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Processing of glutathionylcobalamin by a bovine B_{12} trafficking chaperone bCblC involved in intracellular B_{12} metabolism

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ABSTRACT

Glutathionylcobalamin (GSCbl) is a biologically relevant vitamin B_{12} derivative and contains glutathione as the upper axial ligand thought formation of a cobalt-sulfur bond. GSCbl has been shown to be an effective precursor of enzyme cofactors, however processing of the cobalamin in intracellular B_{12} metabolism has not been fully elucidated. In this study, we discovered that bCblC, a bovine B_{12} trafficking chaperone, catalyzes elimination of the glutathione ligand from GSCbl by using the reduced form of glutathione (GSH). Deglutathionylation products are base-off cob(II)alamin and glutathione disulfide, which are generated stoichiometrically to GSH. Although cob(I)alamin was not detected due to its instability, deglutathionylation is likely analogous to dealkylation of alkylcobalamins, which uses the thiolate of GSH for nucleophilic displacement. The catalytic turnover number for the deglutathionylation of GSCbl is $\geqslant 1.62 \pm 0.13\ min^{-1}$, which is, at least, an order of magnitude higher than that for elimination of upper axial ligands from other cobalamins. Considering the prevalence of GSH at millimolar concentrations in cells, our results explain the previous finding that GSCbl is more effective than other cobalamins for synthesis of enzyme cofactors.

1. Introduction

B_{12} (vitamin B_{12} derivatives, cobalamin) is an essential micronutrient for mammals including humans and serves as an enzyme cofactor. B_{12} contains cobalt, which is coordinated by four equatorial nitrogen atoms in the center of the corrin ring. The fifth ligand to the central cobalt is endogenous dimethylbenzimidazole (DMB) base from the lower side of the corrin ring [1]. The sixth exogenous upper axial ligand varies and determines the biological activities of B_{12}. Methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl, coenzyme B_{12}) are active enzyme cofactors containing methyl and 5′-deoxyadenosyl group, respectively, as the upper axial ligand. In humans, two enzymes have been found use B_{12} as the essential cofactor for their catalytic activities [2,3]. Methionine synthase in the cytosol utilizes MeCbl to catalyze methyl transfer from methyltetrahyrofolate to homocysteine. Methylmalonyl-CoA mutase in mitochondria utilizes AdoCbl to catalyze isomerization of L-methylmalonyl-CoA to succinyl-CoA, which then enters Krebs cycle.

A series of proteins, including the cytosolic protein MMACHC (methylmalonic aciduria type C and homocystinuria, hCblC), is involved in intracellular B_{12} metabolism for enzyme cofactor synthesis from B_{12} precursors. The gene *mmachc* encoding hCblC has been found to be the most frequently mutated in patients with genetic disorders of intracellular B_{12} metabolism [4]. Cells with defective hCblC fail to synthesize both AdoCbl and MeCbl, resulting in accumulation of methylmalonic acid and homocysteine [5]. hCblC has been characterized as a B_{12} trafficking chaperone that binds various cobalamins with different upper axial ligands and transports them to subsequent proteins for enzyme cofactor synthesis [6]. In addition, hCblC catalyzes elimination of cyanide from cyanocobalamin (CNCbl, vitamin B_{12}) and alkyl groups from alkylcobalamins, which are prerequisite processes in enzyme cofactor synthesis. Decyanation uses an electron from free or protein-bound reduced flavin and generates base-off cob(II)alamin, which can be used as a common intermediate for subsequent enzyme cofactor synthesis [6]. On the other hand, dealkylation uses the thiolate of the reduced form of glutathione (GSH) for nucleophilic displacement generating cob(I)alamin with a corresponding glutathione thioether [7,8].

Glutathione (L-γ-glutatmyl-L-cysteinyl-glycine) is the most abundant non-protein thiol (1–10 mM) in mammalian cells existing predominantly in the reduced form GSH [9]. The biological role of GSH is diverse, as it is involved in maintaining intracellular redox states as a major antioxidant, mediating cellular signaling for proliferation and apoptosis, metabolism of xenobiotics and thiol disulfide exchange reactions [10]. GSH is also found in glutathionylcboalamin (GSCbl) as the upper axial ligand thought formation of a cobalt–sulfur bond [11,12]. GSCbl was shown to be more efficient than other cobalamins to reconstitute the activities of methionine synthase and methylmalonyl-CoA mutase [13]. The

