Mechanistic studies on the reaction between glutathionylcobalamin and selenocysteine

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ABSTRACT
The reaction between glutathionylcobalamin (GSCbl), a complex of Co(III)-cobalamin with glutathione, and selenocysteine (Sec) was investigated using ultraviolet-visible (UV–vis) spectroscopy. The interaction results in the formation of cob(II)alamin and proceeds via two pathways: (i) a rapid formation of complex between GSCbl and Sec followed by the rate-determining substitution of glutathionyl-ligand by Sec and rapid electron-transfer from Se-atom to Co(III)-ion and (ii) a nucleophilic attack of Co(III)-S bond by Sec.

ARTICLE HISTORY
Received 8 September 2018
Accepted 9 November 2018

KEYWORDS
Cobalamins; vitamin B12; selenocysteine; glutathione; kinetics

1. Introduction
Cobalamins (Cbls; Figure 1) are a group of cobalt-containing complexes involved in numerous biochemical processes. Their functions in vivo include methyl-transfer reactions, isomerization of C-C bonds, dehalogenations of organic compounds and some other reactions [1–4]. Cbls are a convenient platform for construction of novel derivatives promising for biomedical [5, 6] and catalytic applications [7].
Glutathionylcobalamin (GSCbl) is a Cbl species found in vivo [8], which can be formed during ligand exchange reaction between aquacobalamin (H₂OCo) and glutathione (GSH; Figure 1) [9], ubiquitous in cells tripeptide containing residues of glycine, cysteine, and glutamate. GSCbl is a stable complex under physiological conditions in comparison with several other thiolato-Cbls (e.g. cysteinyl-Cbl decomposes to cob(II)alam in [10]) and undergoes protonation and subsequent hydrolysis to H₂OCo and GSH under acidic conditions [11]. Its structure has been characterized using X-ray diffraction analysis [12] and computationally [13, 14]. GSCbl exhibits superior antioxidant properties over other Cbl-species (e.g. cyano-, methyl-, aqua-Cbls) [15] that can be due to higher stability of GSCbl under oxidative conditions. Indeed, GSCbl is more resistant toward modification by hypochlorite, a neutrophil-derived oxidant, than aqua- and cyano-Cbls [16]. GSCbl can be catalytically reduced to Cbl(I) by GSH in the presence of CblC-protein, whereas in the absence of CblC-protein this reaction does not occur [17, 18]. GSCbl is formed in the course of reaction between dehydroascorbic acid and labile complex of Cbl(II) with GSH [19, 20]. Similar reaction proceeds in the case of cob(II)iminamide (Cbl(II), a nucleotide-free analogue of Cbl(III)), although complex of Cbl(III) with GSH is unstable and undergoes reduction to Cbl(II) [21]. Thus, GSCbl is involved in redox reactions in vivo, which mechanisms need to be more thoroughly investigated.

Selenocysteine (Sec; Figure 1) is a proteinogenic amino acid found in the active site of several enzymes (e.g. glutathione peroxidase, thioredoxin reductase, methionine
sulfoxide reductase, iodothyronine deiodinase) [22]. Its structure resembles cysteine, in which sulfur is replaced by selenium. Sec is highly reactive toward reactive oxygen and nitrogen species [23–25], as well as it can be bound by metal ions [26–29]. Sec is capable of reducing Cbls(III) (viz., H₂OCbl and cysteinyl-Cbl) to Cbl(II) [30]. The reaction between H₂OCbl and Sec proceeds via rate-determining complexation and further rapid decomposition of Sec-Cbl(III) complex to Cbl(II) and selanyl radical [30]. However, the mechanism of the reduction of thiolato-Cbls by Sec remains unclear. This work reports a kinetic and mechanistic study on the interaction between GSCbl and Sec.

