J|A|C|S JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Unusual Aerobic Stabilization of Cob(I)alamin by a B_{12}-Trafficking Protein Allows Chemoenzymatic Synthesis of Organocobalamins

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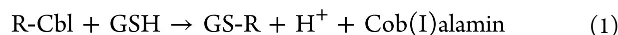
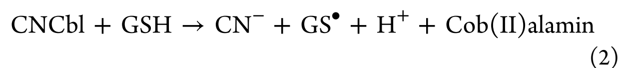
Supporting Information

ABSTRACT: CblC, a B_{12} trafficking protein, exhibits glutathione transferase and reductive decyanase activities for processing alkylcobalamins and cyanocobalamin, respectively, to a common intermediate that is subsequently converted to the biologically active forms of the cofactor. We recently discovered that the *Caenorhabditis elegans* CblC catalyzes thiol-dependent decyanation of CNCbl and reduction of OH_2Cbl and stabilizes the paramagnetic cob(II)alamin product under aerobic conditions. In this study, we report the striking ability of the worm CblC to stabilize the highly reactive cob(I)alamin product of the glutathione transferase reaction. The unprecedented stabilization of the supernucleophilic cob(I)alamin species under aerobic conditions by the intrinsic thiol oxidase activity of CblC, was exploited for the chemoenzymatic synthesis of organocobalamin derivatives under mild conditions.

Derivatives of cobalamin, a complex organometallic cofactor, support the activity of two mammalian enzymes.[1] Unable to synthesize this essential cofactor, mammals use an elaborate pathway for converting dietary cobalamin to its active cofactor forms and for delivering them to the two client enzymes.[2−4] Functional B_{12} deficiency results from genetic defects in the cobalamin trafficking pathway and lead to systemic metabolic defects.[5] An early step in the B_{12} trafficking pathway involves CblC, a versatile protein that exhibits glutathione transferase,[6] reductive decyanase[7] and aquocobalamin (OH_2Cbl) reductase[8] activities and converts incoming cobalamin derivatives to an intermediate that can be partitioned into methylcobalamin (MeCbl) and 5′-deoxyadenosylcobalamin (AdoCbl) synthesis to support cellular needs. The glutathione transferase activity involves an S_N2 attack on the alkyl group of alkylcobalamins (R-Cbl) by the thiolate of glutathione (GSH) and leads to the heterolytic cleavage of the cobalt−carbon bond and formation of cob(I)alamin and the corresponding alkylthioether product (eq 1). The decyanase activity requires a reductant in addition to cyanocobalamin (CNCbl), and leads to homolytic cleavage of the cobalt−carbon bond and formation of cob(II)alamin and cyanide (eq 2). CblC can utilize reduced flavins[7,9] or GSH[8] as an electron source.



$$\text{R-Cbl} + \text{GSH} \rightarrow \text{GS-R} + \text{H}^+ + \text{Cob(I)alamin} \quad (1)$$



$$\text{CNCbl} + \text{GSH} \rightarrow \text{CN}^- + \text{GS}^\bullet + \text{H}^+ + \text{Cob(II)alamin} \quad (2)$$

Both the nucleophilic cob(I)alamin and paramagnetic cob(II)alamin derivatives are susceptible to oxidation and, with human CblC, convert rapidly to OH_2Cbl under aerobic conditions.[6,7] In contrast, the *Caenorhabditis elegans* CblC (*ce*CblC) stabilizes cob(II)alamin formed during GSH-dependent decyanation of CNCbl.[8] The mechanism of stabilization involves efficient reduction of OH_2Cbl by GSH and leads to formation of oxidized glutathione (GSSG). In this study, we report the remarkable aerobic stabilization of cob(I)alamin formed by *ce*CblC in the glutathione transferase reaction, which we have exploited for chemoenzymatic synthesis of organocobalamins under mild conditions.