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glutathione ligand of GSCbl is displaced in intracellular B_{12} metabolism for enzyme cofactor synthesis, which has not yet been elucidated. In this study, we discovered that bCblC, a bovine homolog of hCblC, catalyzes elimination of the glutathione ligand from GSCbl using GSH. The deglutathionylation of GSCbl generates base-off cob(II)alamin and glutathione disulfide (GSSG), which are stoichiometric to GSH. Although cob(I)alamin was not detected due to its instability in the active site of the protein, deglutathionylation likely occurs through nucelophilic displacement using the thiolate of GSH, which is chemically analogous to dealkylation of aklycobalamins [7,8]. In addition, the deglutathionylation rate is much higher than that for elimination of upper axial ligands from other cobalamins. Considering the prevalence of GSH at millimolar concentrations in cells, our results explain the previous finding that GSCbl is more effective than other cobalamins for synthesis of enzyme cofactors.

2. Materials and methods

2.1. Materials and general methods

All chemicals were from Sigma Aldrich, unless otherwise indicated. The recombinant protein bCblC fused with 6xHis-Tag at the C-terminus was prepared as previously described [14]. Anoxic buffer was prepared by boiling, degassing under vacuum and stirring in an anaerobic chamber (Coy Laboratories, Ann Arbor, MI, USA) for at least 24 h. Protein concentrations were determined by Bradford assay [15].

2.2. Glutathionylcobalamin synthesis

GSCbl was synthesized according to a patented method (US patent number 7,030,105). Briefly, OH_2Cbl was mixed with three molar excess GSH in 1.0 ml distilled water and incubated for 3 h at room temperature under dark conditions. Addition of 1.0 ml chilled acetone to the mixture resulted in formation of purple precipitates. After incubation on ice for 30 min, precipitates were collected by centrifugation and extensively washed with >1.0 ml × 10 of 50% (v/v) chilled acetone to remove free glutathione. Dried GSCbl precipitates were dissolved and the concentration was determined by using $\varepsilon 534\ nm = 7.97\ mM^{-1}\ cm^{-1}$ [12].

2.3. Deglutathionylation of GSCbl and GSSG determination

Reactions contained GSCbl and five molar excess bCblC in 50 mM Hepes pH 8.0, 150 mM KCl and 5% glycerol. Deglutathionylation was initiated by addition of GSH at the indicated concentrations and incubated in the dark at room temperature by recording absorption spectra using a Cary 100 UV–Vis spectrophotometer (Varion). Anoxic deglutathionylation was prepared, as described above, in an anaerobic chamber with anoxic buffer, followed by recording absorption spectra using an evolution 260 Bio UV–Vis spectrophotometer (Thermo Scientific).

For GSSG determination, deglutathionylation was terminated immediately after reaction initiation with 5% (w/v) metaphosphoric acid. Protein precipitates were removed by centrifugation and GSSG in the supernatant was determined using a glutathione colorimetric assay kit (Arbor assays, Ann Arbor, MI, USA).

2.4. X-band EPR spectroscopy

EPR samples were prepared with 100 μM GSCbl and 500 μM bCblC under anoxic conditions. Deglutathionylation was initiated by addition of 500 μM GSH and the reaction mixture was immediately frozen in liquid nitrogen. Spectroscopic parameters were: temperature, 100 K; microwave power, 1 mW; microwave frequency, 9.38 GHz; receiver gain, 1×10^5; modulation amplitude, 10.0 G; modulation frequency, 100 kHz.

2.5. Identification of GSSG by HPLC

Anoxic deglutathionylation reactions with 100 μM GSCbl and 500 μM bCblC were initiated by addition of GSH at the indicated concentrations and terminated immediately with 5% (w/v) metaphosphoric acid. Protein precipitates were removed by centrifugation and supernatants were neutralized by addition of saturated potassium carbonate. Glutathione products in supernatants were analyzed by HPLC as previously described [7,16]. Amino groups of glutathione were derivatized with 2,3-dinitrofluorobenzene following reaction of free thiols with monoiodoacetic acid and injected on Phenomenex Bondclone NH_2 column (300 × 3.9 mm, 10 μm) equilibrated with solvent A of 4:1 (v/v) methanol/water. The column was eluted by using solvent B (the mixture of 400 ml of solvent A with a 100 ml solution of 272 g sodium acetate trihydrate, 122 ml water and 373 ml glacial acetic acid) as following conditions: from 0 to 5 min, isocratic 30% solvent B; from 5 to 30 min, linear gradient from 30% to 100% solvent B. Elution peaks were monitored by measuring the absorption at 355 nm. GSSG concentrations were determined by comparing integrated peak areas with the standard curve obtained with commercial GSSG compound.

