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Chloro-cob(II)alamin formation enhances the thiol oxidase activity of the B₁₂-trafficking protein CblC

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Abstract

CblC is a chaperone that catalyzes removal of the β -axial ligand of cobalamin (or B₁₂), generating cob(II)alamin in an early step in the cofactor trafficking pathway. Cob(II)alamin is subsequently partitioned to support cellular needs for the synthesis of the active cobalamin cofactor derivatives. In addition to the β -ligand transferase activity, the *Caenorhabditis elegans* CblC (*ceCblC*) and the clinical R161G/Q variants of the human protein exhibit robust thiol oxidase activity, converting glutathione to glutathione disulfide while concomitantly reducing O₂ to H₂O₂. The chemical efficiency of the thiol oxidase side reaction during *ceCblC*-catalyzed dealkylation of alkyl-cobalamins is noteworthy in that it effectively scrubs ambient oxygen from the reaction mixture, leading to air stabilization of the highly reactive cob(I)alamin product. In this study, we report that the enhanced thiol oxidase activity of *ceCblC* requires the presence of KCl, which explains how the wasteful thiol oxidase activity is potentially curtailed inside cells where the chloride concentration is low. We have captured an unusual chloro-cob(II)alamin intermediate that is formed in the presence of potassium chloride, a common component of the reaction buffer, and have characterized it by EPR, MCD and computational analyses. The ability to form a chloro-cob(II)alamin intermediate could represent an evolutionary vestige in *ceCblC*, which is structurally related to bacterial B₁₂-dependent reductive dehalogenases that form halogen-cob(II)alamin intermediates in their catalytic cycle.

Graphical Abstract

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Author Contributions

ZL designed and performed the biochemical studies. UT and KW performed the EPR analyses. EDG and TCB performed MCD experiments and DFT calculations. MR synthesized AdoCbi and AK helped with some of the biochemical assays. ZL, EDG and RB drafted the manuscript and all authors edited it.

Supporting Information. Additional EPR and MCD data, EPR fit parameters, and further details for the DFT calculations are available in Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

A chloro-cob(II)alamin intermediate is observed in the futile oxidation cycle catalyzed by *ceCblC*, a B₁₂-trafficking protein. Characterization of this reaction by EPR, MCD spectroscopy and by computational analyses unexpectedly revealed its dependence on chloride ion and led to the characterization of a Cl-cob(II)alamin intermediate.

Introduction

The cobalamin cofactor contains a central cobalt ion coordinated to a mono-anionic tetrapyrrolic corrin ring. Humans utilize two biologically active forms of cobalamin: methylcobalamin (MeCbl) for methionine synthase that participates in homocysteine metabolism, and 5'-deoxyadenosylcobalamin (AdoCbl) for methylmalonyl-CoA mutase involved in branched-chain amino acid and odd-chain fatty acid catabolism.¹ The redox state of the cobalt alternates between Co³⁺ in MeCbl and Co¹⁺ in cob(I)alamin in the catalytic cycle of methionine synthase.² Alternatively, the redox state of cobalt alternates between the Co³⁺ in AdoCbl and Co²⁺ in cob(II)alamin in the catalytic cycle of methylmalonyl-CoA mutase.³ Both cob(I)alamin and cob(II)alamin are prone to oxidation, potentially leading to damaging reactive oxygen species formation in the cell.⁴

An intricate trafficking pathway comprising chaperones that process and sequester cobalamin exists in mammals to guide and deliver this essential cofactor to its two target enzymes.^{5–7} CblC (also known as MMACHC) is an early component in this pathway and removes the upper or β -axial ligand of incoming cob(III)alamin derivatives, converting them to a common cob(II)alamin product. Cob(II)alamin is subsequently utilized for MeCbl or AdoCbl synthesis to support the activities of methionine synthase and methylmalonyl-CoA mutase, respectively. Human CblC (hCblC) exhibits great chemical versatility, catalyzing glutathione transferase, reductive decyanase, and aquocobalamin reductase reactions.^{8–10} This list continues to grow with the recently reported denitration (of nitrocobalamin) and nitrite reductase activities.¹¹ Over 400 cases of methylmalonic aciduria and homocystinuria *cbC* type have been reported worldwide.¹² Structurally, CblC belongs to a subfamily within the flavin nitroreductase superfamily,¹³ which also includes reductive dehalogenases that catalyze cobalamin-dependent metabolism of organohalides^{14, 15} as well as a second B₁₂ trafficking protein, CblD.¹⁶

While CblC from *C. elegans* (*ceCblC*) exhibits similar chemical versatility to hCblC, it is notable for its robust thiol oxidase activity. In the presence of the co-substrate, glutathione