Recombinant full-length *ce*CblC was purified as described previously[8] and the thermodynamic parameters and stoichiometry ($n \approx 1$) for binding of AdoCbl (K_D = 13 ± 3 nM) and MeCbl (K_D = 8 ± 1 nM) were determined by isothermal titration calorimetry (Table S1, Figure S1). Dealkylation of AdoCbl was observed in the presence of GSH (5 mM) but not other thiols that were tested (5 mM each of dithiothreitol, β-mercaptoethanol, L-cysteine or L-homocysteine). Strikingly, despite the reaction being conducted under aerobic conditions, formation of cob(I)alamin, characterized by an absorption maximum at 390 nm, was observed with isosbestic points at 354, 415, and 534 nm (Figure 1A). In contrast, the two-electron oxidation product OH_2Cbl, is observed during aerobic dealkylation with human CblC.[6] Upon prolonged incubation, cob(I)alamin was oxidized to cob(II)alamin as evidenced by an increase in absorption at 472 nm with isosbestic points at 358 and 413 nm (Figure 1B). Further oxidation, i.e., conversion of cob(II)alamin to OH_2Cbl, was negligible over the next 2 h. At lower GSH concentrations (<1 mM), oxidation of cob(I)alamin was rapid and a mixture of cob(II)alamin and OH_2Cbl was observed (data not shown). To simplify the analysis, the kinetic parameters for AdoCbl dealkylation were obtained under anaerobic conditions. From the sigmoidal dependence of the reaction rate on GSH concentration (Figure 1C), the following parameters were obtained: $K_{0.5}$ for GSH = 153 ± 4 μM, k_{obs} = 0.19 ± 0.03 min^{-1} at 20 °C and Hill coefficient = 2.3 ± 0.1. The k_{obs} for AdoCbl dealkylation by *ce*CblC is 60-fold faster than that by human CblC.[6]

With MeCbl, cob(I)alamin formation peaked within 1 min following addition of GSH under aerobic conditions (Figure 2A), and was followed by oxidation to cob(II)alamin (*inset*, 471 nm). The kinetic parameters for MeCbl dealkylation by *ce*CblC were determined by stopped-flow spectroscopy under

Received: July 29, 2014
Published: November 4, 2014

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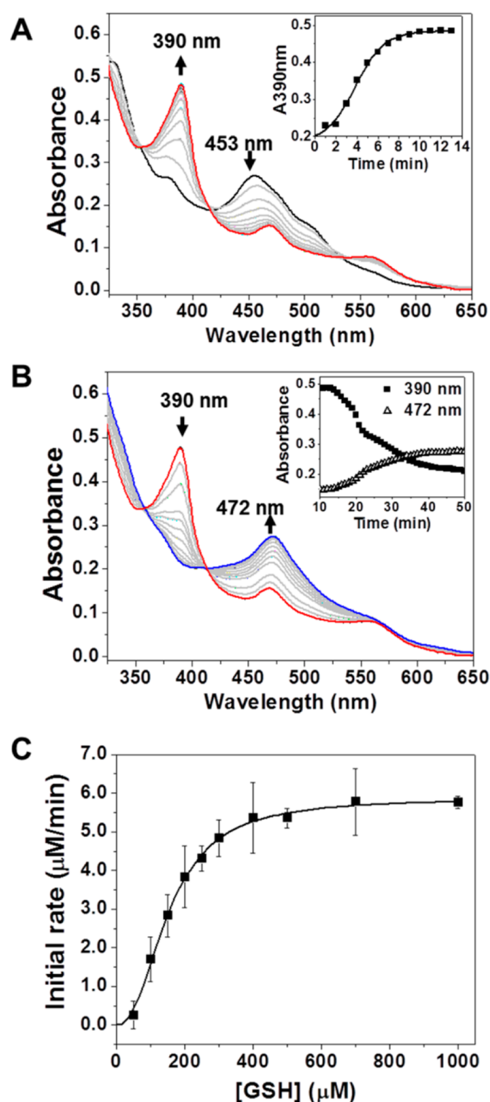


Figure 1. Dealkylation of AdoCbl by *ce*CblC. (A and B) Changes in the UV−visible absorption spectrum of *ce*CblC (40 μM)-bound AdoCbl (30 μM) by GSH (5 mM) in 100 mM HEPES buffer, pH 7.0, containing 150 mM KCl and 10% glycerol at 20 °C under aerobic conditions. (A) Changes observed between 0 (black trace) and 13 min (red trace). The absorbance maxima at 453 and 390 nm correspond to "base-off" AdoCbl and cob(I)alamin, respectively. (B) Changes observed between 13 (red trace) and 40 (blue trace) min of the reaction initiated in (A). The absorption maximum at 472 nm corresponds to cob(II)alamin. (Insets in A and B) Kinetics of cob(I)alamin (■, 390 nm) and cob(II)alamin (△, 472 nm) formation/disappearance. (C) Dependence of the initial rate of AdoCbl dealkylation on GSH concentrations under anaerobic conditions.