3. Results

3.1. Deglutathionylation of GSCbl by bCblC using GSH

Addition of bCblC to GSCbl induced changes in the absorption spectrum indicating binding of GSCbl to bCblC (Fig. 1A). The dissociation constant was estimated to be K_d = 16–25 μM, which is consistent with the previously reported value [14]. Isosbestic points were observed at 514, 386 and 367 nm, although they were not always clear. Incubation of GSCbl-bCblC induced additional slow changes in the absorption spectrum, and the extent of the increase at 355 nm was dependent on GSCbl synthesis (Fig. 1A inset). We assumed that the spectral changes might be due to contamination of GSCbl by free glutathione, since its synthetic reaction includes incubation of OH_2Cbl with a molar excess of GSH, which generates GSSG as a byproduct [17]. Although most of the free glutathione was supposed to be removed by washing, a trace amount of free glutathione (1–5 μM) was detected in GSCbl, which could be removed by extensive washing. In addition, incubation of GSCbl-bCblC prepared with free glutathione-free GSCbl induced no change in the absorption spectrum. This observation prompted us to test the reaction between GSCbl-bCblC and glutathione.

Addition of GSH to GSCbl-bCblC induced immediate changes in the absorption spectrum, whereas no spectral change was observed with GSSG (Fig. 1B). The resulted spectrum showed absorption picks at 355, 499 and 530 nm characteristic of OH/OH_2Cbl, indicating elimination of the glutathione ligand from GSCbl by bCblC. Although the reaction was too rapid to determine its rate by conventional spectroscopy, the apparent K_{ob} was estimated to be $1.62 \pm 0.13\ min^{-1}$ at 355 nm (Fig. 1B inset). Addition of other biologically relevant thiols, cysteine and homocysteine, to GSCbl-bCblC also induced changes in the absorption spectrum generating OH/OH_2Cbl, which was not observed with corresponding disulfide forms of the thiols. These results indicate that bCblC catalyzes elimination of glutathione ligand from GSCbl using thiols. Although deglutathionylation of GSCbl is not specific for GSH, the following experiments were carried out with GSH considering its prevalence in the millimolar range in cells.