(GSH), the thiol oxidase activity leads to rapid O₂ depletion and aerobic stabilization of the highly reactive cob(I)alamin species that is formed during dealkylation of MeCbl or AdoCbl.¹⁷

While this futile redox cycling reaction is suppressed in wild-type hCblC, it is manifest in two pathological variants (R161G and R161Q), which could contribute to the increased oxidative stress associated with the *cbC* type inborn error of cobalamin metabolism.¹⁸

In a previous study of the thiol oxidase activity of *ceCblC*¹⁹ (Fig 1), we had proposed that cob(I)alamin generated from the GSH-dependent dealkylation of alkylcobalamin (*reaction* [i]) is oxidized to form cob(II)alamin and superoxide (O₂^{•-}) (*reaction* [ii]). GSH coordination leads to glutathionyl-cob(II)alamin (*reaction* [iii]), which is subsequently oxidized to glutathionyl-cob(III)alamin (GSCbl, *reaction* [iv]). GSH-dependent dethiolation of GSCbl (*reaction* [v]) regenerates cob(I)alamin, completing the redox cycle. The cycle can also be entered at the cob(II)alamin oxidation state starting with reduction of cyanocobalamin (CNCbl), H₂OCbl or nitrocobalamin (*reaction* [vi]). Direct oxidation of cob(II)alamin by O₂^{•-} (*reaction* [vii]) effectively terminates the catalytic circle. In this mechanism, the relative rates of *reactions* [iii] versus [vii] determine the extent of the thiol-oxidase activity of CblC. The crystal structure of hCblC with GSH and a cobalamin derivative bound²⁰ suggests that Arg-161 could play a key role in favoring *reaction* [vii] over [iii], perhaps by disfavoring thiolate coordination to cob(II)alamin. This interpretation is consistent with the behavior of the hCblC variants in which substitution of Arg161 by glutamine or glycine unleashes the thiol oxidase activity.^{11, 18}

Although GS-cob(II)alamin is postulated to be a key intermediate in the thiol oxidase cycle, it has not been observed. In contrast, the next intermediate, i.e. GSCbl, has been characterized by UV-visible spectroscopy.¹⁹ Accumulation of GSCbl in the thiol oxidase reaction cycle is consistent with the kinetic evidence that *reaction* [v] is rate-determining. In this study, we report the unexpected discovery that potassium chloride present in the reaction buffer leads to a chloride coordinated cob(II)alamin intermediate (Cl-cob(II)alamin) and contributes to the significant thiol oxidase activity of *ceCblC*.

Experimental Section

Expression and purification of CblC

Full-length *ceCblC*-His₆ was expressed and purified as described previously¹⁰ and dialyzed into the Reaction Buffer containing 100 mM HEPES-KOH, pH 7.0, 150 mM KCl and 10% glycerol. All assays were performed in the Reaction Buffer unless otherwise specified. C-terminal His₆-tagged hCblC (spanning residues 1–244) was expressed and purified as described previously.¹³

Preparation of AdoCbi and cob(II)inamide

5'-deoxyadenosylcobinamide (AdoCbi) was prepared by cereous hydroxide hydrolysis of AdoCbl using a modification of the published procedure.²¹ In brief, NaOH (110 mg, 2.75 mmol) was dissolved in 13 mL H₂O followed by addition of cerium nitrate hexahydrate (585 mg, 1.35 mmol). Under reduced illumination, a solution of AdoCbl (30 mg, 12.7 μmol in 2

mL H₂O) was added to the suspension and the reaction mixture was heated to 100 °C with vigorous stirring. After 80 min, the reaction was cooled to room temperature and the pH was adjusted to 8.5 with concentrated ammonium hydroxide. The mixture was centrifuged for 15 min at 2236 × *g*. The supernatant was decanted and the residue was washed twice with 10 mL of water. The supernatants were pooled and the solution was desalted using an RP-18 cartridge (Waters). AdoCbi was eluted with MeOH and lyophilized. Further purification was achieved by preparative HPLC on an RP-18 column (Luna C-18 250×10 mm, Phenomenex) with the following solvent system: A: 10 mM phosphate buffer, pH 6.5; solvent B: acetonitrile; 0–2 min, 2% B isocratic; 2–12 min, 2–15% B; 12–25 min, 15–18% B; 25–30 min, 18–40% B; 30–33 min, 40–60% B; 33–40 min, 60% B isocratic; 40–43 min, 60–2% B; 43–47 min, 2% B. Fractions containing AdoCbi were desalted using an RP-18 cartridge. After lyophilization 9.8 mg AdoCbi (41 % yield) was obtained. Cob(II)inamide was generated by photolysis of AdoCbi in anaerobic Reaction Buffer.

