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Redox turnover of organometallic B_{12} cofactors recycles vitamin C: Sulfur assisted reduction of dehydroascorbic acid by cob(II)alamin

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ABSTRACT

This work reports the reactivity of cob(II)alamin (Cbl(II)) toward reduction of dehydroascorbic (DHA) to ascorbic acid (AA) mediated by sulfur-containing compounds such as glutathione (GSH) and thiocyanate. The reaction supported by GSH proceeded more efficiently than with SCN^-. Our findings demonstrate new aspects of interactions between vitamins B_{12} and C. It has been accepted that simultaneous presence of these vitamins results in their decomposition (viz., irreversible modification of the corrin ring of Cbl and oxidation of AA). We have shown, however, that Cbl(II), the biologically active one-electron reduction product of methyl-Cbl (MeCbl) and adenosyl-Cbl (AdoCbl), is capable of recovering AA in the presence of natural sulfur-containing ligands, within a process that can occur in vivo without glutathione spending, both in a stoichiometric and catalytic manner. Our studies highlight the redox versatility of Cbl(II) and expands the repertoire of reactions whereby redox turnover of the unique B_{12} organometallic cofactors MeCbl and AdoCbl generates Cbl(II), which in turn recycles oxidized vitamin C.

1. Introduction

Dehydroascorbic acid (DHA; Fig. 1A and B) is the product of two-electron oxidation of ascorbic acid (AA; vitamin C; Fig. 1C). Its therapeutic significance attracted a lot of attention, because DHA seems to be the pharmaceutically active form of vitamin C that is preferentially imported by colorectal cancer cells and is responsible for selective toxicity of vitamin C to tumor cells [1]. Upon cell uptake it is reduced to AA, predominantly by glutathione (GSH; Fig. 1D), thioredoxin and NADPH [2–5], which represents an important mechanism of its biological and therapeutic actions [1]. The question remains, whether DHA reduction is always related to GSH consumption or if it can proceed through other pathway, e.g. involving metal centers. The only example of DHA reduction by metal complexes relates to the reaction with iron-nitrosyl-hemoglobin leading to the formation of methemoglobin and NO liberation [6].

Cobalamin (B_{12}, Cbl) is an essential micronutrient required by all cells in the body. An important fraction of the intracellular pool of cobalamin, the only metal-containing vitamin (Fig. 1E), is its one-electron reduced form, i.e. cob(II)alamin (Cbl(II)) [7–9], which can be considered as a reducing agent. The vast majority of cob(II)alamin present in biological systems derives from the one-electron reduction of MeCbl and AdoCbl, as part of the catalytic cycle of cognate enzymes methionine synthase and methylmalonyl-CoA mutase [8,9]. Chemically, the reducing activity of Cbl(II) was observed in the reactions with oxidizing free radicals, e.g. superoxide (O_2^-) [10,11], nitric oxide (NO) [12–14], nitrogen dioxide (NO_2) [15,16] and chlorine dioxide (ClO_2) [17]. Both Cbl(III) and Cbl(II) form complexes with GSH and SCN^- that are abundant compounds in nature [18–23], with concentrations of GSH reaching values of 10 mM in cells [24] and those for SCN^- being 0.5–6.0 mM in mucosal liquids and ca. 10–100-fold lesser in blood [25,26]. In cells, cobalamins are present in relatively low concentrations

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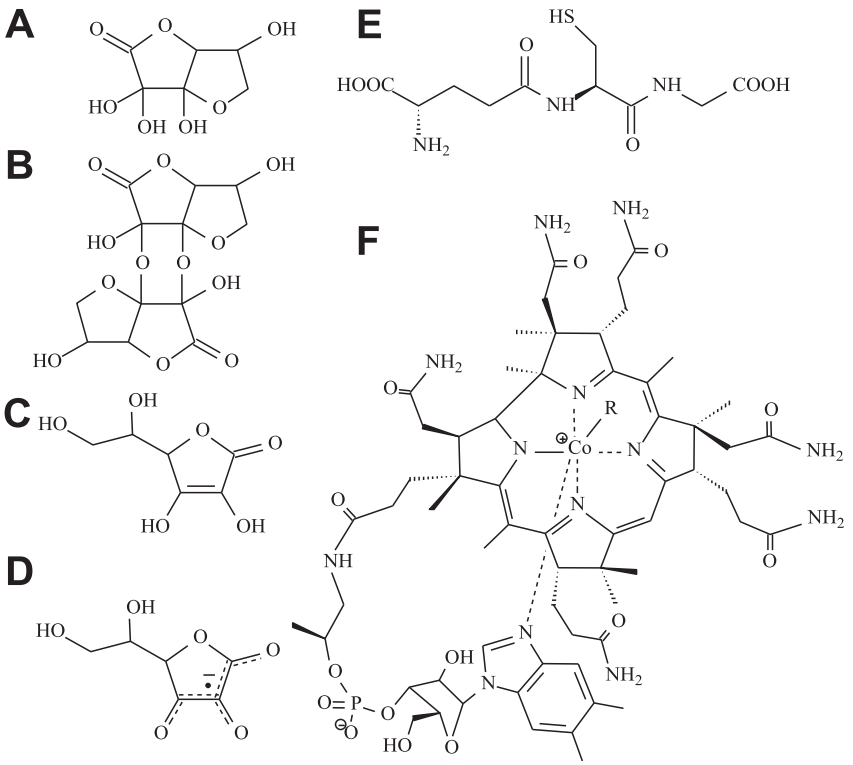