2. Experimental

Hydroxocobalamin hydrochloride (Sigma; HOCbl; ≥96%), seleno-L-cystine (Aldrich; 95%) and L-cysteine (Aldrich; 97%) were used without additional purification. Oxygen-free argon was used to deoxygenate reagent solutions. GSCbl was synthesized by reaction between HOCbl and GSH similarly to preparation of other thiolatocobalamins [31]. The concentrations of the Cbl stock solutions were determined by UV/Vis spectroscopy by the conversion of Cbl into its dicyano form (ε₃₆₈ = 30,400 M⁻¹ cm⁻¹ [32]). Reduction of selenocystine to selenocysteine was performed using sodium borohydride. An excess of borohydride was quenched by adding hydrochloric acid. Buffer solutions (citrate, acetate, phosphate, and borate) were used to maintain the pH during the measurements. Preliminary experiments showed that chloride and buffer components do not affect the kinetics of the reaction. The ionic strength was adjusted to 0.1 M using NaNO₃ in all measurements. The pH values of the solutions were determined by using a Multitest IPL-103 pH-meter (SEMICO) equipped with an ESK-10601/7 electrode (Izmeritel’naya tekhnika) filled with a 3.0 M KCl solution. The electrode was preliminarily calibrated using standard buffer solutions (pH 1.65–12.45). UV/Vis spectra were recorded with a cryothermostated (±0.1 °C) Cary 50 UV/Vis spectrophotometer in quartz cells. Experimental data were analyzed using Origin 7.5 software.
3. Results and discussion

Addition of selenocysteine to GSCbl results in changes in UV–vis spectra shown in Figure 2. The product of the reaction exhibits maxima in UV–vis spectrum at 312 and 475 nm typical for Cbl(II) [33, 34]. Formation of Cbl(II) in the reaction can be explained by two mechanisms: (i) one-electron reduction of GSCbl to Cbl(II) and (ii) nucleophilic attack of Co(III)-S bond by Sec that leads to the formation of Cbl(I), which, due to high reactivity, can be further readily oxidized by GSCbl or Sec/GSH oxidation products to Cbl(II). The nucleophilic-attack route is implemented in the catalytic cycle of CblC-trafficking protein that is capable of reducing of GSCbl to Cbl(I) by GSH [17, 18]. The possibility of the second pathway can be examined by using iodomethane that efficiently methylates Cbl(I) [35]. However, we found no influence of MeI on formation of Cbl(II) in the course of the interaction between GSCbl and Sec (Supporting Information Figure S1). Therefore, the reduction of GSCbl by Sec to Cbl(II) proceeds via one-electron transfer.

It is well-known that the mixture of selenocystine, an oxidized form of Sec, with thiols reversibly produces Sec and disulfides via the transient formation of selenosulfides (Equations 1 and 2) [36–38]. Formation of Sec, which is more reactive than thiols, explains application of this system in catalysis [30, 39–41]. The mixture of selenocystine and GSH is capable of reducing GSCbl with UV–vis spectral changes (Supporting Information Figure S2) similar to those observed in the course of reaction between GSCbl and Sec (Figure 2). Without GSH, selenocystine does not react with GSCbl (Supporting Information Figure S3). Apparently, the reactive species reducing GSCbl is Sec formed in reactions 1 and 2.

\[
R\text{SeSeR} + R'\text{S}^- \rightleftharpoons R\text{SeS}R' + R\text{Se}^- \quad (1)
\]

\[
R\text{SeSR} + R'\text{S}^- \rightleftharpoons R'SR' + R\text{Se}^- \quad (2)
\]

To understand mechanism of the reduction, kinetics of the interaction of GSCbl with Sec was studied. The typical kinetic curve of the process fits well to a first-order

![Figure 3. Typical kinetic curve of the reaction between GSCbl (5.0 \times 10^{-5} \text{ M}) and Sec (5.0 \times 10^{-4} \text{ M}) at \text{pH} 7.1, 25.0 \degree \text{C}.

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rate equation (Figure 3). The presence of excess GSH does not affect the reaction (Supporting Information Figure S4).

The plot of observed rate constants \(k_{\text{obs}}\) determined at \([\text{Sec}] > 0.3\ \text{mM}\) versus \([\text{Sec}]\) is linear, indicating the reaction is first order with respect to Sec and exhibits a positive Y-intercept (Figure 4). However, the kinetics of the reaction change upon lowering \([\text{Sec}]\): the dependence of initial rate \(r_0\) on initial Sec concentration (Figure 5) exhibits saturation behavior and passes through origin at \([\text{Sec}]_0 \leq 0.17\ \text{mM}\).

**Figure 4.** Plot of observed rate constant \(k_{\text{obs}}\) versus \([\text{Sec}]\) for the reaction between GSCbl \((5.0 \times 10^{-5}\ \text{M})\) and Sec at pH 7.1, 25.0 °C. The value of the slope is \((276 \pm 8)\ \text{M}^{-1}\cdot\text{min}^{-1}\).