Electron paramagnetic resonance (EPR) spectroscopy and simulations

EPR samples (250 µL) of *ceCblC* (375 µM) with bound cob(II)alamin (300 µM) were prepared anaerobically in the absence or presence of GSH (i.e. 0–10 mM). GS-cob(II)inamide was prepared anaerobically by mixing cob(II)inamide (300 µM) and GSH (4 mM) in 0.7 M NaOH containing 10% (v/v) glycerol. EPR spectra were collected on a Bruker Elexsys E500 spectrometer interfaced with a Bruker ER 4122SHQE Super High Q Cavity and a Bruker ER4131VT system for temperature control and a nitrogen-flow cooling system. The following experimental conditions were used: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 10 Gauss; microwave power, 20 mW; temperature, 120 K. Samples were transferred to 4 mm outer diameter quartz EPR tubes in an argon-filled glove bag under dim red light. Simulations of the experimental spectra were performed using the EasySpin toolbox²² run in MATLAB (v. R2015a; Mathworks, Natick, MA).

Simulations of the EPR spectra were performed by using MATLAB (Mathworks, Natick, MA) programs that utilized the EasySpin (v. 5.2.23) tool box.²² The calculations were based on the following spin Hamiltonian:

$$\hat{H} = \beta_e \hat{\mathbf{S}} \cdot \mathbf{g} \cdot \mathbf{H} + h \hat{\mathbf{S}} \cdot \mathbf{A}_1 \cdot \hat{\mathbf{I}}_1 + h \hat{\mathbf{S}} \cdot \mathbf{A}_2 \cdot \hat{\mathbf{I}}_2$$

The first term on the right-hand side represents the Zeeman interaction of the electron spin ($S=1/2$) on Co²⁺, the second term (subscript, 1) represents the hyperfine interaction of the electron spin with the ⁵⁹Co nucleus ($I=7/2$), and the third term (subscript, 2) represents the superhyperfine interaction of the electron spin with the ³⁵Cl nucleus ($I=3/2$; isotopic abundance, 76%). In Eq. S1, $\mathbf{g}$ is the electron g tensor, $\mathbf{H}$ is the magnetic field vector, $\mathbf{A}_1$ and $\mathbf{A}_2$ are the hyperfine and superhyperfine tensors, respectively, $\hat{\mathbf{S}}$ and $\hat{\mathbf{I}}$ are electron and nuclear spin operators, respectively, β_e is the Bohr magneton, and h is Planck's constant. The principal axes of $\mathbf{g}$ and the $\mathbf{A}$ were assumed to be aligned. The varied parameters included the principal values of the g tensor and the principal values of the hyperfine and superhyperfine tensors. The linewidth parameter ("lw") was fixed at the value corresponding to the experimental modulation amplitude and was tailored by using g strain. In the

simulations, an iterative global scanning of the varied parameters, followed by Nelder-Mead simplex optimization (performed on the derivative lineshape), was used to converge on a set of parameters, that satisfied the combination of statistical (root mean square deviation, rmsd) and visual criteria. Best-fit values are included in Table 1.

Magnetic circular dichroism (MCD) spectroscopy

Samples containing *ce*CblC (250 μ M), cob(II)alamin (200 μ M) and GSH (4 mM) were prepared anaerobically in buffer containing 0.1 M HEPES pH 7.0, 0 or 150 mM KCl and 55% (v/v) glycerol. GS-cob(II)inamide was prepared anaerobically by mixing cob(II)inamide (200 μ M) and GSH (4 mM) in 0.2 M NaOH followed by addition of 55% (v/v) glycerol. MCD and low-temperature absorption spectra were collected on a Jasco J-715 spectropolarimeter in conjunction with an Oxford Instruments SM-4000 8T magnetocryostat. All MCD spectra presented were obtained by taking the difference between spectra collected with the magnetic field oriented parallel and antiparallel to the light propagation axis to remove contributions from the natural CD and glass strain.

Quantification of GSSG

Dealkylation of *ce*CblC (16 μ M)-bound AdoCbl (8 μ M) in the presence of GSH (4 mM) was carried out under aerobic conditions, in buffer containing 0.1 M HEPES-KOH, pH 7.0, 10% (v/v) glycerol and various amounts of potassium halide salts (as described in the text). The reactions were stopped at the desired time point by mixing with an equal volume of metaphosphoric acid solution (16.8 mg/ml metaphosphoric acid, 2 mg/ml EDTA and 9 mg/ml NaCl) to precipitate proteins. The supernatant (80 μ L) was mixed with glutathione reductase (0.7 units/mL, recombinant Bakers yeast, Sigma-Aldrich) and NADPH (0.2 mM) in 50 mM Tris-HCl, pH 7.5 to a total volume of 400 μ L, and incubated for 20 min at room temperature. The concentration of GSSG produced in the reaction was quantified based on the oxidation of NADPH ($\epsilon_{340\text{nm}} = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$). The initial velocities of reactions were obtained by linear fits of the initial phases.