Fig. 1. Structures of (A) monomeric, MDHA, and (B) dimeric, DDHA, forms of dehydroascorbic acid, (C) ascorbic acid, (D) ascorbyl free radical, (E) glutathione and (F) cobalamin ($R = H_2O$, CN^-).

(0.15–0.20 nM), but their reduced forms cob(II)alamin and cob(I)alamin are highly reactive [27]. Glutathionylcob(III)alamin (GS^--Cbl(III)) was found in vivo [27]. Important characteristic of these complexes is the existence of a Co–S bond, strengthening the notion that Co–S bonded cobalamins, including the fraction of SCN^- that binds through the sulfur atom partake in the complex map of cellular cobalamin species [20,21].

Knowing that extra-ligands may influence redox behavior of the metal centers we were interested to reveal the reactivity of Cbl(II)/GSH-system toward DHA. Therefore, in this work we report kinetic and mechanistic studies of reaction of Cbl(II) with dehydroascorbic acid in acidic and neutral medium (at room temperature) in the presence of glutathione. For comparison, the studies were performed with thiocyanate as well, which differs from GSH in that it is a redox inactive (under applied experimental conditions) S-donating ligand. Interestingly, we have observed a catalytic effect of S-containing ligands on the reduction of DHA by Cbl(II). This has important mechanistic implications related to physiological and therapeutic aspects of vitamin C: (1) Cbl(II) formed during normal redox cycling of organometallic derivatives MeCbl and AdoCbl can recycle vitamin C in a process assisted by natural sulfur-containing species, and (2) the intracellular conversion of DHA to vitamin C mediated by Cbl(II) does not occur at the expense of glutathione consumption.

2. Experimental

2.1. Chemicals

Hydroxocobalamin hydrochloride (Fluka; ≥ 95%), dehydro-L-(+)-ascorbic acid dimer (Sigma-Aldrich; ≥ 80%), sodium borohydride (Aldrich; ≥ 96%), glutathione (Sigma-Aldrich; ≥ 98%), potassium thiocyanate (Sigma-Aldrich; ≥ 99%) were used as received. Concentrations of Cbl were found by means of conversion to dicyano form ($\varepsilon_{367} = 30{,}400\ M^{-1}\ cm^{-1}$) [28]. Cbl(II) was prepared using sodium borohydride according to a published procedure [29]. Excess borohydride was destroyed by adding acetone. Stock solutions of DHA were prepared by dissolving the commercial compound in diluted H_3PO_4 (pH 2.3), kept at 5 °C and used within 3 h. Concentrations of DHA were determined after its reduction to AA ([GSH] = 50 mM, pH 5–6) using published extinction coefficients [30]. Air-free argon was used to deoxygenate solutions. Phosphoric acid, mono- and disubstituted potassium phosphate and sodium acetate were used to adjust pH. All experiments were performed in 0.1 M buffers under anaerobic conditions.

2.2. Methods

UV–vis spectra were recorded on thermostated (±0.1 °C) Cary 50 spectrophotometer in gas-tight quartz cells with optical pathlength 1.00 cm. Stopped-flow experiments were performed on μSFM-20 BioLogic device equipped with J&D TIDAS detector.

Cyclic voltammetry (CV) experiments were carried out using an Autolab instrument with a PGSTAT 30 potentiostat. A conventional three electrode arrangement was employed consisting of a glassy carbon working electrode (Metrohm), a platinum wire as the counter electrode and silver chloride (in 3 M NaCl) reference electrode (0.222 V vs NHE at 20 °C). Prior to the measurements, the surface of the glass-carbon electrode was polished with alumina powder. Lithium perchlorate (0.1 M) was used as the supporting electrolyte.

Analysis of reaction products was performed anaerobically using water as a solvent (the resulting pH of the samples was 5.3). Starting material, adducts and products were identified by cryo-