**Figure 5.** Plot of initial rate \(r_0\) vs. \([\text{Sec}]_0\) for the reaction between GSCbl \((5.0 \times 10^{-5}\ \text{M})\) and Sec in the range of low \([\text{Sec}]_0\) at pH 7.1, 25.0 °C.
We examined the influence of pH on the reaction kinetics in the presence of excess Sec. The plot of the slope of concentration dependence \( k_{sl} \) versus pH is shown in Figure 6, which indicates substantial increase in the reaction rate in acidic (pH < 4) and alkaline (pH > 9) media and a slightly pronounced inflection at pH ~ 6. Increase in rate at pH > 9 as well as the presence of the inflection point can be explained by the involvement of three Sec species in the interaction with GSCbl (equilibria between Sec species are shown by reactions 3 and 4; \( pK_{a1} = 5.4 \) and \( pK_{a2} = 10.7 \) at 25 \(^\circ\)C [42]). The increase in the rate at pH < 4 can be explained by the protonation of sulfur of GSCbl (\( pK_a = 1.3 \) at 25 \(^\circ\)C [11]) that increases electrophilicity of Co(III)-S motif and facilitates interaction with Sec.

\[
\begin{align*}
(\text{I}) & \quad \text{HSe}^+ \quad \text{NH}_3^+ \quad \text{O}^- \quad \text{O}^- \\
(\text{II}) & \quad \text{Se}^- \quad \text{NH}_3^+ \quad \text{O}^- \quad \text{O}^- \\
(\text{III}) & \quad \text{Se}^- \quad \text{NH}_2^- \quad \text{O}^- \quad \text{O}^- \\
(4; pK_{a2}) & \\
(\text{V}) & \quad \text{GSH} \quad \text{Co}^{II} \quad \text{N} \quad \text{N} \\
(\text{VI}) & \quad \text{GS}^- \quad \text{Co}^{III} \quad \text{N} \quad \text{N} \\
(5; pK_a(GSCbl)) & 
\end{align*}
\]
To describe dependence of $k_{sl}$ on pH (Figure 6), Equation 6 accounting for the acid-base properties of both reactants was expressed:

$$k_{sl} = \left( \frac{k_1}{10^{-pH} + 10^{-pK_{a1}(GSCbl)}} + \frac{k_2}{10^{-pK_{a1}+pH}} \right) \frac{10^{-pK_{a2}}}{10^{-pH} + 10^{-pK_{a2}}}$$

$$+ \frac{k_3}{10^{-pK_{a2}} + 10^{-2pH} + 10^{-pK_{a1}+pK_{a2}}}$$

where $k_{1-1}$ and $k_{1-2}$ are rate constants of reactions of protonated and deprotonated GSCbl species with (I) (Equation 3), $M^{-1} s^{-1}$; $k_2$ and $k_3$ are rate constants of reactions of deprotonated GSCbl species with (II) and (III) (Equations 3 and 4), $M^{-1} s^{-1}$; $pK_{a1}$ and $pK_{a2}$ are acid dissociation constants corresponding to equilibria (3) and (4); $pK_{a1}(GSCbl)$ is the acid dissociation constant of protonated GSCbl (Equation 5). The following values of rate constants were obtained using Equation 6: $k_{1-1} = (4.2 \pm 0.7) \times 10^4 M^{-1} min^{-1}$, $k_{1-2} \sim 70 M^{-1} min^{-1}$, $k_2 = (2.7 \pm 0.3) \times 10^2 M^{-1} min^{-1}$, $k_3 = (8.8 \pm 0.3) \times 10^3 M^{-1} min^{-1}$ (25°C).

The mechanisms of GSCbl reduction by Sec are different in ranges of low and high Sec concentrations. Probably, the first route of the reduction proceeds via rapid complex formation between GSCbl and Sec, further slow substitution of bound glutathionyl-ligand and subsequent rapid electron transfer from Se- to Co(III)-ion. The complexation of Sec may involve lower (α) and upper (β) sides of GSCbl, although the reaction with α-side (i.e. substitution of 5,6-dimethylbenzimidazole) must be accompanied by pronounced changes in the UV–vis spectrum at the beginning of the reduction that is not observed. Upon increasing [Sec], the rate of the route reaches saturation and contribution of the second pathway in the kinetics becomes significant; the second route proceeds via nucleophilic attack of the Co(III)-S bond by Sec. Both pathways are shown in Scheme 1.