Dependence of the thiol oxidase activity on the halide concentration

Dealkylation of *ce*CblC was conducted as described above for GSSG quantification with the exception that 0–400 mM KCl or KBr was added to the reaction mixture. The GSSG production rate (V) was determined using the assay described above and the data were fitted with the equation below, where x is the concentration of the salt.

$$V = V_{\text{max}} \times \frac{x}{x + K_{\text{app}}} \times \frac{1}{1 + x/K_{\text{app}}}$$

Computational analyses

A Cl–cob(II)inamide model was generated from the highest-resolution X-ray crystal structure of AdoCbl (CCDC file: DADCBL) with ligand changes made in silico and all side chains truncated after the first carbon. The geometry was optimized via spin-unrestricted density functional theory (DFT) with the Orca 4.0 software package²³ using the BP86 functional^{24,25} with the TZVP basis set²⁶ applied to Co and all ligating atoms and the def2-

SVP²⁷ basis set for all remaining atoms. An EPR parameter calculation was performed on the optimized geometry using the B3LYP functional.^{24,28} The CP(PPP) basis set²⁹ was applied to Co, IGLO-III³⁰ to all ligating atoms, and SVP to the remaining atoms in the model. The EPR g values as well as $A(^{59}\text{Co})$ and $A(^{35}\text{Cl})$ tensors were computed using coupled-perturbed self-consistent field (CP-SCF) theory,³¹ with a complete mean-field treatment of spin-orbit coupling and the IGLO gauge origin. A H_2O -cob(II)inamide model was generated following the same method with the identical functionals and approximations as listed above. The energies and absorption intensities of the electronic transitions for the Cl-cob(II)inamide and H_2O -cob(II)inamide models were calculated using time-dependent DFT (TD-DFT) in conjunction with the camB3LYP functional³² and basis sets as described for the geometry optimizations. A total of 40 excited states were computed with TD-DFT by including all single excitations between molecular orbitals (MOs) with orbital energies within a window of ± 3 Hartree around the highest occupied MO and lowest unoccupied MO gap using the RI and Tamm-Dancoff approximations.^{33–35}

Results

EPR spectroscopy reveals Cl-cob(II)alamin bound to ceCblC

We used EPR spectroscopy in an attempt to detect the putative GS-cob(II)alamin intermediate that is predicted to form in the thiol oxidase cycle (Fig 1, *reaction iii*).¹⁹ The EPR spectrum of cob(II)alamin bound to ceCblC is characteristic of a H_2O -ligated species with a residual ($g = 2.0$) signal from $\text{O}_2^{\bullet-}$ -cob(III)alamin,³⁶ indicating some O_2 contamination (Fig 2A), consistent with the base-off conformation of ceCblC -bound B_{12} .¹⁹ The presence of The 5-coordinate cob(II)alamin with a water ligand is signaled by the characteristic singlet hyperfine lines in the high-field, g_z region of the spectrum, which result from the coupling between the unpaired cobalt electron and the cobalt nucleus ($I = 7/2$). A superhyperfine triplet structure would be observed if a nitrogen ($I = 1$) from the intramolecular dimethylbenzimidazole base were coordinated to the cobalt ion, which is not expected to be the case based on the crystal structures of CblC .¹⁹ Addition of GSH resulted in the appearance of a new EPR line shape (Fig 2B), distinguished by a quartet superhyperfine splitting of each cobalt hyperfine line around g_z , indicating coupling between the unpaired electron on cobalt and an $I = 3/2$ nucleus. It is likely that the lower spectral quality in our earlier study contributed to this species being missed previously.¹⁹ To better characterize this new species, we performed a titration experiment in which the concentration of GSH was varied from 0.1–10 mM (Fig S1). An overall ~50% decrease in the intensity of the base-off cob(II)alamin spectrum was seen in the presence of 10 versus 0 mM GSH. Therefore, the EPR spectrum of the new species was deconvoluted by subtraction using this scaling factor (Fig 2C). The difference spectrum clearly revealed the quartet hyperfine structure on each cobalt A_z hyperfine feature. The two nuclear isotopes of chlorine, ^{35}Cl and ^{37}Cl , both have $I = 3/2$ and similar magnetogyric ratios. Therefore, we interpret the difference spectrum as representing axial ligation of Co^{2+} in cob(II)alamin by a chloride ion (Cl-cob(II)alamin). The line shape is similar to that reported for Cl-cob(II)alamin in the reductive dehalogenase *RdhA* from *Nitratireductor pacificus* (*NpRdhA*).¹⁴ The Cl-cob(II)alamin assignment is further supported by spectral simulation (Table 1) and the spectrum is clearly different from that of a model base-off GS-

