Spectroscopic Studies of the Salmonella enterica Adenosyltransferase Enzyme SeCobA: Molecular-Level Insight into the Mechanism of Substrate Cob(II)alamin Activation

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ABSTRACT: CobA from Salmonella enterica (SeCobA) is a member of the family of ATP:Co(I)rrinoid adenosyltransferase (ACAT) enzymes that participate in the biosynthesis of adenosylcobalamin by catalyzing the transfer of the adenosyl group from an ATP molecule to a reactive Co(I)rrinoid species transiently generated in the enzyme active site. This reaction is thermodynamically challenging, as the reduction potential of the Co(II)rrinoid precursor in solution is far more negative than that of available reducing agents in the cell (e.g., flavodoxin), precluding nonenzymic reduction to the Co(I) oxidation state. However, in the active sites of ACATs, the Co(II)/Co(I) redox potential is increased by >250 mV via the formation of a unique four-coordinate (4c) Co(II)rrinoid species. In the case of the SeCobA ACAT, crystallographic and kinetic studies have revealed that the phenylalanine 91 (P91) and tryptophan 93 (W93) residues are critical for in vivo activity, presumably by blocking access to the lower axial ligand site of the Co(I)rrinoid substrate. To further assess the importance of the P91 and W93 residues with respect to enzymatic function, we have characterized various SeCobA active-site variants using electronic absorption, magnetic circular dichroism, and electron paramagnetic resonance spectroscopies. Our data provide unprecedented insight into the mechanism by which SeCobA converts the Co(II)rrinoid substrate to 4c species, with the hydrophobicity, size, and ability to participate in offset π-stacking interactions of key active-site residues all being critical for activity. The structural changes that occur upon Co(II)rrinoid binding also appear to be crucial for properly orienting the transiently generated Co(I) “supernucleophile” for rapid reaction with cosubstrate ATP.

Adenosylcobalamin (AdoCbl) is one of Nature’s most complex cofactors, employed by biological systems as a controlled source of radical species.1,2 It is composed of a redox-active cobalt ion coordinated equatorially by the four nitrogen atoms of a tetrapyrrole macrocycle known as the corrin ring. A pendant 5,6-dimethylbenzimidazole (DMB) base attached to the corrin macrocycle by an intramolecular loop occupies the “lower” (Coβ) axial position, while an ATP-derived S′-deoxyadenosyl moiety is bound to the Co ion in the upper (Coα) position via a unique organometallic bond (Figure 1).3 AdoCbl serves as the cofactor for a class of enzymes that catalyze various 1,2-rearrangement reactions.4 These AdoCbl-dependent enzymes can be grouped into three families: (i) enzymes that form aldehydes via dehydration or deamination of substrates, which include diol dehydratase, glycerol dehydratase, and ethanolamine ammonia lyase;5,6 (ii) aminomutases, such as d-ornithine 4,5-aminomutase and l-leucine 2,3-aminomutase, which facilitate the migration of primary amine groups;7 and (iii) mutases, such as methylmalonyl-CoA mutase and glutamate mutase, which catalyze carbon skeleton rearrangements.8,9 A common feature shared by all of these enzymes is the controlled homolytic cleavage of the Co–C(Ado) bond of AdoCbl in response to substrate binding, to yield a reactive Ado-based radical capable of abstracting a hydrogen atom from the substrate.4

