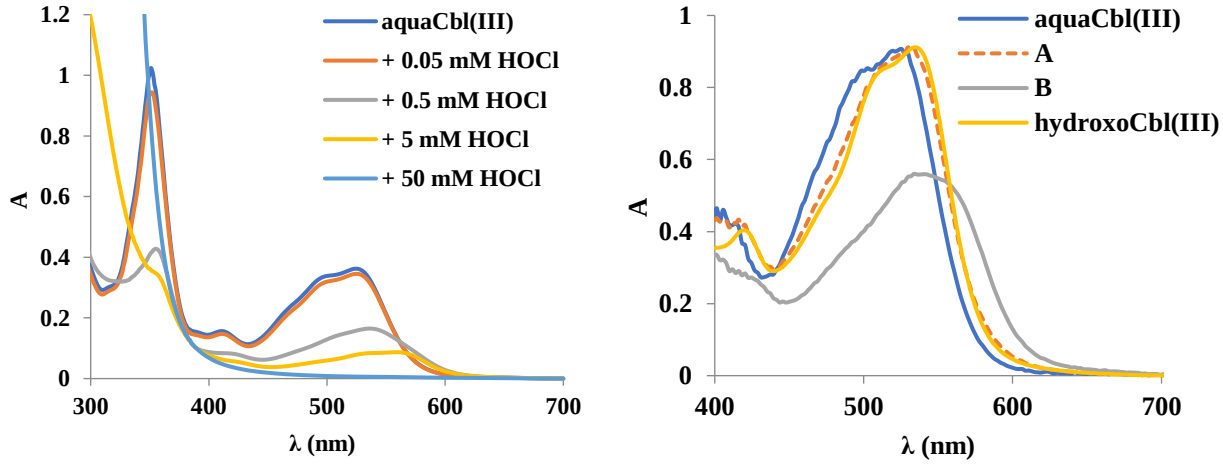

Figure 11. Top left: UV-vis spectra of aquaCbl treated with various concentrations of hypochlorous acid (manual mixing, ~1 minute reaction time). Top right: representative spectra of species A and B resulted from fitting of the stopped-flow data upon reaction of aquaCbl with 40 mM hypochlorite; also shown for reference are the static/equilibrium spectra of aqua and of hydroxoCbl in the absence of HOCl.