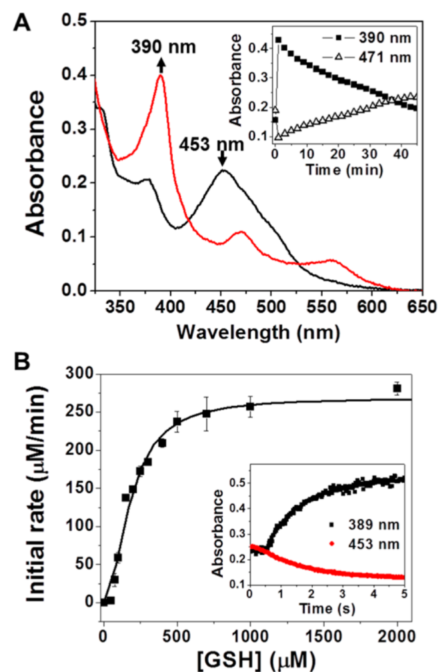
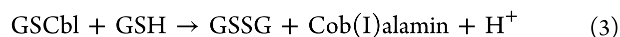


Figure 2. Dealkylation of MeCbl by *ce*CblC. (A) Changes in the absorption spectrum of *ce*CblC (40 μM)-bound MeCbl (30 μM) in aerobic 100 mM HEPES buffer, pH 7.0, containing 150 mM KCl and 10% glycerol at 20 °C following addition of 5 mM GSH. The black and red traces were recorded at 0 and 1 min, respectively. (Inset) Kinetics of cob(I)alamin (■, 390 nm) disappearance and cob(II)-alamin (△, 471 nm) formation. (B) Dependence of the initial rate of MeCbl dealkylation on GSH concentrations under anaerobic conditions. (Inset) Time-dependent changes in absorbance at 389 nm (black) and 453 nm (red).

anaerobic conditions (Figure 2B) and yielded values for $K_{0.5}$ for GSH = 183 ± 11 μM, k_{obs} = 13.5 ± 0.4 min^{-1} at 20 °C and Hill coefficient = 1.9 ± 0.2. While the absorbance at 453 nm for MeCbl decreased immediately following addition of GSH, a lag-phase of ∼0.5 s was observed at 389 nm for cob(I)alamin formation. The origin of the lag phase is not clear. Dealkylation of MeCbl by *ce*CblC was ∼70-fold faster than that of AdoCbl and ∼70-fold faster than that of the corresponding reaction catalyzed by human CblC.[6]

The directional characteristics and high charge density of the lone pair of electrons in the highest occupied and weakly antibonding d_{z^2} orbital in cob(I)alamin are responsible for its supernucleophilic character.[10] In fact, with a nucleophilic reactivity constant of 14.4[10], cob(I)alamin is the most powerful nucleophile known in biology and its reaction with alkylating agents represents a method of choice for the synthesis of organocobalamins.[11−14] Given the unexpected aerobic stability of *ce*CblC-bound cob(I)alamin, we explored the chemo-enzymatic synthetic potential using iodoethane and iodopropane as proof of principle. For ease of differentiation from the product alkylcobalamin, glutathionyl-cobalamin (GSCbl) with an absorbance maximum at 533 nm was initially used to generate cob(I)alamin in the presence of GSH as described[15] (eq 3, Figure 3A,B). In the presence of iodoethane, GSCbl converted rapidly to ethylcobalamin (EtCbl),



$$\text{GSCbl} + \text{GSH} \rightarrow \text{GSSG} + \text{Cob(I)alamin} + \text{H}^+ \quad (3)$$

which exhibited an absorbance maximum at 457 nm, characteristic of "base-off" alkylcobalamins bound to *ce*CblC. EtCbl formation was confirmed by mass spectrometric (Figure 3C) and HPLC (Figure 3D) analyses. On the basis of the absorbance at 254 nm, ∼70% of GSCbl was recovered as EtCbl in 1 min at 20 °C under both aerobic and anaerobic conditions (Figure 3D). Dethiolation of GSCbl by GSH is not observed in the absence of *ce*CblC under our experimental conditions (data not shown). When GSCbl was substituted by MeCbl, EtCbl was also obtained and in comparable yield (Figure S2). In the presence of iodopropane, the expected propylcobalamin (PrCbl) product was formed (Figure S3).