While only some bacteria and archaea possess the complete enzymatic machinery to synthesize AdoCbl from small molecule precursors, all organisms that require AdoCbl in their metabolism must produce ATP:Co(I)rrinoid adenosyltransferase (ACAT) enzymes.10 ACATs catalyze the formation of the Co–C(Ado) bond via the transfer of the S′-deoxyadenosyl moiety of ATP to a cobalamin substrate.11 To date, three nonhomologous, structurally distinct classes of ACATs have been identified and classified according to their roles in Salmonella enterica sv Typhimurium LT2 (hereafter S. enterica), which contains a member of each class in its genome.12–14 The S. enterica CobA (SeCobA) enzyme is involved in the de novo synthetic pathway of AdoCbl and in scavenging various corrinoids from the environment. One prominent substrate for SeCobA is cob(II)inamide [Co(II)-Cbl1], which features the cobalt-containing, tetrapyrrolic corrin ring present in all corrinoids but lacks the nucleotide loop and...
DMB base found in Co(II)Cbl and instead binds a water molecule in the (Coα) axial position (Figure 1 and Figure S8 of the Supporting Information). Corrinoids such as cobinamide must be adenosylated before they can be converted to cobalamins, and thus, the cobA gene is constitutively expressed by the cell to maintain basal levels of AdoCbl. Alternatively, the gene encoding the PduO ACAT is expressed only when 1,2-propanediol is present, whereas expression of the gene encoding EutT requires the presence of ethanolamine and AdoCbl. Note that the single ACAT employed by humans (generally termed hATR) is homologous to the PduO enzyme, and malfunctioning of hATR has been linked to diseases related to cobalamin deficiency, such as methylmalonic aciduria. Previous studies of ACATs have led to the proposal that these enzymes employ a common mechanism for the biosynthesis of AdoCbl. This mechanism involves the one-electron reduction of a Co(II)corrinoid precursor to form a “supernucleophilic” Co(I) species, which performs a nucleophilic attack on the S’-carbon of ATP to yield the adenosylated product (Figure 2). The reduction of Co(II)corrinoid to produce the key Co(I) intermediate is thermodynamically challenging, as the Co(II)/Co(I) reduction potential for the naturally encountered substrates \(E^0 = -610\, \text{mV vs NHE}\) for cob(II)alamine [Co(II)Cbl], and \(E^0 = -490\, \text{mV vs NHE}\) for cob(II)inamide [Co(II)Cbi⁺], a Co(II)Cbl precursor missing the nucleotide loop and DMB base, is too negative for the reducing agents available in the cell (the semiquinone/reduced flavin couple in FldA, the purported physiological partner to at least one ACAT, CobA, is \(E^0 = -440\, \text{mV vs NHE}\)). Early spectroscopic studies of ScCobA, as well as of the Lactobacillus reuteri PduO-type ACAT (LrPduO), have provided strong evidence of the formation of a structurally unique Co(II)corrinoid species in the active sites of these enzymes. In particular, electron paramagnetic resonance (EPR) characterization of Co(II)corrinoids bound to these enzymes complexed with ATP revealed unusually large g shifts and A(Co) hyperfine coupling constants, consistent with the Co(II) ion residing in an effectively square planar, four-coordinate (4c) ligand environment. Formation of a 4c intermediate was shown to stabilize the singly occupied redox-active Co 3d₂-based molecular orbital and thus to raise the reduction potential by an estimated \(\geq 250\, \text{mV}\), to within the range of those of biologically available reductants. Further evidence of enzymatic tuning of the Co(II)/Co(I) redox potential was obtained from magnetic circular dichroism (MCD) studies, which revealed the appearance of a series of sharp, positively signed features between \(\sim 10000\) and 20000 cm⁻¹ when Co(II)Cbi⁺ binds to ScCobA/ATP or LrPduO/ATP that are unique among Co(II)corrinoid species. Subsequent crystallographic studies confirmed the presence of 4c Co(II)Cbl species bound to the active sites of ScCobA and LrPduO in the presence of ATP and provided insights into the mechanism by which ACATs generate 4c Co(II)corrinoids. Specifically, it was found that a noncoordinating Phe residue occupies the lower axial position of the Co(II)Cbl cofactor where the DMB ligand would normally be found (F112 in LrPduO and F91 in ScCobA). A subsequent kinetic and spectroscopic study of LrPduO revealed that F112 and adjacent F187 and V186 residues form a hydrophobic “wall” in response to the one-electron reduction of Co(II)Cbl. Previous studies of ACATs have led to the proposal that these enzymes employ a common mechanism for the

**Figure 1.** Chemical structure of adenosylcobalamin (AdoCbl), the final product of the reaction catalyzed by ATP-Co(I)corrinoid adenosyltransferases (ACATs). In the case of adenosylcobinamide (AdoCbi⁺) and related species, the DMB moiety and nucleotide loop are absent.