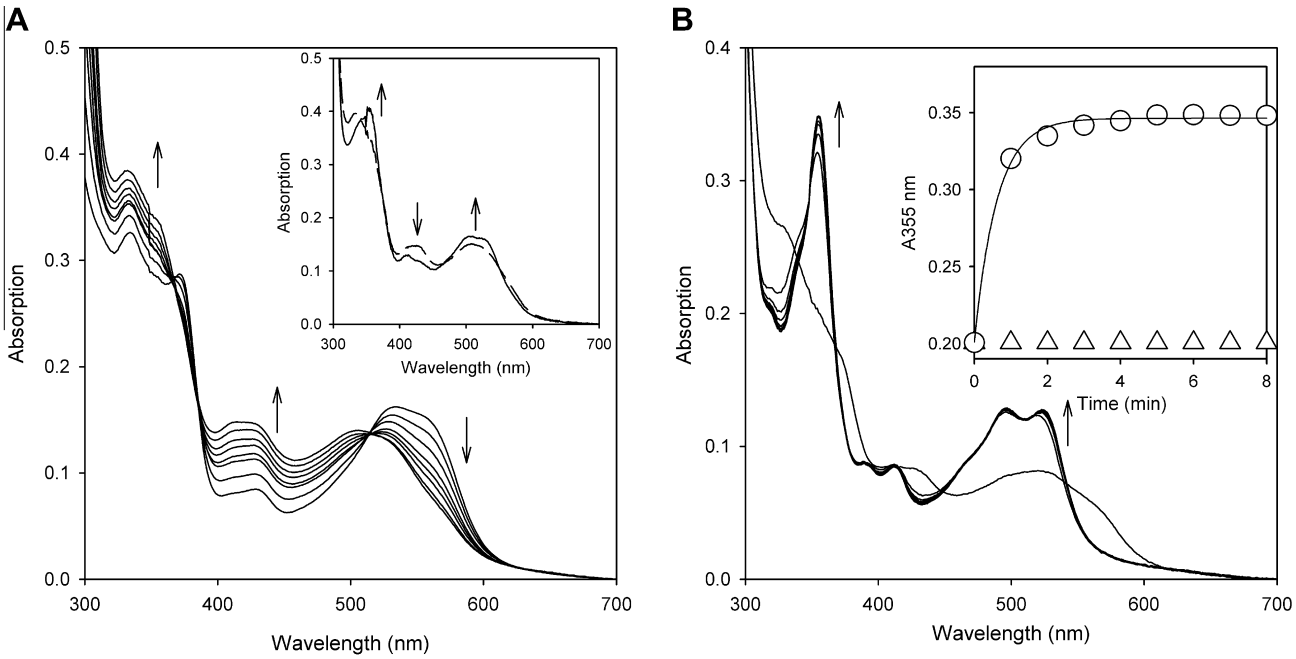

Fig. 1. Binding of GSCbl to bCblC and GSH-dependent deglutathionylation. (A), UV–Vis absorption spectra were recorded by addition of bCblC in aliquots to 20 μM GSCbl in 50 mM Hepes pH 8.0, 150 mM KCl and 5% glycerol. The dissociation constant was determined as described in a previous report [14]. The inset shows changes in the spectrum of 20 μM GSCbl-bCblC containing a trace amount of free glutathione after 3 h incubation. (B), Deglutathionylation was initiated by addition of 100 μM GSH to 15 μM GSCbl-bCblC and absorption spectra were recorded by incubation. The reaction rate K_{ob} = 1.62 ± 0.13 min^{-1} was determined by fitting the incubation time-dependent increase in A355 nm to a first order equation (inset). Arrows indicate changes in the absorption spectrum.

3.2. Deglutathionylation is stoichiometric to GSH

Deglutathionylation of GSCbl was examined at different GSH concentrations. There was no significant difference in reaction rate at all tested GSH concentrations. However, at sub-stoichiometric GSH concentrations to the concentration of GSCbl-bCblC, final absorption spectra were for the mixture of OH/OH_2Cbl and GSCbl-bCblC, indicating partial deglutathionylation (Fig. 2A). The increase at 355 nm for OH/OH_2Cbl generation was proportional to GSH concentrations, until the molar ratio of [GSH]/[GSCbl-bCblC] = 1.1 ± 0.1 was reached (Fig. 2A inset). The plot in 2B shows clear one-to-one stoichiometry between GSH and OH/OH_2Cbl generated by deglutathionylation ([GSH]/[OH/OH_2Cbl] = 0.95 ± 0.2) (Fig. 2B). We also measured GSSG, which may be the other product of deglutathionylation. As shown in Fig. 2C, GSSG was detected as a deglutathionylation product and GSSG generation appeared to be stoichiometric to GSH added in the deglutathionylation reactions. These results indicate that bCblC uses a stoichiometric amount of GSH to GSCbl for deglutathionylation. However, in a coupled assay involving glutathione reductase and NADPH for GSH regeneration from GSSG, only a catalytic amount of GSH was sufficient to complete deglutathionylation of GSCbl (Fig. 2D).

3.3. Identification of base-off cob(II)alamin generated by deglutathionylation

Anoxic deglutathionylation was carried out as described in the materials and method, followed by UV–Vis spectroscopy (Fig. 3A). Addition of GSH to GSCbl-bCblC induced immediate changes in the absorption spectrum with the absorption increase at 473 nm characteristic of cob(II)alamin. The anoxic deglutathionylation rate was estimated to be K_{ob} = 1.64 ± 0.12 min^{-1}, which is similar with the aerobic reaction rate. Cob(II)alamin generated by anoxic deglutathionylation was confirmed by EPR spectroscopy (Fig. 3B). The spectrum was characteristic of cob(II)alamin in the base-off state showing eight-line hyperfine splitting patterns as triplets in the high-field region and the 140 G spacing between hyperfine lines [6]. This result indicates that cob(II)alamin generated by deglutathionylation is bound to bCblC in the base-off state, since free cob(II)alamin in neutral or slightly alkaline solution is in the base-on state.