cob(II)alamin derivative formed by mixing GSH and cob(II)inamide (i.e. lacking the nucleotide loop containing the intramolecular base) under alkaline conditions (Fig 2F). These data support the conclusion that Cl–cob(II)alamin rather than the expected GS–cob(II)alamin intermediate, was captured. The chloride anion is presumably derived from the reaction buffer, which contains 150 mM KCl that is routinely added to stabilize the protein. Notably, a Cl–cob(II)alamin species was not observed with hCblC under the same conditions (Fig 2E; the low-amplitude “triplet” superhyperfine features at g_z arise from a minor fraction of free base-on cob(II)alamin).

Chloride enhances the thiol oxidase activity of *ceCblC*

To assess the relevance of the Cl–cob(II)alamin species with respect to the thiol oxidase activity of *ceCblC*, we compared the rates of GSSG production in buffers with or without KCl (Fig 3A). The presence of KCl increased GSSG production >10-fold, consistent with a role for chloride in stimulating the thiol oxidase activity of *ceCblC*.

Next, we tested whether other potassium halides also enhanced the thiol oxidase activity and found that while KBr was equally effective, KI and KF were less effective (Fig 3B). In principle, the chloro intermediate observed by EPR spectroscopy could be formed via reaction [ii] or [iii] in Fig 1, since each of these two steps includes cob(II)alamin. For instance, Cl–cob(II)alamin could form during the oxidation of cob(I)alamin and serve as a precursor for GS–cob(II)alamin as described in equations 1 and 2. Alternatively, the chloro intermediate could result from β -ligand exchange of H₂O–cob(II)alamin as described in equation 3.

The thiol oxidation rate exhibited a biphasic dependence on the concentration of KCl and KBr. Thus, the reaction rate increased at low but decreased at high salt concentrations (Fig 3C). If Cl–cob(II)alamin is formed from cob(I)alamin [Eq 1], then the subsequent ligand exchange reaction with GSH [Eq 2] would be disfavored at increasing concentrations of halide ions, inhibiting thiol oxidation. However, this explanation would not account for the inhibition data if Cl–cob(II)alamin were formed from H₂O–cob(II)alamin via ligand exchange [Eq 3]. The kinetic data were fitted to a modified Michaelis-Menten equation (described in the Experimental Section) and yielded values for $K_{act(app)}$ for KCl and KBr of 76 ± 4 mM and 35 ± 3 mM, respectively.

MCD analysis of chloro- and thiolato-cob(II)alamin

We had previously reported that the lowest-energy feature in the MCD spectrum of cob(II)alamin bound to *ceCblC* shifts from 16,134 to 15,659 cm^{−1} upon addition of GSH,

which we attributed to a lengthening of the Co-OH₂ bond.¹⁹ Since these samples also contained KCl, and in light of the new EPR data (Fig 2), we asked whether the GSH-induced shift might instead reflect formation of Cl-cob(II)alamin. To test this possibility, we compared the MCD spectra of samples containing *ceCblC* bound cob(II)alamin and GSH in the absence and presence of KCl (Fig 4A,B). In the absence of KCl, the spectrum was virtually identical to that reported for cob(II)alamin bound to *ceCblC* without GSH¹⁹ (Fig 4A). Consistent with the EPR spectra presented in Fig 2, addition of KCl caused a perturbation of the axial ligand-cob(II)alamin bonding interaction, as evidenced by the ~460 cm⁻¹ red shift of the lowest energy, positively signed feature relative to the sample lacking KCl (Fig 4A versus B). The MCD spectrum of Cl-cob(II)alamin (Fig 4C) in the protein matrix was resolved by subtraction of the spectrum without KCl (scaled by 0.5 as determined from the EPR spectrum collected for this MCD sample) from the spectrum of the sample with KCl to remove the contributions from H₂O-cob(II)alamin. The 4.5 and 15 K difference spectra (Fig. 4C) are only marginally different from those of *ceCblC*-bound cob(II)alamin + GSH in the absence of KCl, signaling a relatively minor change in the energies of the cob(II)alamin frontier molecular orbitals upon substitution of the axially bound water molecule by a chloride ion. This finding is consistent with the close similarity between the EPR *g* values and Co hyperfine coupling constants displayed by *ceCblC*-bound H₂O-cob(II)alamin and Cl-cob(II)alamin (Table 1). To confirm that the MCD difference spectra in Fig. 4C are due to *ceCblC*-bound Cl-cob(II)alamin rather than thiolato-cob(II)alamin, variable-temperature MCD spectra of a model base-off GS-cob(II)alamin species were also recorded. For this, GSH was combined with cob(II)inamide under alkaline conditions (Fig 4D). The resulting spectrum contains unique features extending into the IR region (also see Fig S3) that are not observed in the spectrum of cob(II)alamin bound to *ceCblC*, ruling out accumulation of GS-cob(II)alamin.