**Figure 2.** Proposed mechanism for the reaction catalyzed by ACATs, adapted from refs 24 and 67. Complexation of the enzyme active site with cosubstrate ATP promotes the binding of Co(II)Cbl and its conversion to a 4c species via removal of the axial ligand. One-electron reduction of this species produces a Co(I)Cbl intermediate that is properly oriented for nucleophilic attack on the S’-carbon of ATP to form AdoCbl.
to Co(II)rrinoid binding, blocking ligand access to the Coax face of the corrin ring,\textsuperscript{35} while a salt bridge interaction between residues D35 and R128 near the corrin ring was found to be important for properly positioning the Co(II)rrinoid substrate.\textsuperscript{30} Although the detailed mechanism of 4c Co(II)rrinoid formation employed by ScCobA is less well understood, the most recently published X-ray crystal structure of this enzyme provided similar information about the conformation of the active site during catalysis. Notably, this structure revealed the active-site geometry at the catalytic site containing 4c Co(II)Cbi species (the “closed” conformation), as well as the binding geometry of pentacoordinate (5c) Co(II)Cbl prior to enzyme activation (the “open” conformation).\textsuperscript{32} It also confirmed the unique binding motif of ATP,\textsuperscript{36} oriented toward the corrin ring for nucleophilic attack by the Co ion, and identified additional amino acid residues responsible for displacing the lower axial ligand of the bound corrinoid in the “closed” conformation of the enzyme. In analogy to the previously characterized LrPduO ACAT, a set of hydrophobic residues, namely, F91, W93, V13, and V17, in ScCobA are positioned near the lower face of the cofactor, thus providing a wall of hydrophobic residues between the Co(II) ion and the solvent. However, unlike in LrPduO where a single aromatic residue is present at the location where the DMB coordinates to the Co(II) ion in solution,\textsuperscript{37,52} two bulky, aromatic amino acids in an offset π-stacking conformation are positioned at this location in ScCobA (Figure 3). This pair of residues is adjacent only to the V13 and V17 residues of the N-terminal helix that caps the active site, while the remaining interactions are with solvent molecules and pendant groups from the corrin ring. Preliminary studies of the Methanosarcina mazei CobA enzyme, a ScCobA homologue lacking the N-terminal helix, revealed that these Val residues are important for increasing the yield of 4c Co(II)rrinoid species but are not essential for activity.\textsuperscript{18} Alternatively, amino acid substitutions at the F91 and W93 positions were shown to have a drastic effect on the catalytic efficiency of ScCobA.\textsuperscript{32}

Previously, we have employed MCD and EPR spectroscopies to probe the coordination environment of the Co center in Co(II)rrinoids\textsuperscript{38,39} and to monitor the structural changes that occur in the catalytic cycles of various cobalamin-dependent enzymes and ACATs.\textsuperscript{29–32} MCD spectroscopy offers a particularly sensitive probe of Co(II)rrinoid species formed during enzymatic turnover, as with this technique ligand field (LF) and charge transfer (CT) transitions can be observed that are masked by intense corrin π−π* transitions in the corresponding absorption spectra. In the study presented here, we have used electronic absorption, MCD, and EPR spectroscopies to characterize several ScCobA variants with substitutions of residues F91 and W93. These variants were chosen to assess the importance of specific intermolecular interactions with respect to the formation of 4c Co(II)rrinoids by varying the size of residues 91 and 93 (F91Y, W93F, F91W, and W93A), their relative positioning (F91W/W93F), and polarity (F91Y and W93H). By conducting studies with Co(II)Cbl and Co(II)Cbi\textsuperscript{+}, both of which are substrates of enzymes and ACATs,\textsuperscript{29} we have gained significant insights into how the strength of the axial ligand–Co bonding interaction modulates the relative yield of 4c Co(II)rrinoid species in ScCobA.