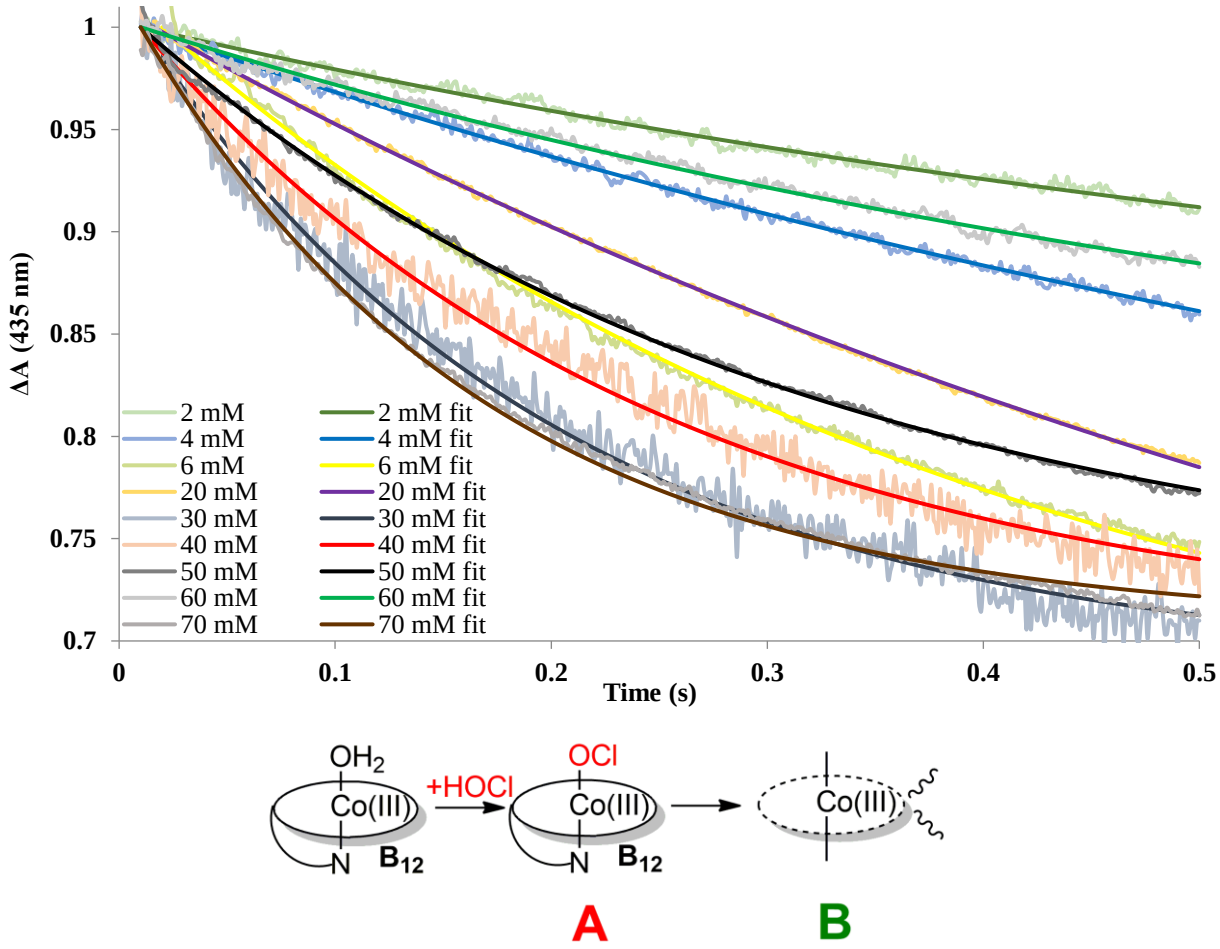

Figure 12. Normalized absorbance (ΔA) time course from stopped-flow UV-vis experiments. Conditions: 0.5 mM Cbl, pH 7, unless otherwise specified.

Species A in Figure 11 and 12 is hence assigned as a Co(III)-hypochlorite adduct. As also illustrated in Figure 11, the apparent rate constants for A→B do not appear to show a clear dependence on hypochlorite concentration (R^2=0.2), even though larger concentrations do lead to faster reactions. This situation is consistent with a reaction mechanism where, at [HOCL]>4 mM, aquaCbl reacts with hypochlorite within the dead-time of the instrument (~2 ms), so that the first observable species is a Cbl(III)-hypochlorite adduct.

7. The chlorite adduct of aquacobalamin: contrast with chlorite dismutase[6]

Previous chapters have demonstrated that vitamin B_{12} exhibits a previously unknown domain of coordination chemistry, with peroxidative agents and with hypochlorite. The present chapter explores the extent to which hypochlorite is no exception - and hence other chlorine oxyanions can coordinate to cobalamin. For this purpose, chlorite is used as a ligand.

Figure 13 and 14 shows that chlorite up to 100 mM has no clear effect on the UV-vis spectrum of aquacobalamin above 450 nm. In the 250-350 nm, an increase in absorbance is seen due to chlorite, but no change in the 350-nm band of Cbl. The slight changes in the 400-450 nm region may partially be due to chlorite as well; however, the 415-nm maximum appears slightly redshifted and less defined at higher chlorite concentrations.