3.4. Identification of GSSG generated by deglutathionylation

Generation of GSSG by anoxic deglutathionylation of GSCbl with GSH was confirmed by HPLC analysis. Separation of the glutathione product showed a clear elution peak for GSSG compared with a reference compound (Fig. 4A). On the other hand, only minor elution peaks for GSH and GSSG were detected with GSCbl-bCblC in the absence of GSH, which might be released from GSCbl and spontaneously oxidized under acidic conditions of analysis. Generation of GSSG was quantified with anoxic deglutathionylation at different GSH concentrations (Fig. 4B). GSSG were determined to proportionally increase by increasing GSH concentrations, until GSH reached an equimolar concentration with GSCbl-bCblC. The plot of GSSG against GSH consistently indicates that GSSG generation is stoichiometric to GSH in deglutathionylation reactions ([GSH]/[GSSG] = 1.1 ± 0.1).

4. Discussion

Ever since GSCbl was found to be an effective precursor of coenzymes [13], its natural occurrence in cells had been controversial, as extraction of non-alkyl cobalamins from biological samples causes exchange of upper axial ligands. In particular, a major intracellular B_{12}, OH_2Cbl, is highly reactive with GSH abundant in cells, which can generate GSCbl during extraction of intracellular cobalamins. On the other hand, it was suggested that any free OH_2Cbl in cells would be converted into GSCbl, according to the high reactivity between OH_2Cbl and GSH generating GSCbl almost irreversibly