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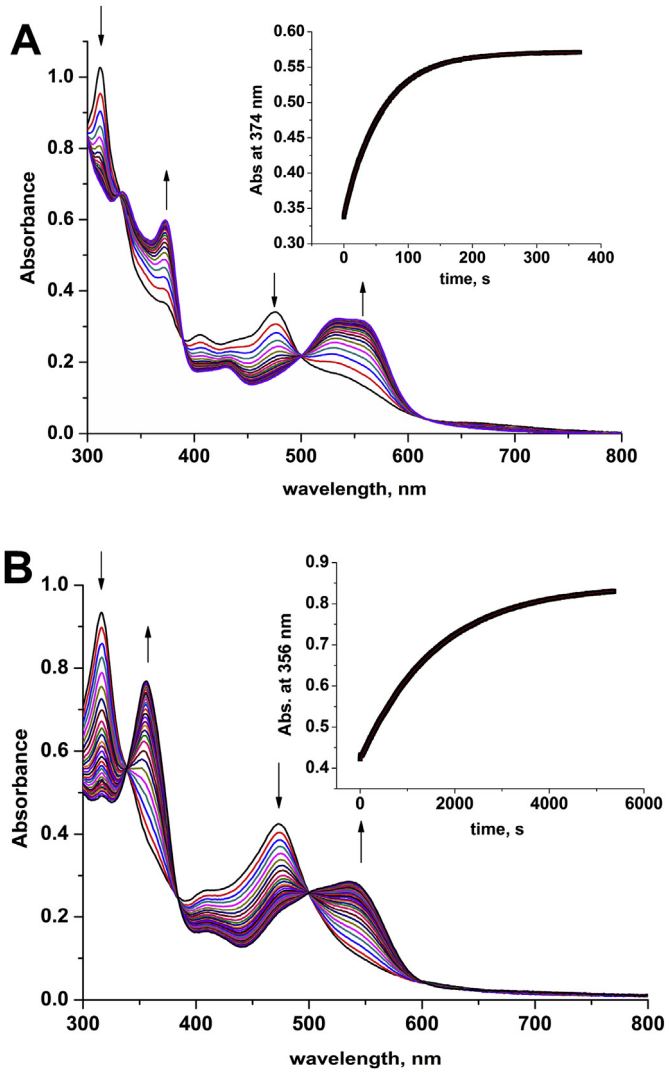

Fig. 2. UV/Vis spectra for the reaction between Cbl(II) (4×10^{-5} M) and DHA (5.9×10^{-4} M (A), 2.7×10^{-3} M (B)) in the presence of GSH (1×10^{-3} M) at pH 5.0 (A), and SCN^- (1×10^{-2} M) at pH 1.55 (B), 25 °C, and typical kinetic curves of these reactions (insets).

mass spectrometry. Hydroxocobalamin (final concentration 1×10^{-5} M) was reduced with sodium borohydride to produce Cbl(II). The excess of reducing agent was eliminated by addition of acetone. The reactions of Cbl(II) with DHA (final concentration 5×10^{-4} M) in the presence or absence of glutathione (final concentration 1×10^{-4} M) were followed continuously for 10 min, by cryospray-ionization MS (CSI-MS) measurements. A dead-time of 3 min was estimated between the rapid mixing of the reagents inside the anaerobic glovebox and data collection in the mass spectrometry instrument. CSI-MS measurements were performed on a UHR-TOF Bruker Daltonik (Bremen, Germany) maXis plus 5G, an ESI-quadrupole time-of-flight (qToF) mass spectrometer capable of resolution of at least 60,000 FWHM, which was coupled to a Bruker Daltonik Cryospray unit. Detection was in positive ion mode, the source voltage was 3.8 kV. The flow rates were 280 μL/h. The drying gas (N_2), to aid solvent removal, was held at +5 °C and the spray gas was held at +5 °C. The machine was calibrated prior to every experiment via direct infusion of the Agilent ESI-TOF low concentration tuning mixture, which provided an m/z range of singly charged peaks up to 2700 Da in both ion modes.

Determination of equilibrium constants was performed on a thermostated Cary 50 UV−vis spectrophotometer under anaerobic conditions. Equilibrium constants were calculated using Eq. (1) [21].

$$A = \frac{A_0 + A_\infty K[L]}{1 + K[L]}, \quad (1)$$

[L] is the total (free + bound) ligand concentration in solution, M; A, A_0, A_∞ are absorbances at the monitoring wavelength for the metallocomplex at a particular ligand concentration, for the starting complex, and for the final complex, respectively; K is equilibrium constant at a given pH, M^{-1}.

3. Results and discussion

At first the effect of biologically relevant sulfur-containing ligands on the oxidation of Cbl(II) by DHA was monitored by UV/Vis spectroscopy. In the absence of GSH or SCN^- the reaction between Cbl(II) and DHA does not proceed in acidic and neutral medium. However, in their presence it clearly takes place, resulting in the formation of GS^--Cbl(III) (appearance of absorbance maxima at 374, 532 and 556 nm [23]; Fig. 2A) and thiocyanato-Cbl(III) (increase of absorbance at 356 and 537 nm [21]; Fig. 2B) adducts.