Computational model of Cl-cob(II)inamide

The Cl-cob(II)alamin species generated in the active site of *ceCblC* in the presence of GSH and KCl was further characterized by DFT and TD (time-dependent)-DFT computational analyses (Table S2). In the DFT-optimized model of Cl-cob(II)inamide (Fig S4), the Co-Cl bond length is estimated to be 2.5 Å, which is longer than the 2.3 Å Co-O bond distance in H₂O-cob(II)inamide and correlates with the difference in the covalent radii of Cl versus O. Additionally, the 2.5 Å Co-Cl bond length is consistent with X-ray crystallographic data for the Cl-cob(II)alamin species in *NpRdhA*.¹⁴

The Cl-cob(II)inamide model was further evaluated on the basis of our spectroscopic data of *ceCblC*-bound Cl-cob(II)alamin by calculating EPR parameters and electronic transition energies using DFT in conjunction with the CP-SCF (coupled-perturbed self-consistent field) approach and TD-DFT, respectively. The computed EPR *g*-values and ⁵⁹Co hyperfine coupling constants for Cl-cob(II)inamide are smaller than those predicted for H₂O-cob(II)inamide (Table 1). These predictions are in reasonable agreement with the trends observed experimentally, though the *g_x* and *g_y* values are considerably underestimated. This discrepancy may reflect interactions between the chloro ligand and active-site residues in *ceCblC*•Cl-cob(II)alamin (e.g. a ring of arginine residues, see below) that are not accounted for in the computational model. Indeed, the *g_x* and *g_y* displayed by *ceCblC*•Cl-

cob(II)alamin and Np*RdhA*•Cl–cob(II)alamin are significantly different, highlighting the role of second-sphere interactions in modulating these parameters. Importantly, the $A_z(^{35}\text{Cl})$ superhyperfine coupling constant, which is nearly identical for these two enzymes and thus appears to be relatively insensitive to outer-sphere interactions, is well reproduced by the computation.

Analysis of the TD-DFT computational results reveals a small red-shift of nearly all transitions upon substitution of H₂O for Cl in the axial position of cob(II)inamide (Table S1), consistent with the experimental MCD spectra (Fig 4). In particular, the majority of the ligand-field transitions, which are responsible for the dominant features in the low-energy region of the MCD spectrum, are red-shifted in Cl–cob(II)inamide due to bonding contributions from the Cl 3p atomic orbitals to Co 3d_{z²} and 3d_{xy} orbitals (note that in the experimental spectra, these shifts may be slightly attenuated by outer-sphere interactions of the chloro ligand). The TD-DFT computations also predict a minor overall red shift of the corrin $\pi \rightarrow \pi^*$ transitions associated with the features in the 23,000–28,000 cm^{–1} range, in good agreement with the experimental MCD spectra. Collectively, the reasonable agreement between the DFT/CP-SCF and TD-DFT computational results and experimental data lends further confidence to our assignment of the *ce*CblC-bound cob(II)alamin species in the presence of GSH and KCl as Cl–cob(II)alamin. We note that while the chloride ion is bound on the β -face in our Cl–cob(II)inamide model, we cannot rule out the possibility of chloride coordination on the α -face.

DISCUSSION

The intriguing difference between the human and *C. elegans* orthologs of CblC in their propensity to catalyze a futile thiol oxidation reaction,¹⁷¹⁹ raises questions about the structural underpinnings that support or suppress this activity. It also raises the question as to how this wasteful activity is suppressed in cells to avoid generation of reactive oxygen species and depletion of the GSH pool. Analysis of clinical variants of hCblC at Arg-161 suggests that this residue plays a key role in partitioning the cob(II)alamin intermediate towards oxidation (Fig 1, *reaction* vii) and away from the ligand exchange reaction (*reaction* iii), thereby subduing oxidative cycling.¹¹ While GS–cob(II)alamin was postulated as a key intermediate in the thiol oxidation cycle, it had not been detected. Instead, we had previously assigned spectral changes in H₂O–cob(II)alamin bound to *ce*CblC that were induced by GSH as evidence of Co–O bond lengthening,¹⁹ as a prelude to ligand exchange and GS–cob(II)alamin formation. In this study, we report the unexpected capture of a Cl–cob(II)alamin intermediate, which leads us to revise the proposed mechanism of thiol oxidation and explain how this activity is suppressed in cells.