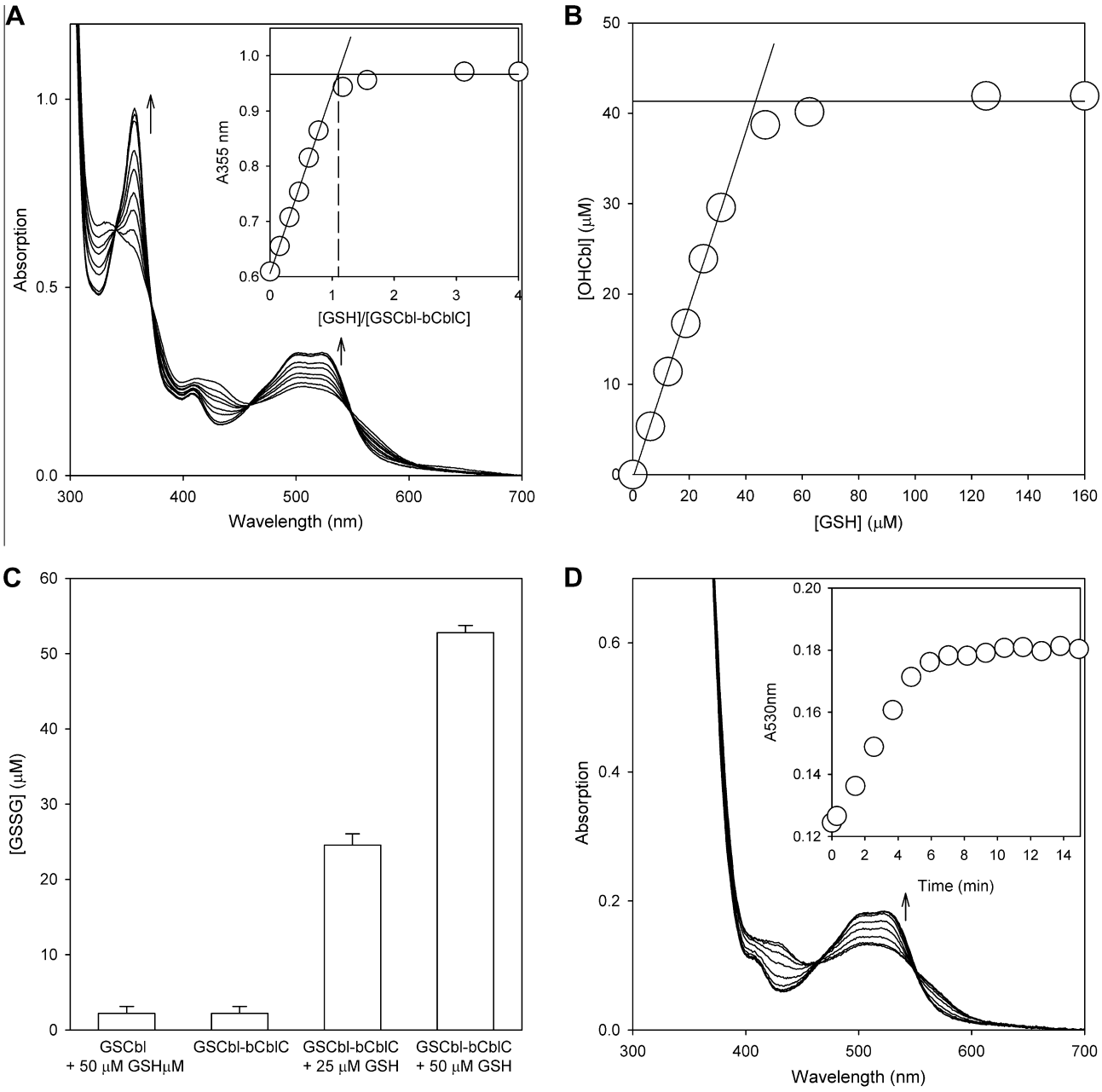

Fig. 2. Stoichiometric generation of OH_2Cbl and GSSG to GSH by deglutathionylation. (A), Deglutathionylation was examined at increasing GSH concentrations (0–160 μM) with 40 μM GSCbl-bCblC and resulted absorption spectra were recorded. The inset shows the plot of A355 nm against the molar ratio of [GSH]/[GSCbl-bCblC] and fitting (lines) the data to a zero order equation. (B), The plot in the A inset is expressed as OH_2Cbl concentrations vs. GSH concentrations using $\Delta\varepsilon 355\ nm = 8.9\ mM^{-1}\ cm^{-1}$ between OH_2Cbl and GSCbl-bCblC. (C), GSSG concentrations were determined, as described in the materials and methods, for deglutathionylation with 50 μM GSCbl-bCblC and the indicated GSH concentrations. (D), Deglutathionylation was initiated by addition of 1 μM GSH to 20 μM GSCbl-blCblC in the presence of 1 U glutathione reductase and 0.2 mM NADPH, and absorption spectra were recorded by incubation. The inset shows the increase in A550 nm for OH_2Cbl generation.

at neutral pH [17]. In fact, a recent study identified GSCbl as an intracellular B_{12} by assessing cobalamin profiles of cells using an improved method [18]. However accurate determination of GSCbl concentrations in cells seems challenging due to the acidic conditions of HPLC analysis, which may cause dissociation of the glutathione ligand from GSCbl.

The bovine protein bCblC shows 88% amino acid sequence identity with the human B_{12} trafficking chaperon protein hCblC, which is well conserved in mammals [5,19]. We previously showed that bCblC binds cobalamins with various upper axial ligands, as well as exhibits decyanation and dealkylation activities, as shown for hCblC [6,14,20]. bCblC binds alkylcobalamins and CNCbl, inducing characteristic changes in the absorption spectra for the conformational transition from the base-onto base-off state [14]. Binding of GSCbl to bCblC also induces significant changes in the absorption spectrum, which may implicate the conformational transition of the cobalamin (Fig. 1A). However, the conformation of GSCbl bound to bCblC could not be defined as the base-off sate due to the absence of a reference base-off GSCbl. The base-off transition of free cobalamins is governed by the p*K*a for protonation of the DMB base (~4–7 units below physiological pH), which occurs under acidic conditions [21]. However the base-off transition of GSCbl could not be obtained, since the glutathione ligand spontaneously dissociates from the cobalt at pH < 4.0, although GSCbl exhibits unique stability at a neutral pH compared with other thiolatocobalamins [11,17].