In the case of GSH, the rate of oxidation of Cbl(II) was higher than in the presence of SCN^- (insets in Fig. 2A and B; Table 1) and the linear dependence of the observed rate constants (k_{obs}) on [DHA] was observed (Fig. 3A) with a positive intercept, whose origin is discussed below. This behavior indicates that the reaction is first order with respect to the oxidant. The pH dependence of the second order rate constants (k', obtained from slopes of DHA concentration dependencies) demonstrates substantial increase of the reaction rate upon a pH increase from 3.6 to 7.0 (Fig. S1) and this is related to the deprotonation of GSH (see SI). Importantly, reduction of DHA by a Cbl(II)/GSH mixture proceeds much faster ($k' = 1977 \pm 66\ M^{-1}\ s^{-1}$ at pH 7.0) than the direct reaction between DHA and GSH ($0.43 \pm 0.01\ M^{-1}\ s^{-1}$ at pH 7.0; Fig. S2), thus, the latter being negligible under our experimental conditions (Table 1). Titration of Cbl(II) with DHA in the presence of GSH (1 mM) (Fig. S3) demonstrated that the ratio of $[DHA]_0$: $[Cbl(II)]_0 = 1:2$ is sufficient for the complete conversion of Cbl(II) into GS^--Cbl(III), suggesting

Table 1
Rate constants of reactions related to this study.

Reaction	Conditions	Rate constant, $M^{-1}\ s^{-1}$
DHA + GSH → AFR^- + $GS^\bullet$ + H^+	pH 7.0, 25 °C	0.43
Cbl(II) + DHA + GSH → GSCbl(III) + AFR^- + H^+	[GSH] = 1 mM, pH 7.0, 25 °C	1977
	[GSH] ≥ 10 mM, pH 7.0, 25 °C	ca. 6000
Cbl(II) + DHA + SCN^- → SCNCbl(III) + AFR^-	[SCN^-] = 0.5 M, pH 6.4, 25 °C	3.1
H_2OCbl^+ + HAA^- → Cbl(II) + AFR^- + H^+ + H_2O	pH 7.0, 21 °C	29.8
GSCbl(III) + HAA^- → Cbl(II) + AFR^- + GSH	[GSH] = 1 mM, pH 7.0, 21 °C	not detectable

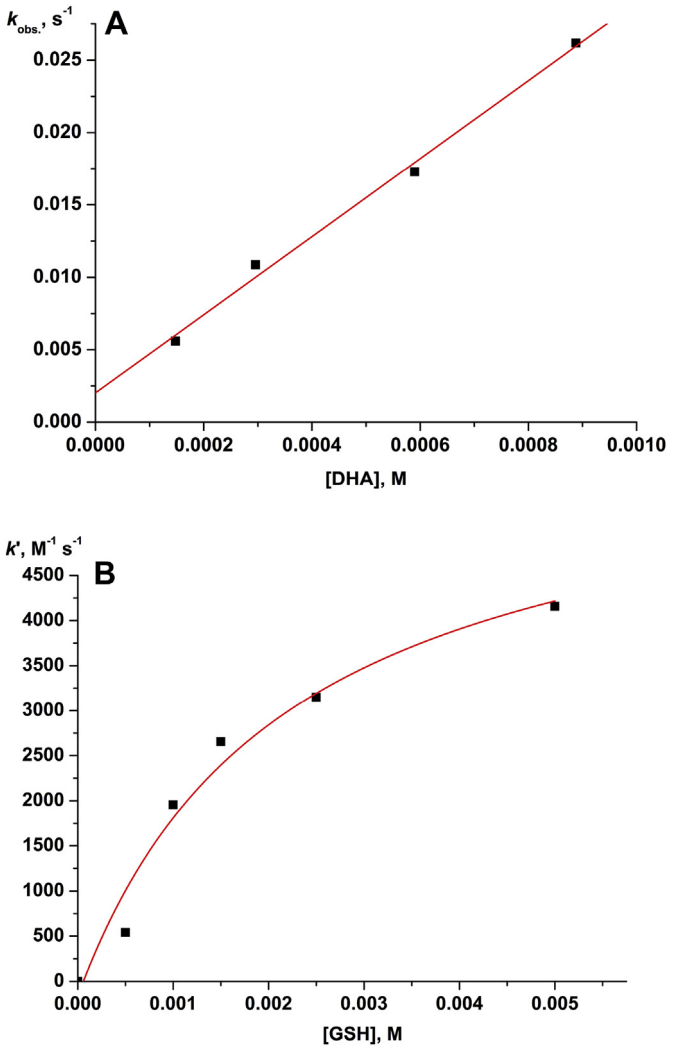

Fig. 3. (A) Plots of $k_{obs.}$ vs. [DHA] for the reaction of oxidation of Cbl(II) by DHA in the presence of GSH (1×10^{-3} M) at pH 5.0, 25 °C. (B) Dependence of the second order rate constant k' on [GSH] at pH 7.0, room temperature (k' was determined as a slope of the linear dependence of $k_{obs.}$ on [DHA]).

that DHA accepts two electrons and is transformed to AA.