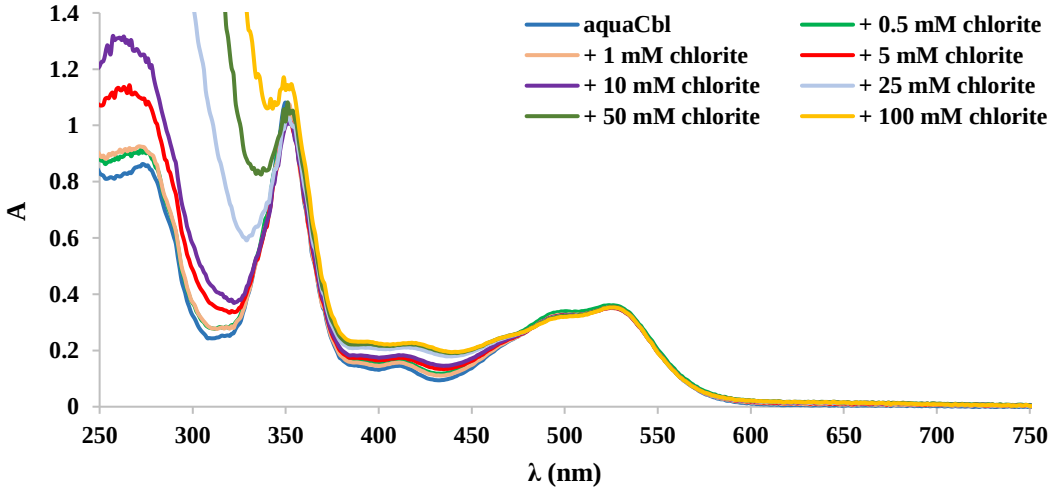

Figure 13. UV-vis spectra of the reaction between 50 uM aquaCbl and 0.5 mM-100 mM chlorite at pH 7, room temperature. Lower panel, left: titration curve for aquacobalamin reacting with chlorite at pH 7, monitored at 430 nm.

[6] Submitted to the Journal of Biological Inorganic Chemistry, 2024.

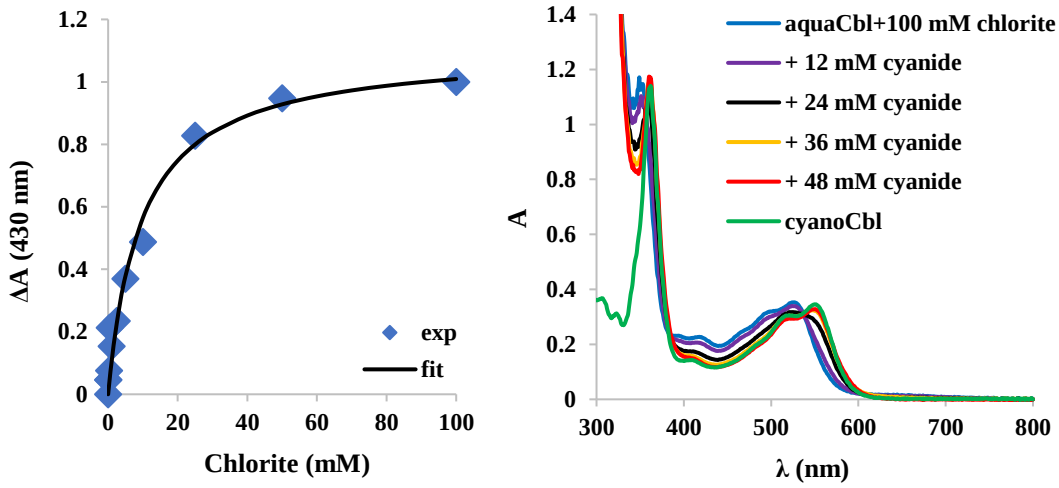

Figure 14. Left panel: titration curve for aquacobalamin reacting with chlorite at pH 7, monitored at 430 nm. Right panel: UV-vis spectra of aquaCbl reacting with 100 mM chlorite, compared to aquaCbl reacting with 12 mM-14 mM cyanide and cyanoCbl.

Somewhat similar changes induced by hydrogen peroxide on the cobalamin UV-vis spectra had previously been ignored until our more detailed investigations showed that they were due to peroxide complexation to Co(III) Cbl. Figure 13 also shows that these changes show a saturation behavior with an apparent K_d of 9.6 mM, which may be taken as evidence for formation of a Co(III)-chlorite complex. As also shown in Figure 13 and 14, addition of cyanide to the proposed Co(III)-chlorite cobalamin complex leads to formation of cyanocobalamin; these spectra may be interpreted as evidence that cyanide displaces chlorite from Co(III) – and that chlorite has not induced any detectable degradation of Cbl at these concentrations. These observations were corroborated with NMR, Raman resonance, mass spectrometry and DFT data.