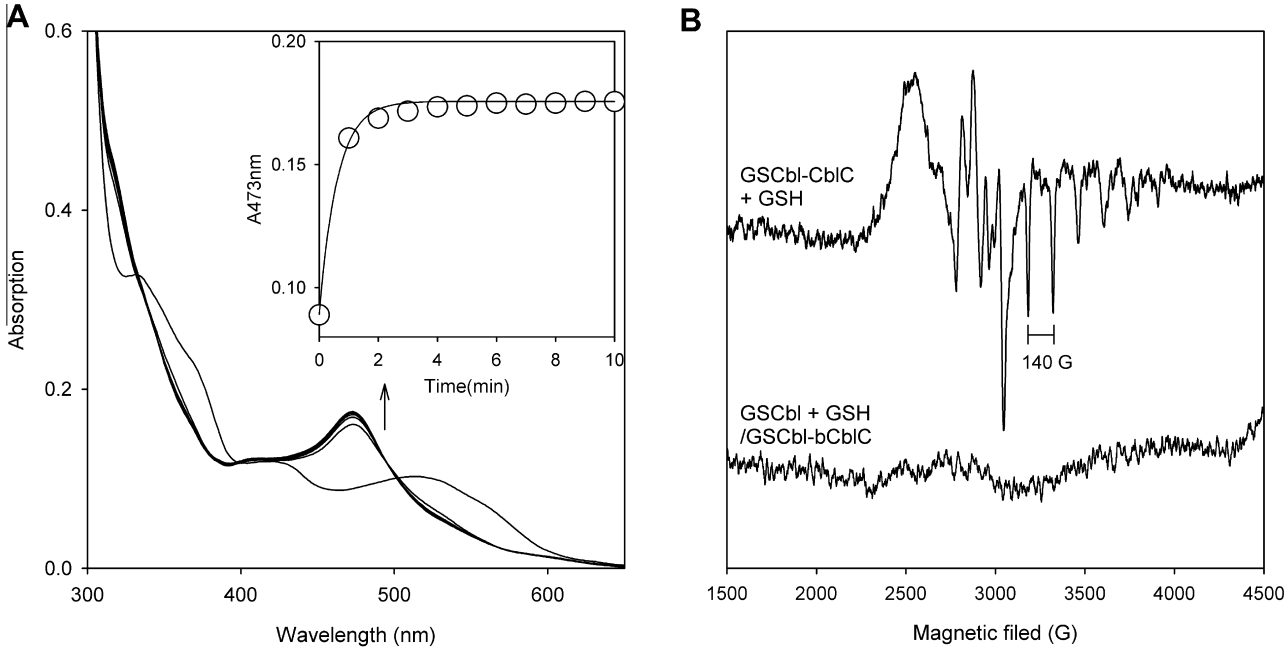

Fig. 3. Identification of base-off cob(II)alamin generated by anoxic deglutathionylation. (A), Anoxic deglutathionylation reaction was initiated by addition of 100 μM GSH to 20 μM GSCbl-bCblC and absorption spectra were recorded by incubation. The inset shows the increase in A473 nm for cob(II)alamin generation. (B), EPR sample was prepared with 100 μM GSCbl-bCblC or 100 μM GSCbl. Deglutathionylation was initiated by addition of 500 μM GSH and immediately terminated by freezing in liquid nitrogen. Spectroscopic parameters are as described in Section 2.