**MATERIALS AND METHODS**

**Cofactors and Chemicals.** The chloride salt of aquacobalamin ([H\textsubscript{2}O]Cbi\textsuperscript{2+}), dicyanocobinamide ([CN],Cbi\textsuperscript{−}), and potassium formate (HCOOK) were purchased from Sigma and used as obtained. Diaquaobinamide ([H\textsubscript{2}O],Cbi\textsuperscript{2−}) was prepared by adding NaBH\textsubscript{4} to an aqueous solution of (CN),Cbi, loading the reaction mixture onto a C18 SepPack column, washing it with doubly distilled \text{H}_2\text{O}, and eluting the product with methanol, as described in previous reports.\textsuperscript{24,30} Co(II)Cbl and Co(II)Cbi\textsuperscript{+} were prepared by adding a small volume of saturated HCOOK to degassed solutions of H\textsubscript{2}OCbi\textsuperscript{−} and (H\textsubscript{2}O)\textsubscript{2}Cbi\textsuperscript{2−}, respectively, and the progress of the reduction was monitored spectrophotometrically.

**Protein Preparation and Purification.** Wild-type and variant CobA from *S. enterica* sv. Typhimurium LT2 was purified as described elsewhere.\textsuperscript{34} Briefly, the wild-type cobA gene was cloned into the pTEV5 overexpression plasmid,\textsuperscript{42} which includes a cleavable, N-terminal hexahistidine tag. ScCobA variants were generated using the QuikChange II site-directed mutagenesis kit (Stratagene). All proteins were overexpressed in *Escherichia coli* BL21 and purified on a HisTrap nickel affinity column (GE Life Sciences). The N-terminal hexahistidine tag was cleaved using recombinant tobacco etch virus (rTEV) protease.\textsuperscript{43} Proteins were purified to homogeneity as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).\textsuperscript{44}

**Sample Preparation.** Purified ∼300–500 μM ScCobA in 50 mM Tris buffer (pH 8) containing 0.5 mM DTT was

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**Figure 3.** X-ray crystal structure of the “closed” subunit of ScCobA in the region of the active site featuring 4c Co(II)Cbi (orange), cobalate ATP (purple), and Mg(II) (green), based on Protein Data Bank entry 4HUT. The subunit containing the F91 and W93 residues (shown as sticks) is colored cyan. The N-terminal helix of the adjacent “open” subunit, which caps the active site and contains the V13, V17, and V21 residues, is colored green.
complexed with Co(II)Cbl or Co(II)Cbi+ under anoxic conditions in an ∼0.8:1 cofactor:protein ratio (see the Supporting Information for details). If appropriate, MgATP was added in a 10-fold molar excess over protein as the source of ATP. Solutions were then injected into the appropriate sample cells in an oxygen-free glovebox. Samples were immediately frozen and stored in liquid nitrogen.

**Spectroscopy.** Magnetic circular dichroism (MCD) spectra were collected on a Jasco J-715 spectropolarimeter in conjunction with an Oxford Instruments SM-4000 8T magnetocryostat. All MCD spectra 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. X-Band EPR spectra were obtained by using a Bruker ESP 300E spectrometer in conjunction with an Oxford ESR 900 continuous-flow liquid helium cryostat and an Oxford ITC4 temperature controller. The microwave frequency was measured with a Varian EIP model 62SA CW frequency counter. All spectra were collected using a modulation amplitude of 10 G and a modulation frequency of 100 kHz. EPR spectral simulations were performed using the WEPR program developed by F. Neese.

**Computations.** Initial atomic coordinates for the structure of wild-type SeCobA in complex with ATP and Co(II)Cbl were obtained from the most recently published crystal structure [Protein Data Bank (PDB) entry 4HUT].34 Pymol was used to introduce in silico amino acid substitutions into the SeCobA subunit containing 4c Co(II)Cbl and ATP. The newly introduced residues were positioned so as to minimize steric clashes, while preserving the orientation of the original residue as closely as possible. Molecular mechanics as implemented in GROMACS version 4.5 was then employed to minimize the energy of the protein model in the presence of water solvent [using the simple point charge (SPC) model for water molecules],45 with a box size of 5 nm. The Amber98 force field was used for the protein residues and supplemented with parameters for ATP by Carson et al.46 and for cobalamin by Marques et al.47,48 To accelerate the calculations, the other subunit of the SeCobA dimer containing 5c Co(II)Cbl was removed, except for the N-terminal helix that interacts with the subunit of interest. No significant differences in the secondary structure were observed among the energy-minimized models of the variants, and computed Ramachandran plots for the optimized structures indicated that no misoriented amino acids or unreasonable conformations were present. From these optimized structures, the residues at positions 91 and 93 were excised and used in subsequent density functional theory (DFT) single-point calculations with Orca version 3.0 to evaluate the magnitude of dispersion interactions involving these two residues (while ignoring all other residues). These computations employed the B3LYP functional and TZVP basis set for all atoms and were conducted by choosing the dispersion correction developed by Grimme and co-workers.49−52 Although the absolute dispersion energies obtained in these calculations may be subject to systematic errors because substitutions of residues 91 and 93 may also alter the interactions with nearby amino acid residues, the computed values should properly reproduce the general trend in dispersion energies.