8. General conclusions

This thesis has explored the coordination chemistry of cobalamin with oxidizing agents. Cobalamin shows some structural similarity with heme. Hemes and hemoproteins display, among others, biologically relevant reactions with oxidizing agents leading to Fe(IV) or Fe(V) and possibly free radicals, by mechanisms including or resembling Fenton chemistry. On the other hand, prior to our studies, the field of cobalamin coordination chemistry with oxidizing agents was entirely void. Our data has now shown that several oxidizing agents – hydrogen peroxide, organic peroxides, and halide oxyanions – in fact form complexes with Co(III) cobalamin that are similar to, but more stable than, those seen in the related active centers of heme proteins.

A stable and reversible adduct of Co(III) cobalamin with hydrogen peroxide is described here for the first time, using UV-vis and supported by NMR, Raman, and DFT data. It is proposed that the ligand is deprotonated and binds in a monodentate manner that leads to the reversible formation of a relatively stable Co(III)-hydroperoxide adduct.

The early stages of the reaction between Cbl(II) and hydrogen peroxide revealed in our studies a bifurcated reaction mechanism. The initial outer-sphere oxidation of Cbl(II) by H_2O_2 results in the formation of Cbl(III) and a hydroxyl radical. This hydroxyl radical can either attack the corrin ring edge, leading to the formation of a stable yellow corrinoid (SYC) or it can diffuse into the solution, leaving behind an intact Co(III). The pentacoordinated Co(III) intermediate was not detected.

Cbl(III) forms a peroxoacid complex with deprotonated m-chloroperoxybenzoic acid, as evidenced by UV-vis and supported by ^{1}H-NMR, resonance Raman spectroscopic data, density functional calculations, and mass spectrometry. In contrast, tBuOOH does not seem capable of forming a peroxo complex with Cbl(III). These findings indicate that the peroxide coordination chemistry of cobalamin extends beyond just hydrogen peroxide, but not all peroxo compounds should be expected to bind to Cbl(III).

Transient intermediates have been detected in the reaction of aqua as well as of CNCbl with hypochlorite using stopped-flow UV-vis kinetics. Supported by DFT calculations, the intermediate in aquaCbl is attributed to the substitution of the aqua ligand with hypochlorite, forming a Co(III)-OCl- adduct. For cyanoCbl, the intermediate is proposed to arise from

substitution of the DMBZ trans to the cyanide – hence a ClO^--Co(III)-CN^- adduct. These Co(III)-hypochlorite adducts serve as an intriguing analogue to the Fe(III)-hypochlorite adduct proposed as Compound X in hemoproteins. Particularly in the cyano adduct, they present opportunities for further experimental exploration as potentially more stable counterparts to Compound X. [39,40]

Last but not least, chlorite forms a stable Co(III) complex with cobalamin as evidenced by UV-vis, NMR, MS, resonance Raman and DFT. The stability of this complex is in contrast to what is seen in ferric hemes, where (for chlorite dismutase, at least) O-Cl bond cleavage appears to take less than 100 μs. Similar to the case of O-O bond cleavage in Fe/Co-peroxide complexes, the increased ease of O-Cl bond cleavage in Fe(III) compared to Co(III) is probably related to the difference in M(III)/M(IV) redox potentials.

10. References

1. Karlson, P. The structure of vitamin B1. *Trends Biochem. Sci.* **9**, 536–537 (1984).
2. Padmanabhan, S., Jost, M. & Drennan, C. L. A New Facet of Vitamin B 12 : Gene Regulation by Photoreceptors. *Annu. Rev. Biochem.* **86**, 485–514 (2017).
3. Dereven'kov, I. A., Salnikov, D. S. & Makarov, S. V. Interaction between super-reduced cobalamin and selenite. *Russ. J. Phys. Chem. A* **91**, 2404–2408 (2017).
4. Wolak, M., Stochel, G., Hamza, M. & Van Eldik, R. Aquacobalamin (vitamin B(12a)) does not bind NO in aqueous solution. Nitrite impurities account for observed reaction. *Inorg. Chem.* **39**, 2018–2019 (2000).
5. Spitler, R. *et al.* Nitrosyl-cobinamide (NO-Cbi), a new nitric oxide donor, improves wound healing through cGMP/cGMP-dependent protein kinase. *Cell. Signal.* **25**, 2374–2382 (2013).
6. Wolak, M., Zahl, A., Schneppensieper, T., Stochel, G. & van Eldik, R. Kinetics and mechanism of the reversible binding of nitric oxide to reduced cobalamin B(12r) (Cob(II)alamin). *J. Am. Chem. Soc.* **123**, 9780–9791 (2001).
7. Selçuki, C., Van Eldik, R. & Clark, T. NO Binding to Cobalamin: Influence of the Metal Oxidation State. *Inorg. Chem.* **43**, 2828–2833 (2004).
8. Subedi, H., Hassanin, H. A. & Brasch, N. E. Kinetic and mechanistic studies on the reaction