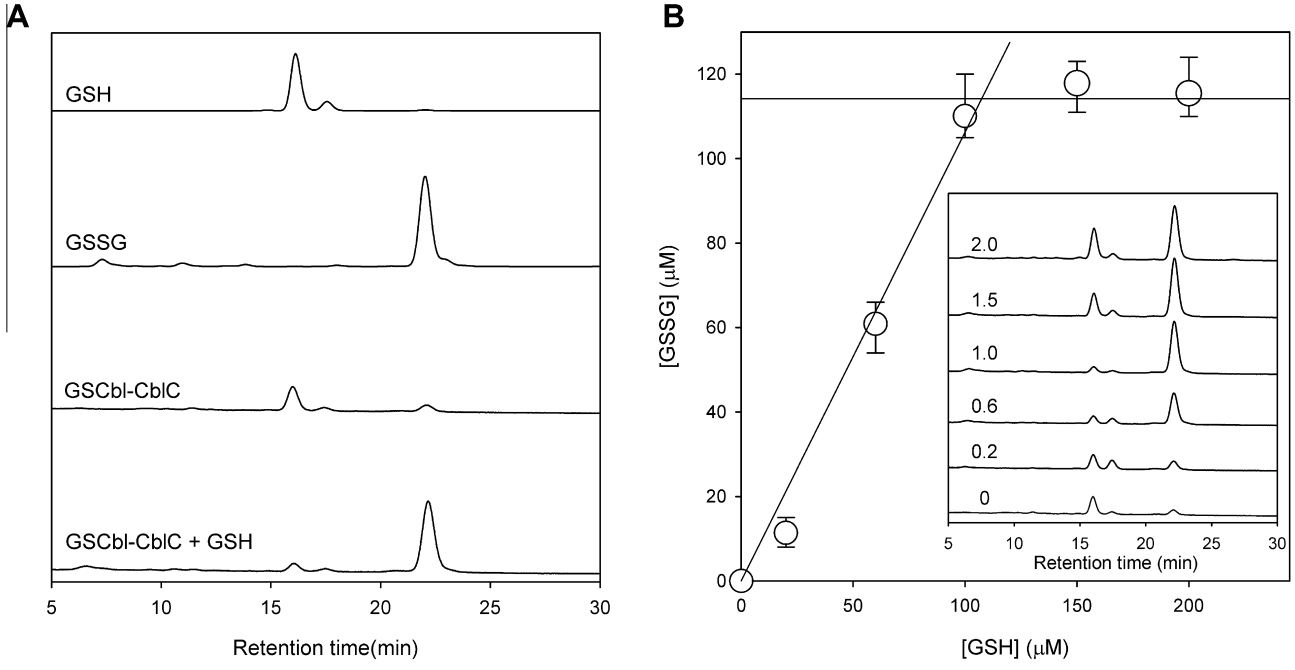

Fig. 4. Identification and quantification of GSSG generation by HPLC. Glutathione products of deglutathionylation were separated by HPLC as described in the materials and methods. (A), HPLC samples contained 100 μM GSCbl-bCblC with/without 100 μM GSH or commercial glutathione compound as indicated. (B), Concentrations of GSSG generated by deglutathionylation at increasing GSH (0–200 μM) were determined and plotted against GSH concentrations. The inset shows HPLC separation profiles at the indicated molar ratios of [GSH]/[GSCbl-bCblC].

The most important discovery in this study is that bCblC catalyzes elimination of the glutathione ligand from GSCbl using GSH and generates base-off cob(II)alamin with GSSG, which has not yet been described with hCblC. Considering the high amino acid sequence identity between bCblC and hCblC as well as their similar catalytic activities, deglutathionylation of GSCbl is unlikely to be specific for bCblC, although this needs to be examined for the human homolog. bCblC uses not only GSH, but also other biologically relevant thiols for deglutathionylation of GSCbl, which differs from the GSH-dependent dealkylation of alkylcobalamins [7,8,14]. This different thiol specificity can be explained by different conformation of bCblC bound with GSCbl compared to that bound with alkylcobalamins. It was previously shown that binding of cobalamins changes the conformation of bCblC differently depending of

the upper axial ligands [22]. Nevertheless, cysteine and homocysteine are unstable and their concentrations are an order of magnitude lower than GSH concentrations in cells [9,23]. Therefore, GSH would be the most probable physiological thiol used by bCblC for deglutathionylation of GSCbl. In addition, since cobalamin concentrations are estimated to be relatively low (0.03–0.7 mM) [24], deglutathionylation of GSCbl would efficiently occur in vivo. In fact, in the presence of glutathione reductase, only a catalytic amount of GSH is sufficient for deglutathionylation of GSCbl (Fig. 2D). The stoichiometric generation of GSSG to GSH strongly indicates that deglutathonylation of GSCbl is chemically analogous to dealkylation of alkylcobalamins [7,8]. It was demonstrated that hCblC catalyzes dealkylation by using the thiolate of GSH for nucleophilic displacement generating cob(I)alamin with corresponding glutathione thioethers. However, we detected only cob(II)alamin, but not cob(I)alamin, for bCblC-catalyzed dealkylation of MeCbl and AdoCbl, although corresponding glutathione thioethers were identified (unpublished data). It is hard to think that dealkylation catalyzed by two highly homologous proteins occurs though different reaction mechanism. Therefore, we concluded that cob(I)alamin in the active site bCblC is less stable than that in the active site of hCblC, which is spontaneously oxidized to cob(II)alamin. Hence, generation of base-off cob(II)alamin by deglutathionylation of GSCbl by bCblC is likely via cob(I)alamin and its spontaneous oxidation in the active site of the protein.

In conclusion, we discovered that bCblC catalyzes elimination of the glutathione ligand from GSCbl using GSH. The deglutathionylation of GSCbl generates base-off cob(II)alamin and GSSG, which are stoichiometric to GSH. Although cob(I)alamin was not detected due to its instability in the active site of the protein, deglutathionylation is likely analogous with the dealkylation of alkylcobalamins, which uses the thiolate of GSH for nucleophilic displacement. The catalytic turnover number for the deglutathionylation of GSCbl is $\geqslant 1.62 \pm 0.13\ min^{-1}$, which is, at least, an order of magnitude higher than that for elimination of upper axial ligands from other cobalamins [6,7]. Considering the prevalence of GSH at millimolar concentrations in cells, our results explain the previous finding that GSCbl is more effective than other cobalamins for synthesis of enzyme cofactors.

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