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**RESULTS**

**Corrinoid Binding to Wild-Type SeCobA (SeCobA^WT).** The low-temperature (LT) absorption spectrum of Co(II)Cbi+ (Figure 4A, gray trace) is characterized by an intense feature centered at ∼21000 cm⁻¹, the so-called α-band that has previously been assigned to a corrin-based π→π* transition on the basis of its high extinction coefficient and time-dependent DFT results.53,54 This feature is blue-shifted by ∼150 cm⁻¹ from its position in the absorption spectrum of Co(II)Cbl (Figure 5A, gray trace), as reported previously.38 In the presence of the SeCobA^WT/ATP complex (Figure 4B, gray trace), the α-band undergoes a further (∼350 cm⁻¹) shift to a higher energy. As the strength of the metal–ligand interaction in Co(II)rrinoids was previously found to modulate the relative energies of the corrin π/π* frontier molecular orbitals (MOs),50 the observed blue-shift of the α-band is consistent with large perturbations to the axial ligand environment of Co(II)Cbi+ in the presence of the SeCobA^WT/ATP complex. Further insight into the nature of these perturbations is obtained by MCD spectroscopy. Most importantly, a series of sharp, positively signed intense bands appear in the low-energy region (∼10000 to 20000 cm⁻¹) of the MCD spectrum upon binding of Co(II)Cbi+ to the SeCobA^WT/ATP complex (Figure 4B, color traces) that are characteristic of Co(II)rrinoid species bound to ACATs.29,31,38 From their large MCD:absorption intensity ratios [alternatively, C:D ratios (see Figure S5 of the Supporting Information)] and relatively narrow bandwidths,
As in the case of Co(II)Cbl\textsuperscript{+}, the absorption spectrum of Co(II)Cbl also changes in the presence of the ScCobA\textsuperscript{WT}/ATP complex, with the α-band undergoing an ~450 cm\textsuperscript{-1} blue-shift (Figure 5, gray traces). Intriguingly, in the MCD spectrum of Co(II)Cbl and the ScCobA\textsuperscript{WT}/ATP complex, the intensity of the δ-band is considerably weaker than in the analogous Co(II)Cbl\textsuperscript{+} spectrum (Figure 5B), indicating a large (~5-fold) decrease in the relative yield of 4c species when Co(II)Cbl serves as the substrate of ScCobA\textsuperscript{WT}. Thus, the magnitude of the blue-shift of the α-band observed in the absorption spectrum does not correlate directly with the yield of 4c species formed in the ScCobA\textsuperscript{WT} active site, indicating that absorption spectroscopy is not suitable for quantifying the relative yields of 4c Co(II)rrinoid species. A further analysis of the MCD spectrum of Co(II)Cbl and the ScCobA\textsuperscript{WT}/ATP complex reveals that the remaining spectral contributions are consistent with the presence of a 5c Co(II)Cbl species with N(DBM) bound to the Co(II) ion. However, the MCD features of this 5c species are significantly different from those observed for free Co(II)Cbl. Specifically, the positive feature at ~19000 cm\textsuperscript{-1} in the MCD spectrum of Co(II)Cbl red-shifts by ~200 cm\textsuperscript{-1} in the presence of the ScCobA\textsuperscript{WT}/ATP complex, while the positive feature at 21000 cm\textsuperscript{-1} blue-shifts by ~400 cm\textsuperscript{-1} (Figure 7, top). Because features in this region of the MCD spectrum of Co(II)Cbl have previously been assigned to LF and CT transitions that are sensitive to changes in the axial ligand environment,\textsuperscript{56} the band shifts induced by the addition of the ScCobA\textsuperscript{WT}/ATP complex are consistent with perturbations to the DMB base via interactions with the protein scaffold. Lastly, the high-energy region (>22000 cm\textsuperscript{-1}) of the MCD spectrum of Co(II)Cbl in the presence of the ScCobA\textsuperscript{WT}/ATP complex is reminiscent of that of free Co(II)Cbl, in particular with regard to the intense derivative-shaped feature at 31000 cm\textsuperscript{-1} (also see Figure S7 of the Supporting Information). Inspection of the remaining bands in this region, however, reveals sizable differences in terms of their positions and relative intensities, suggesting that the conformation of the corrin ring in the 5c Co(II)Cbl fraction is significantly altered from that of free Co(II)Cbl. These results are in agreement with the bond distances and angles of the enzyme-bound 5c and 4c Co(II)Cbl species derived from the most recent crystal structure of ScCobA\textsuperscript{WT} (Table 1). A comparison of the relevant structural parameters of free Co(II)Cbl\textsuperscript{+} and the 5c Co(II)Cbl fraction bound to the ScCobA\textsuperscript{WT}/ATP complex indicates that the Co–N(DBM) bond length increases by ~0.2 Å upon enzyme binding, concurrent with an ~6° increase in the long axis, butterfly fold angle, θ(LA).\textsuperscript{54} Upon removal of the DMB ligand, θ(LA) decreases by ~6°, φ(SA) increases by ~3°, and the Co···S- C(ATH) distance is shortened by ~0.4 Å, highlighting the effect