We further investigated the effect of AA on possible reverse or side reactions. Neither Cbl(II) nor a Cbl(II)/DHA mixture reacted with AA during the timescale of all measurements performed in this work (Fig. S4). Although AA can reduce Cbl(III), the corresponding rate constant is so low (29.8 ± 0.2 M^{-1} s^{-1} and pH 7.0; Table 1 and Fig. S5), thus, considering the formation of AA in the concentration range of 10^{-5}-10^{-4} M (based on the above mentioned reaction stoichiometry) it cannot contribute to the value of the experimentally observed intercept. Furthermore, the presence of GSH additionally decreases the rate of Cbl(III) reduction by AA (Table 1; Fig. S6). Importantly, addition of 1 mM AA shifted the Y-intercept to zero, while the slope remained unchanged compared to experiments carried out without addition of AA (Fig. S7). This may suggest the involvement of a transient radical species (e.g. complex of glutathionyl-Cbl(III) with ascorbyl free radical (AFR), GS^{-}-Cbl(III)-AFR^{-}, that is produced after one-electron reduction of DHA, *vide infra*) in the process responsible for the intercept, which can be scavenged by AA. Similarly, GSH can also scavenge AFR^{-}. And indeed an increase in [GSH] decreased the Y-intercept (Fig. S8; at [GSH] $\geq$ 2.5 mM the intercept is equal to zero, within the experimental error limits). However, an increase in [GSH] accelerated the reaction (increase in slope; Fig. S8) reaching a maximum of the second order rate constant k' at high GSH concentrations (app. 6300 ± 900 M^{-1} s^{-1} at [GSH] $\geq$ 10 mM; Fig. 3B). This saturation kinetic behavior indicates involvement of a pre-equilibrium in the reaction mechanism (*vide infra*), with formation of a precursor adduct GS^{-}-Cbl(II) that has been earlier confirmed by EPR [22]. Herein, we provide further evidence for the existence of glutathionylcob(II)alamin by application of cryo ESI-MS analysis (Fig. 4) and UV/Vis spectroscopy (Fig. S9). The formation and properties of glutathionylcob(II)alamin have been demonstrated by two independent groups by EPR [22,31]. Herein, we adopted an ultra-high resolution, anaerobic, low-temperature mass spectrometry approach to detect glutathionylcob(II)alamin, a species predicted to be both short-lived and produced in low-yields in our reaction. Cryo ESI-MS analysis has revealed that Cbl(II) (experimental mass/charge ratio of $[Cbl(II) + H^{+} + Na^{+}]^{2+}$ is 676.28, Table S1) as well as Cbl(III) (experimental mass/charge ratio of $[Cbl(III) + Na^{+}]^{2+}$ is 675.77, Table S1) (presence of Cbl(III) was not possible to avoid under conditions of MS experiments) are capable of binding GSH (mass/charge ratio of $[GSCbl(II) + 2H^{+} + Na^{+}]^{2+}$ is 829.82, and the ratio of $[GSCbl(III) + H^{+} + Na^{+}]^{2+}$ is 829.32, Table S1) (Fig. 4; Table S1). This is the first study to document direct structural information (mass) demonstrating formation of glutathionylcob(II)alamin. Our results show that coordination of GSH to Cbl(II) occurs independently of DHA (Fig. 4A) and further suggests that this process represents the pre-equilibrium preceding the electron transfer to DHA. The generation of GS^{-}-Cbl(II) was also detected in the presence of DHA (Fig. 4B), though in lower quantity, in agreement with its intermediate nature in the overall reaction mechanism (*vide infra*). Titration of Cbl(II) by GSH results in UV/Vis spectral changes shown in Fig. S9. UV/Vis spectra are established immediately after mixing of reactants that is typical for rapid ligand exchange on soft Co(II)-center and absorbance at 550 nm reaches maximum value at [GSH] > 15 mM. The value of equilibrium constant for GS^{-}-Cbl(II) formation was found using Eq. (1): $K(GSH) = 287.1 \pm 17.5$ M^{-1} at pH 6.6, 25 °C, $I(NaNO_3) = 0.3$ M.

Cryo ESI-MS was also used to characterize reaction products. In the mixture of Cbl(II) and DHA without GSH no reduction of DHA could be observed (Fig. 4C). However, immediately after addition of GSH to the Cbl(II)/DHA mixture, formation of AA was detected (Fig. 4B). Approximately 2% of DHA was converted to AA (corresponding to ca. 10^{-5} M of AA, obtained from the mixture of 2×10^{-5} M Cbl(II) and 5×10^{-4} M DHA in the presence of 10^{-4} M GSH), in good correlation with the expected reaction stoichiometry ($[Cbl(II)]_0/[DHA]_0 = 2$). Importantly, transformation of GSH, e.g. its oxidation to GSSG, was not detected by ultra high resolution ESI-MS. In addition, we performed oxidation of Cbl(II) by excess of DHA in the presence of deficient quantities of GSH (as compared to Cbl(II)) and found that the ratio of $[GSH]_0/[Cbl(II)]_0 \geq 1$ is necessary for complete oxidation of Cbl(II) to GS^{-}-Cbl(III) (Fig. S10).