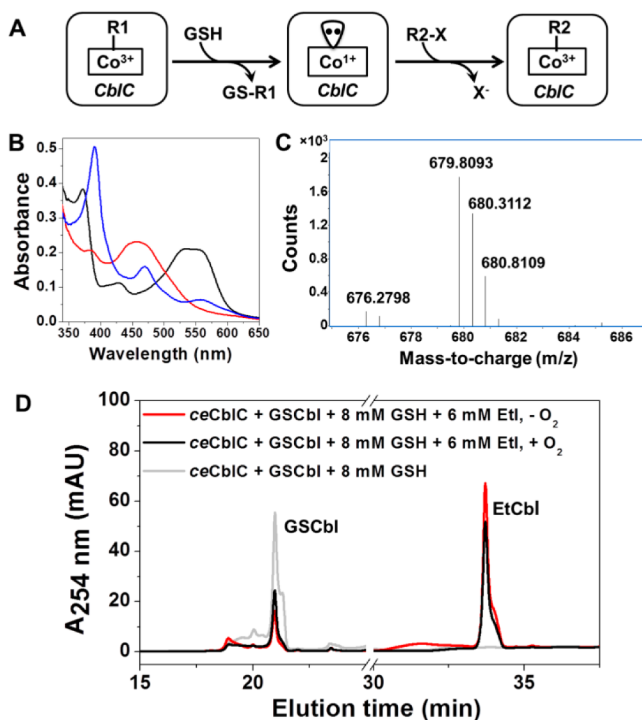


Figure 3. Chemoenzymatic synthesis of EtCbl using *ce*CblC. (A) Proposed scheme for synthesis of alkylcobalamins using *ce*CblC. R1 = Me or GS. R2 = any other alkyl group. X = I. (B) Changes in the UV−visible absorption spectrum of GSCbl (30 μM) mixed with 8 mM GSH in the presence or absence of 6 mM iodoethane following addition of *ce*CblC (40 μM) in aerobic 100 mM HEPES buffer, pH 7.0, containing 150 mM KCl and 10% glycerol at 20 °C. *Black trace*, free GSCbl mixed with 8 mM GSH; *blue trace*, 1 min after addition of *ce*CblC to GSCbl and 8 mM GSH; the absorption maximum at 391 nm corresponds to cob(I)alamin formation; *red trace*, 2 min after addition of *ce*CblC to GSCbl, 6 mM iodoethane and 8 mM GSH, the absorption maximum at 457 nm is indicative of "base-off" alkylcobalamin formation. (C) Mass spectrometric analysis of EtCbl generated as in (B). The predicted *m*/*z* value of the $[M + 2H]^{+2}$ ion for EtCbl is 679.8090. (D) HPLC analysis of samples containing 40 μM *ce*CblC + 30 μM GSCbl +8 mM GSH + 6 mM iodoethane after 1 min incubation: (1) under aerobic conditions (black line), (2) under anaerobic conditions (red line) or (3) in the absence of iodoethane under aerobic conditions (gray line) at 20 °C. In the absence of an alkylating agent, cob(I)alamin is oxidized to OH_2Cbl once *ce*CblC is denatured and reacts with excess GSH to form GSCbl. GSCbl and EtCbl eluted at 21 and 34 min, respectively.

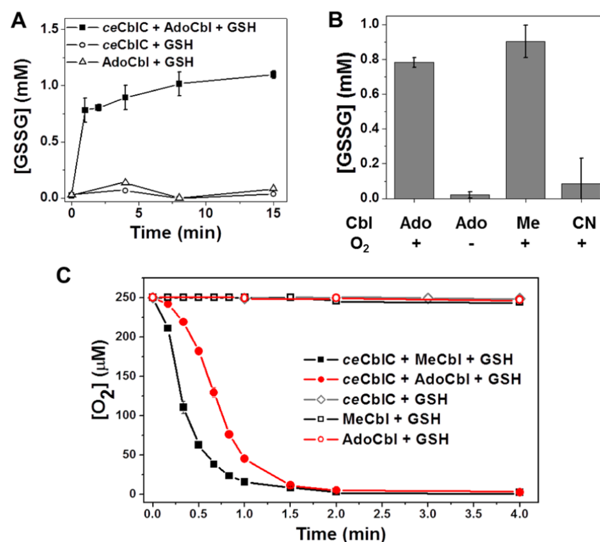


Figure 4. Oxygen-dependent conversion of GSH to GSSG catalyzed by *ce*CblC in the presence of alkylcobalamins. *ce*CblC (40 μM) was incubated with 20 μM each of MeCbl, AdoCbl, or CNCbl in the presence of 4 mM GSH at 20 °C in buffer containing 100 mM HEPES, pH 7.0, 150 mM KCl and 10% glycerol. (A) Concentration of GSSG in samples during dealkylation of AdoCbl. (B) Concentration of GSSG in samples after 2 min incubation ± O_2. (C) Kinetics of O_2 consumption during the dealkylation reactions catalyzed by *ce*CblC.