of the vitamin B 12 complex aquacobalamin with the HNO donor angeli's salt: Angeli's salt and HNO react with aquacobalamin. *Inorg. Chem.* **53**, 1570–1577 (2014).

9. Subedi, H. & Brasch, N. E. Mechanistic studies of the reactions of the reduced Vitamin B12 derivatives with the HNO donor Piloty's acid: Further evidence for oxidation of cob(i)alamin by (H)NO. *Dalt. Trans.* **45**, 352–360 (2015).
10. Chan, A. *et al.* Nitrocobinamide, a new cyanide antidote that can be administered by intramuscular injection. *J. Med. Chem.* **58**, 1750–1759 (2015).
11. Dereven'kov, I. A. *et al.* Comparative study of reaction of cobalamin and cobinamide with thiocyanate. *J. Inorg. Biochem.* **125**, 32–39 (2013).
12. Cameron, E., Pauling, L. & Leibovitz, B. Ascorbic Acid and Cancer: A Review. *Cancer Res.* **39**, 663–681 (1979).
13. Arabi, Y. M., Fowler, R. & Hayden, F. G. Critical care management of adults with community-acquired severe respiratory viral infection. *Intensive Care Med.* **46**, 315–328 (2020).
14. Bischin, C. *et al.* Free-radical reactions: the fine line between the anti- and pro-oxidant reactivities. *Oxid. Commun.* **41**, 130–140 (2018).
15. Silaghi-Dumitrescu, R. Redox activation of small molecules at biological metal centers. *Struct. Bond.* **150**, 97–118 (2013).
16. Zhang, Y. & Gascon, J. A. QM/MM investigation of structure and spectroscopic properties of a vanadium-containing peroxidase. *J. Inorg. Biochem.* **102**, 1684–1690 (2008).
17. de Visser, S. P., Kumar, D., Neumann, R. & Shaik, S. Computer-generated high-valent iron--oxo and manganese--oxo species with polyoxometalate ligands: how do they compare with the iron--oxo active species of heme enzymes? *Angew. Chem. Int. Ed. Engl.* **43**, 5661–5665 (2004).
18. Hersleth, H. P., Ryde, U., Rydberg, P., Gorbitz, C. H. & Andersson, K. K. Structures of the high-valent metal-ion haem-oxygen intermediates in peroxidases, oxygenases and catalases. *J. Inorg. Biochem.* **100**, 460–476 (2006).
19. Carrascoza, F., Surducan, M., Eriksson, L. A. & Silaghi-Dumitrescu, R. Interaction of cobalt and iron hydroperoxo bleomycin with deoxyribonucleic acid (DNA): Dynamic vs. electronic structure considerations. *Inorganica Chim. Acta* **509**, 119682 (2020).
20. Dereven'Kov, I. A. A. *et al.* Comparative study of reaction of cobalamin and cobinamide

with thiocyanate. *J. Inorg. Biochem.* **125**, 32–39 (2013).