Taken together, our results support a general mechanism whereby GSH acts as a catalyst for the reduction of DHA to vitamin C by cob(II)alamin (Eq. (2)), GSH accelerates this process from its non-detectable rate in its absence to the rate of up to ca. 6000 M^{-1} s^{-1}, while remaining unaffected.

$$DHA + 2Cbl(II) \xrightarrow{GSH} AA + 2\ GS - Cbl(III)/Cbl(III) \qquad (2)$$

By way of comparison, we further investigated kinetics of the reaction in the presence of redox silent S-donor, SCN^{-}. Observed rate constants linearly depend on [DHA] indicating the first order

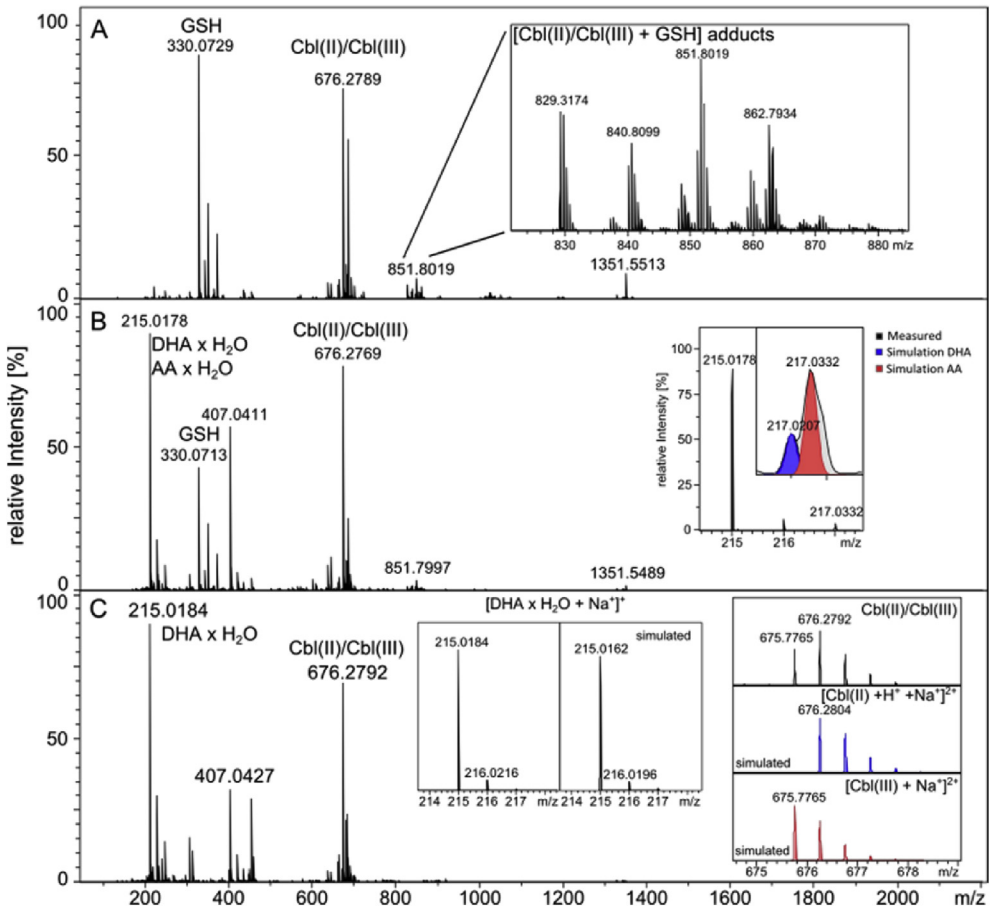

Fig. 4. Cryo ESI-MS spectra of (A) mixture of Cbl(II) (2×10^{-5} M) with GSH (10^{-4} M), (B) reaction mixture of Cbl(II), GSH and DHA (5×10^{-4} M) and (C) mixture of Cbl(II) and DHA (without GSH).

kinetics with respect to oxidant (Fig. S11), as observed in the case of GSH. However, the corresponding second order rate constants (k'; Table 1) are significantly lower ($k' = 274.1\ M^{-1}\ s^{-1}$ for 1 mM GSH and $k' = 3.1\ M^{-1}\ s^{-1}$ for 500 mM SCN^- at pH = 6.4). Importantly, as observed with GSH, k' is a function of [DHA] and exhibits a saturation behavior, achieving a maximal value at $[SCN^-] > 0.7$ M (Fig. S12). This is again in agreement with the formation of a thiocyanato-Cbl(II) precursor described earlier [21], in a pre-equilibrium step characterized by the equilibrium constant $K(SCN) = 14.7 \pm 4.6\ M^{-1}$ (obtained by fitting the equation $k' = k_{sat} \cdot K(SCN) \cdot [SCN^-]/(1 + K(SCN) \cdot [SCN^-])$ to data in Fig. S12). The obtained value corresponds well to the formation constant of Co(II)–SCN^- determined previously ($23.8 \pm 1.1\ M^{-1}$ at 25 °C, I = 1 M) [21]. The pH dependence of k' is described by a sigmoidal curve with an inflection point at pH = 4.41 ± 0.03 (25 °C, $[SCN^-] = 0.5$ M; Fig. S13). Such pH profile can be explained by transformation of DHA dimeric form (DDHA) to monomeric one (MDHA) (Fig. 1A and B) that occurs at pH ~4 and the fact that MDHA exhibits more pronounced oxidizing properties than DDHA [32].