Cob(I)alamin is a strong reducing reagent ($E°$ = −500 mV for the "base-off" cob(I)alamin/cob(II)alamin couple) and undergoes ready oxidation in air to cob(II)alamin.[16] In principle, aerobic stabilization of cob(I)alamin could occur via its sequestration in the active site of *ce*CblC or via scrubbing of dissolved O_2 in the reaction mixture by oxidation of GSH. We have previously shown that *ce*CblC-dependent stabilization of cob(II)alamin, formed during the decyanation reaction, comes at the price of GSH oxidation.[8] To assess whether cob(I)alamin is similarly stabilized, GSH and GSSG were monitored during the time course of AdoCbl dealkylation by *ce*CblC under aerobic conditions (Figure 4A). Within 2 min, ∼1.6 mM GSH was converted to GSSG. The time course of GSSG formation was more rapid than for cob(I)alamin formation from AdoCbl (Figure 1A *inset*). The percent conversion of GSH to GSSG during AdoCbl or MeCbl dealkylation was comparable (Figure 4B). Under anaerobic conditions, GSSG production was significantly diminished. Under aerobic conditions, the rapid production of GSSG resulted in complete depletion of dissolved O_2 in the reaction solution (Figure 4C). The rapid oxidation of *ce*CblC-bound cob(I)alamin upon gentle agitation of the reaction mixture (not shown) argues against its stabilization by sequestration in the active site. Instead, our results are consistent with the model that stabilization of cob(I)alamin occurs primarily via the cobalamin-dependent thiol oxidase activity of *ce*CblC, which results in efficient scrubbing of O_2. The mechanism of GSH oxidation catalyzed by *ce*CblC is currently under investigation.

In summary, we have demonstrated that GSH-dependent dealkylation of AdoCbl and MeCbl occurs 60- to 70-fold more rapidly than previously observed for the human enzyme and is accompanied by the remarkable stabilization of cob(I)alamin, which has previously only been observed under rigorously anaerobic conditions.[6,16,17] The stabilization of cob(I)alamin by the thiol oxidase activity of *ce*CblC affords a novel strategy for the synthesis of alkycobalamins under mild conditions and in the presence of air, which is unprecedented.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and supporting data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Support for this work was provided in part by the National Institutes of Health (DK45776). We acknowledge support from

the mass spectrometry facility in the Chemistry Department, (University of Michigan) for analysis of alkylcobalamins.

■ REFERENCES

(1) Banerjee, R.; Ragsdale, S. W. *Annu. Rev. Biochem.* **2003**, *72*, 209.
(2) Banerjee, R. *ACS Chem. Biol.* **2006**, *1*, 149.
(3) Banerjee, R.; Gherasim, C.; Padovani, D. *Curr. Opin. Chem. Biol.* **2009**, *13*, 484.
(4) Gherasim, C.; Lofgren, M.; Banerjee, R. *J. Biol. Chem.* **2013**, *288*, 13186.
(5) Shevell, M. I.; Rosenblatt, D. S. *Can. J. Neurol. Sci.* **1992**, *19*, 472.
(6) Kim, J.; Hannibal, L.; Gherasim, C.; Jacobsen, D. W.; Banerjee, R. *J. Biol. Chem.* **2009**, *284*, 33418.
(7) Kim, J.; Gherasim, C.; Banerjee, R. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 14551.
(8) Li, Z.; Gherasim, C.; Lesniak, N. A.; Banerjee, R. *J. Biol. Chem.* **2014**, *289*, 16487.
(9) Koutmos, M.; Gherasim, C.; Smith, J. L.; Banerjee, R. *J. Biol. Chem.* **2011**, *286*, 29780.
(10) Schrauzer, G. N.; Deutsch, E.; Windgassen, R. J. *J. Am. Chem. Soc.* **1968**, *90*, 2441.
(11) Schrauzer, G. N.; Deutsch, E. *J. Am. Chem. Soc.* **1969**, *91*, 3341.
(12) Hannibal, L.; Kim, J.; Brasch, N. E.; Wang, S.; Rosenblatt, D.; Banerjee, R.; Jacobsen, D. W. *Mol. Genet. Metab.* **2009**, *97*, 260.
(13) oProinsias, K.; Giedyk, M.; Gryko, D. *Chem. Soc. Rev.* **2013**, *42*, 6605.
(14) Kim, S. H.; Chen, H. L.; Feilchenfeld, N.; Halpern, J. *J. Am. Chem. Soc.* **1988**, *110*, 3120.
(15) Jeong, J.; Park, J.; Kim, J. *Biochem. Biophys. Res. Commun.* **2014**, *443*, 173.
(16) Lexa, D.; Saveant, J.-M. *Acc. Chem. Res.* **1983**, *16*, 235.
(17) Banerjee, R.; Frasca, V.; Ballou, D. P.; Matthews, R. G. *Biochemistry* **1990**, *29*, 11101.