EPR and MCD spectroscopy revealed formation of a Cl–cob(II)alamin intermediate when cob(II)alamin bound to *ce*CblC was mixed with GSH (Fig 2, 4). DFT calculations predict that the Co–Cl bond in Cl–cob(II)alamin (2.5 Å) is longer, and slightly weaker, than the Co–O(H₂) bond in H₂O–cob(II)alamin (2.3 Å), which is consistent with the small red-shift of the lowest-energy features in the MCD spectra from *ce*CblC-bound H₂O–cob(II)alamin to Cl–cob(II)alamin (Fig 4). We hypothesize that formation of the Cl–cob(II)alamin intermediate facilitates ligand switching to form the catalytically essential GSCbl species

(Fig. 1, *reactions* [ix] and [iv]), and contributes to the 10-fold enhancement of thiol oxidase activity in the presence of 50–100 mM chloride. In contrast, hCblC, which does not accumulate Cl–cob(II)alamin under comparable conditions, exhibits a 30-fold lower thiol oxidase activity than the worm ortholog.^{11, 19} In this revised mechanism, a GS–cob(II)alamin intermediate while not essential (Fig. 1, *reaction* [ix]), is likely to form since dissociative ligand exchange at the cob(III)alamin oxidation state would be kinetically disfavored. Interestingly, the formation of Cl–cob(II)alamin is dependent on GSH (Fig 2), suggesting that the energy of GSH binding is utilized to promote the H₂O-to-Cl ligand switch (Fig. 1, *reaction* [viii]). Additionally, since our EPR data indicate that switching to the chloro ligand can occur from H₂O–cob(II)alamin (equation 3), it establishes that cob(I)alamin is not essential for Cl–cob(II)alamin formation as described in equation 1. Like GSH, chloride is predicted to bind to the upper or β -face of the corrin ring²⁰ (Fig 5A) since the α -side of cobalamin bound to ϵ CblC faces a crowded hydrophobic pocket.¹⁹ A ring of arginine residues frames the β -face of the corrin and could contribute to stabilizing the negative charges on the chloride and thiolate anion of GSH to facilitate ligand substitutions. The distance between the Co atom and the side chain of the closest residue on the α -face, Ile172, is 4.7 Å (Fig S5), which would disfavor chloride binding. Although we cannot rule out the possibility that the active site undergoes a conformational change to accommodate the chloride ion on the α -face, accommodating an even larger bromide ion on this side would be even more challenging.

Cob(II)alamin serves as a cofactor for various enzymes,³⁷ including *Np*RdhA, a B₁₂-dependent reductive dehalogenase whose closest structural homolog is CblC¹⁴. Intermediates with Co–Cl and Co–Br ligands are proposed to be involved in the reductive haloelimination reactions catalyzed by *Np*RdhA¹⁴ and a Co–Cl bond distance of 2.5 Å has been modeled in the 2.3 Å resolution crystal structure of this protein (Fig 5B). A Br–cob(II)alamin intermediate was identified by EPR spectroscopy in the presence of KBr and the substrate, 3,5-dibromo-4-hydroxybenzoic acid. Characterizations of Co–Cl and Co–Br coordination in the dehalogenases have been limited so far possibly due to the difficulty of reconstituting the enzymes with the full complement of cofactors. Biochemical analyses of ϵ CblC confirm the importance of chloride and by inference, the spectroscopically characterized Cl–cob(II)alamin species, in promoting the satellite thiol-oxidase activity (Fig 3). Since KBr also supports the thiol oxidation activity, it follows that a Br–cob(II)alamin intermediate forms in the ϵ CblC active site, although this was not characterized in the present study.

It is tempting to speculate that the ability of ϵ CblC to form Co–Cl and Co–Br bonds represents an evolutionary vestige of the divergent evolution of reductive dehalogenases and CblC, which are structurally related. However, the mechanisms by which the Co–halogen bond is formed in these two proteins are distinct. The Co–halogen bond in RdhA is predicted to form following reduction of cob(II)alamin to cob(I)alamin, which subsequently dehalogenates the substrate in an S_N² reaction.³⁸ In contrast, the Co–halogen bond in CblC is formed via a ligand exchange reaction in the cob(II)alamin oxidation state. The high $K_{\text{act(app)}}$ for chloride (76 ± 4 mM) for ϵ CblC relative to the intracellular chloride concentration, estimated to be 4–15 mM,³⁹ suggests how this deleterious activity is curtailed.