Based on the saturation dependence of the rate constants (k') on [GSH] and $[SCN^-]$ (vide supra), respectively, and characterization of the GS^--Cbl(II) adduct, we can conclude that coordination of GSH or SCN^- to initial five-coordinate Cbl(II) (through its vacant β–axial site) modifies redox properties of Co^{2+}-ion and activates an electron-transfer from Co^{2+} to DHA. To probe such paradigm, we examined the redox behavior of the Cbl(II)/SCN^- and Cbl(II)/GSH systems by cyclic voltammetry (CV). Nevertheless, CV studies of GS^--Cbl(III) have been performed earlier [33], but applied relatively low GSH concentrations showed no influence on Cbl(II) oxidation.

Addition of SCN^- and GSH, respectively, shifted the position of the oxidation wave of Cbl(II) (0.075 V vs. Ag/AgCl) to more negative values. GSH exerts stronger effect than SCN^-, by shifting the oxidation wave to −0.25 V (at [GSH] = 0.14 M; Fig. 5A) in comparison to −0.099 V vs. Ag/AgCl in the presence of 1 M SCN^- (Fig. 5B). These results clearly demonstrate that GS^--Cbl(II) and thiocyanato-Cbl(II) adducts are stronger reducing agents than Cbl(II) explaining the activation effects of S-donating ligands, GSH and SCN^-, respectively. (Redox potentials relevant to this study are summarized in Table S2). Thus, the less prominent efficiency of SCN^- as compared to GSH results from its lower influence on the Co(II) redox potential.

According to the presented results an overall mechanistic picture can be visualized (Schemes 1 and S1). It is important that coordination of GS^- or SCN^- within the fast pre-equilibrium induces either cleavage or labilization of the axial Co^{2+}-N_{ax}(DMBI) bond, respectively (Fig. 1E, Schemes 1 and S1), as suggested in the literature based on the EPR studies [21,22]. This additional activation effect of S-donating ligands permits a closer interaction between the Co(II) center and DHA to yield ascorbyl free radical (AFR^-) and thiocyanato-Cbl(III) or GS^--Cbl(III), respectively (Schemes 1 and S1). Probably, AFR^- remain bound in transient L–Cbl(III)–AFR^- complex and capable to reduce Co(III) to Co(II).

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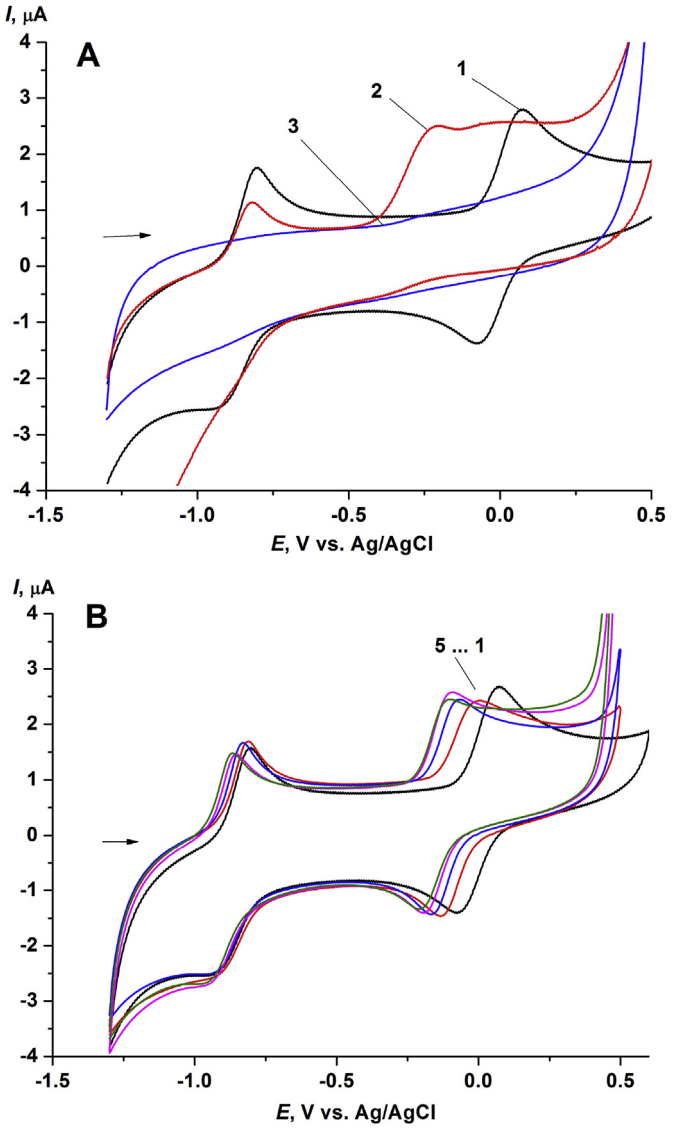