21. Lexa, D., Savéant, J. M. & Zickler, J. Electrochemistry of Vitamin B_{12}. 5. Cyanocobalamins. *J. Am. Chem. Soc.* **102**, 2654–2663 (1980).
22. Wolak, M., Stochel, G. & Van Eldik, R. Mechanistic studies on the interaction of reduced cobalamin (vitamin B_{12r}) with nitroprusside. *J. Am. Chem. Soc.* **125**, 1334–1351 (2003).
23. Lehene, M. *et al.* Adduct of Aquacobalamin with Hydrogen Peroxide. *Inorg. Chem.* **60**, 12681–12684 (2021).
24. Ensing, B., Buda, F., Blochl, P. & Baerends, E. J. Chemical Involvement of Solvent Water Molecules in Elementary Steps of the Fenton Oxidation Reaction. *Angew. Chemie - Int. Ed.* **40**, 2893–2895 (2001).
25. Sadrzadeh, S. M., Graf, E., Panter, S. S., Hallaway, P. E. & Eaton, J. W. Hemoglobin. A biological fenton reagent. *J. Biol. Chem.* **259**, 14354–14356 (1984).
26. Buda, F., Ensing, B., Gribnau, M. C. & Baerends, E. J. DFT study of the active intermediate in the Fenton reaction. *Chemistry (Easton).* **7**, 2775–2783 (2001).
27. Sharpe, M. A., Robb, S. J. & Clark, J. B. nitric oxide and Fenton/Haber-Weiss chemistry: nitric oxide is a potent antioxidant at physiological concentrations. *J. Neurochem.* **87**, 386–394 (2003).
28. Marcus, S. R. & Dharmalingam, M. Iron, Oxidative Stress and Diabetes. in *Diabetes: Oxidative Stress and Dietary Antioxidants* 51–64 (2013).
29. Nichol, A. W., Hendry, I. & Morell, D. B. Mechanism of formation of sulphhaemoglobin. *Biochim. Biophys. Acta* **156**, 97–108 (1969).
30. Oliver, J. & Brittain, T. A comparison between the ease of haemoglobin oxidation a n d modification by sulphide. *Comp. Biochem. Physiol.* **76B**, 579–583 (1983).
31. Pietri, R., Roman-Morales, E. & Lopez-Garriga, J. Hydrogen sulfide and hemeproteins: knowledge and mysteries. *Antioxid Redox Signal* **15**, 393–404 (2011).
32. Román-Morales, E. *et al.* Structural determinants for the formation of sulfhemeprotein complexes. *Biochem. Biophys. Reports* **400**, 489–492 (2010).
33. Ríos-González, B. B., Román-Morales, E. M., Pietri, R. & López-Garriga, J. Hydrogen Sulfide Activation in Hemeproteins: The Sulfheme Scenario. *J. Inorg. Biochem.* **133**, 78–86 (2015).
34. Makarov, S. V., Horváth, A. K., Silaghi-Dumitrescu, R. & Gao, Q. *Sodium Dithionite,*

Rongalite and Thiourea Oxides. *Sodium Dithionite, Rongalite and Thiourea Oxides: Chemistry and Application* (World Scientific (Europe), 2016). doi:10.1142/q0028.

35. Rudenco, O. *et al.* Versatility of thiourea dioxide as redox agent in globins: case study with myoglobin. *Inorganica Chim. Acta* 121474 (2023).
36. Salnikov, D. S., Makarov, S. V. & Koifman, O. I. The radical: versus ionic mechanisms of reduced cobalamin inactivation by tert -butyl hydroperoxide and hydrogen peroxide in aqueous solution. *New J. Chem.* **45**, 535–543 (2021).
37. Dereven'kov, I. A., Salnikov, D. S. D. S., Silaghi-Dumitrescu, R., Makarov, S. V. S. V. & Koiffman, O. I. Redox chemistry of cobalamin and its derivatives. *Coord. Chem. Rev.* **309**, 68–83 (2016).
38. Gebicka, L. & Banasiak, E. Hypochlorous acid-induced heme damage of hemoglobin and its inhibition by flavonoids. *Toxicol. Vitr.* **26**, 924–929 (2012).
39. Silaghi-Dumitrescu, R. Halide activation by heme peroxidases: Theoretical predictions on putative adducts of halides with compound I. *Eur. J. Inorg. Chem.* 5404–5407 (2008) doi:DOI 10.1002/ejic.200800732.
40. Chiang, R., Rand Meir, T., Makino, R. & Hager, L. P. Compound X. An intermediate in enzymatic halogenation. *J. Biol. Chem.* **251**, 6340–6346 (1976).