**Scheme 1.** Suggested mechanism of the reaction between aquacobalamin and selenocysteine.
To describe the dependence of \( r_0 \) versus \([\text{Sec}]_0\) for the first reduction route, Equation 7 was expressed:

\[
 r_0 = \frac{k_{\text{sub}} \cdot [\text{GSCbl}]_0 \cdot K_{\text{compl}} \cdot [\text{Sec}]_0}{1 + K_{\text{compl}} [\text{Sec}]_0}
\]  
(7)

where \( k_{\text{sub}} \) is the rate constants of the substitution of glutathionyl-ligand by Sec, M\(^{-1}\) min\(^{-1}\); \( K_{\text{compl}} \) is the equilibrium constant of the complexation between GSCbl and Sec, M\(^{-1}\); \([\text{GSCbl}]_0\) and \([\text{Sec}]_0\) are initial concentrations of GSCbl and Sec, M. The following values of constants were obtained using Equation 7: \( k_{\text{sub}} = (3.0 \pm 0.5) \times 10^{-1} \) M\(^{-1}\) min\(^{-1}\), \( K_{\text{compl}} = (2.0 \pm 0.4) \times 10^{3} \) M\(^{-1}\) (pH 7.1; 25°C).

**4. Conclusion**

This work showed that reduction of glutathionylcobalamin by selenocysteine to give cob(II)alamin represents a complex process. The mechanism of reaction includes two pathways, i.e. by (i) the rapid complexation between Sec and GSCbl, further rate-determining substitution of bound GSH by Sec and electron transfer from Sec on Co(III)-ion and (ii) nucleophilic attack of Co(III)-S bond by Sec.

**Disclosure statement**

The authors declare no competing financial interests.

**Funding**

This work was supported by the Russian Science Foundation, Agreement No. 14-23-00204 P.

**References**

[1] R.G. Matthews. *Met. Ions Life Sci.*, 6, 53 (2009).
[2] B. Kräutler. *Biochem. Soc. Trans.*, 33, 806 (2005).
[3] I.A. Dereven’kov, D.S. Salnikov, R. Silaghi-Dumitrescu, S.V. Makarov, O.I. Koifman. *Coord. Chem. Rev.*, 309, 68 (2016).
[4] J. Bridwell-Rabb, C.L. Drennan. *Curr. Opin. Chem. Biol.*, 37, 63 (2017).
[5] F. Zelder. *Chem. Commun. (Camb).* 51, 14004 (2015).
[6] F. Zelder. *J. Porphyrins Phthalocyanines*, 22, 1 (2018).
[7] M. Giedyk, K. Goliszewska, D. Gryko. *Chem. Soc. Rev.*, 44, 3391 (2015).
[8] L. Hannibal, A. Axhemi, A.V. Glushchenko, E.S. Moreira, N.E. Brasch, D.W. Jacobsen. *Clin. Chem. Lab. Med.*, 46, 1739 (2008).
[9] L. Xia, A.G. Cregan, L.A. Berben, N.E. Brasch. *Inorg. Chem.*, 43, 6848 (2004).
[10] R.K. Suto, N.E. Brasch, O.P. Anderson, R.G. Finke. *Inorg. Chem.*, 40, 2686 (2001).
[11] L.A. Schumacher, R. Mukherjee, J.M. Brown, H. Subedi, N.E. Brasch. *Eur. J. Inorg. Chem.*, 4717 (2011).
[12] L. Hannibal, C.A. Smith, D.W. Jacobsen. *Inorg. Chem.*, 49, 9921 (2010).
[13] K.S. Conrad, T.C. Brunold. *Inorg. Chem.*, 50, 8755 (2011).
[14] A.S. Eisenberg, I.V. Likhtina, V.S. Znamenskiy, R.L. Birke. *J. Phys. Chem. A*, 116, 6851 (2012).
[15] C.S. Birch, N.E. Brasch, A. McCaddon, J.H.H. Williams. *Free Radic. Biol. Med.*, 47, 184 (2009).