Fig. 5. (A) Cyclic voltammograms of Cbl(II) (0.3 mM) (1), Cbl(II) (0.3 mM) + GSH (0.14 M) (2) and GSH (0.14 M) (3) at pH 7.0; 21 °C. A poorly resolved oxidation wave observed at −0.1 V in the red curve (2) probably relates to the catalytic oxidation of GSH that occurs under conditions of the electrochemical experiment. (B) Cyclic voltammograms of Cbl(II) + SCN^- mixtures. [Cbl(II)] = 0.3 mM; [SCN^-] = 0 (1), 0.01 (2), 0.1 (3), 0.5 (4), 1.0 (5) M; pH 7.0; 21 °C. Arrows indicates direction of scan. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

scavengers (e.g., AA and GSH) and acidification of medium (AFR^- rapidly disproportionates in acidic medium to DHA and HAA^- [34]) that is observed in studied system (viz., the value of intercept is decreased upon addition of AA and GSH and acidification). Experimental evidences for the formation of a Cbl(II)-DHA adduct were not found, but it is expected to be labile and short lived due to the rapid inner-sphere electron transfer resulting in unstable radical species.

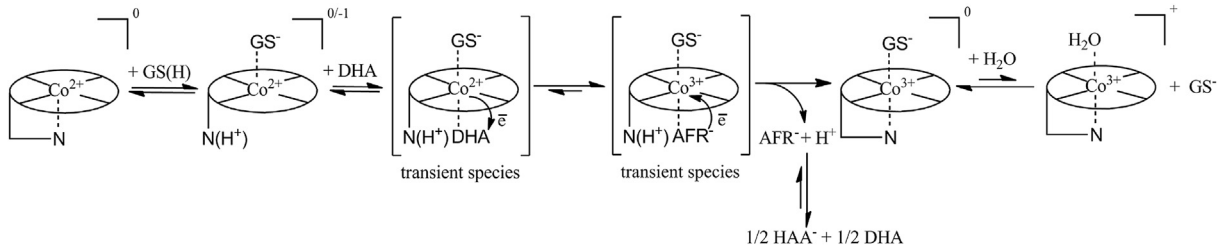
Another potential representation of reaction mechanism can be performed via generation of highly reactive cob(I)alamin (Cbl(I)) and its further reaction with DHA. Indeed, generation of Cbl(I) in the presence of thiols was proven in several Cbl-systems [35,36]. To access a role of Cbl(I) in reduction of DHA in Cbl(II)/GSH system, we attempted to perform the oxidation of Cbl(II) by nitrite and thiosulfate (efficient oxidants of Cbl(I) [37,38]) in the presence of GSH in neutral medium and observed no detectable reactions. Latter discriminates formation of Cbl(I) as a necessary step in reaction mechanism of studied system.

Collectively, the reported process represents a stoichiometric reduction of DHA by the S-ligated Cbl(II) adducts that results in vitamin C and Cbl(III) (i.e. its S-ligated adducts), without consumption of S-donors that serve rather as catalysts (Eq. (2)).

All biological cob(II)alamin derives from the processing of dietary MeCbl and AdoCbl by the glutathione-dependent dealkylase MMACHC (CblC) [39,40], and from the continuous redox cycling alternating cob(I)alamin, cob(II)alamin and Co–C bond formation leading to re-formation of MeCbl and AdoCbl in the active site of the two cobalamin-dependent enzymes, methionine synthase and methylmalonyl-CoA mutase [8,9]. Oxidation of cob(II)alamin is repaired by dedicated reductases, that re-couple cobalamin metabolism [8,9], thus, providing a sustainable source of cob(II)alamin that can participate in non-canonical reactions, such as the reduction of DHA to regenerate vitamin C. Herein, we presented evidence that cob(II)alamin, an essential intermediate in biological organometallic chemistry, can be side-tracked to catalytically and stoichiometrically rescue oxidized vitamin C.

4. Conclusion

For the first time we have demonstrated that AA can be recovered from DHA within a process involving cob(II)alamin in a stoichiometric manner (Eq. (2)). This process does not expend GSH, whose binding to the Co(II) center modulates the underlying redox mechanism. These findings not only open up a completely new network of the biological interplay between vitamin C, glutathione and metal centers, but also new possibilities for therapeutic approaches.



Scheme 1. Mechanism of the reduction of DHA by Cbl(II) in the presence of glutathione.

This reverse reaction can provide an explanation of intercepts on concentration dependencies (Fig. 3A). In this case, an extent of reverse reaction is expected to be decreased after addition of AFR^-

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Abbreviations

AA	Ascorbic acid
AFR^{-}	Ascorbyl free radical
Cbl	Cobalamin
DMBI	5,6-Dimethylbenzimidazole
DHA	Dehydroascorbic acid
DDHA	Dehydroascorbic acid dimer
GSH	Glutathione
MDHA	Dehydroascorbic acid monomer

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jorganchem.2017.01.002.

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