In summary, we have identified a chloride ion dependence of the thiol-oxidase activity of *ceCblC* and used spectroscopic and computational analyses to characterize the unexpected Cl-cob(II)alamin species that is formed. This study expands the repertoire of cobalt coordination chemistry that is available to proteins involved in the cobalamin trafficking pathway and reveals how reaction promiscuity is potentially suppressed by the available ligand concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AdoCbl	5'-deoxyadenosylcobalamin
MeCbl	methylcobalamin
GSCbl	glutathionylcobalamin
AdoCbi	5'-deoxyadenosylcobinamide
OH₂Cbl	aquo-cob(III)alamin
GSH	reduced glutathione
GSSG	oxidized glutathione

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FIGURE 1.

Proposed mechanism for the thiol-oxidase activity of CblC. X = CN, H₂O or NO₂; R = Me or Ado. The blue arrows show the formation and decay of the newly discovered Cl-cob(II)alamin intermediate. For clarity, only a subset of ligands is specified.

FIGURE 2.

EPR spectra of cob(II)alamin bound to *cCblC*. (A) Spectrum of H₂O-cob(II)alamin bound to *cCblC* in the absence of GSH; (B) In the presence of 10 mM GSH, the EPR spectrum is due to a mixture of ~50% H₂O-cob(II)alamin and ~50% Cl-cob(II)alamin; (C) the difference spectrum representing (B) – 0.5 × (A) reveals the spectrum of Cl-cob(II)alamin featuring the quartet superhyperfine splitting in the high-field, *g_z* region (vertical dashed lines); (D) simulated spectrum of (C). (E) spectrum cob(II)alamin bound to *hCblC* in the presence of 5 mM GSH; (F) spectrum of cob(II)inamide in the presence of GSH (4 mM) under alkaline conditions. *Bar above (A) shows position and extent of a minor O₂^{•-} – cob(III)alamin species.

FIGURE 3.

Stimulation of thiol oxidation by halide ions. (A) Time course for GSSG formation by αCblC (16 μM) in the presence of AdoCbl (8 μM) and GSH (4 mM) in 0.1 M HEPES pH 7.4, 10% (v/v) glycerol in the presence (red) and absence (black) of 150 mM KCl. (B) GSSG production rate in the presence of halide salts (150 mM each). (C) Dependence of the GSSG production rate on the concentration of KCl and KBr. The data represent the mean $\pm$ SD of 3 independent experiments.

FIGURE 4.

MCD spectra of α CblC-bound cob(II)alamin + GSH in the absence (A) and presence (B) of 150 mM KCl at 4.5 K (solid line) and 15 K (dotted line). The difference spectrum (C) was obtained by subtracting the spectra in B (scaled by 0.5) from that in A. (D) MCD spectrum of cob(II)inamide + GSH under alkaline conditions. The vertical red dashes highlight the small red-shift of the prominent lowest-energy feature in response to axial coordination of a chloride ion to α CblC-bound cob(II)alamin

FIGURE 5.

Structures of *ceCblC* (PDB ID: 5UJC) and *NpRdhA* (PDB ID: 4RAS). (A) Structure of *ceCblC* with MeCbl (purple) bound. Two GSH molecules (navy and cyan) can be modeled in the structure supporting the feasibility of GSH-dependent dethiolation of GSCbl (Fig 1, *reaction* [v]).¹⁹ The GSH corresponding to the navy molecule has been observed in hCblC (PDB ID: 5UOS). Arginine residues on the upper face of cobalamin are shown in red. (B) Co-Cl coordination observed in the cobalamin binding domain (244–505) of *NpRdhA*. Cobalamin is shown in blue while the chloride ion is shown as a red sphere.

Table 1.

EPR parameters for Cl-, thiolato- and H₂O–cob(II)inamides and cob(II)alamins from DFT calculations and simulations of experimental EPR spectra

	Species	<i>g</i>			⁵⁹ Co (MHz)			³⁵ Cl (MHz)		
		<i>g_x</i>	<i>g_y</i>	<i>g_z</i>	<i>A_x</i>	<i>A_y</i>	<i>A_z</i>	<i>A_x</i>	<i>A_y</i>	<i>A_z</i>
DFT calculations	H ₂ O–cob(II)inamide	2.224	2.206	2.008	169	175	558	-	-	-
	Cl–cob(II)inamide	2.164	2.142	2.004	3	28	424	13	12	76
	Cys–cob(II)inamide ^a	2.134	2.101	1.998	–58	–141	293	-	-	-
Experimental values	<i>ce</i> CblC•H ₂ O–cob(II)alamin ^b	2.429	2.324	1.998	262	231	401	-	-	-
	<i>ce</i> CblC•Cl–cob(II)alamin	2.441	2.309	1.995	275	243	401	<10	<10	65.3
	GS–cob(II)inamide	2.234	2.234	2.007	49.2	49.2	300	-	-	-

^aDFT calculations from Reference¹⁹

^bSimulation data are shown in Fig. S2