Table 1. Relevant Structural Parameters of Free and ScCobA-Bound Co(II)Cbl Species As Determined by X-ray Crystallography

| Co(II)Cbl species | Co–C_{ATP} (Å) | Co–N_{CB1} (Å) | θ(LA) (deg) | φ(SA) (deg) |
|-------------------|----------------|----------------|--------------|-------------|
| no protein\textsuperscript{a} | n/a\textsuperscript{a} | 2.13 | 19.8 | 6.3 |
| 5c ScCobA site ("open")\textsuperscript{b} | 3.42 | 2.32 | 26.2 | 7.7 |
| 4c ScCobA site ("closed")\textsuperscript{c} | 3.06 | n/a\textsuperscript{a} | 19.5 | 10.7 |

\textsuperscript{a}From ref S7. \textsuperscript{b}From PDB entry 4HUT. \textsuperscript{c}Not applicable.

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Figure 5. Absorption spectra collected at 4.5 K (gray traces) and variable-temperature MCD spectra at 7 T of (A) free Co(II)Cbl and (B) Co(II)Cbl in the presence of ScCobA\textsuperscript{WT} and ATP.
of the rearrangement of the F91 and W93 active-site residues on the relative positioning and conformation of the Co(II)-ncinoid substrate.

Point Substitutions at the W93 Position. W93A. Previous studies have indicated that the tryptophan residue at position 93 (W93) is critical for retaining enzyme activity,18 thus, it was originally postulated that this residue played a role similar to that of F112 in the LrPduO ACAT. However, the most recent crystal structure of ScCobAWT with Co(II)Cbl and MgATP bound shows instead that a nearby phenylalanine (F91) residue is positioned on the face of the corrin ring where the DMB group is usually found. It is possible that the aromatic side chain of the W93 residue is associated with this F91 residue in an offset π-stacking fashion during catalysis. Because replacement of W93 with alanine completely abolishes the catalytic activity of the enzyme with Co(II)Cbl, while modest activity is retained with Co(I)Cbl,34 the W93A substitution likely affects the Co(II)/Co(I) reduction step. Indeed, the MCD spectra of Co(II)Cbl and Co(II)Cbl in the presence of the ScCobAW93A/ATP complex are almost identical to the corresponding MCD spectra in the absence of ATP (Figures 6 and 7, trace G) and the MCD spectra of the free cofactors (Figures 6 and 7, trace H), indicating that the variant is unable to generate 4c Co(II)ncinoids.

W93F. Substitution of Trp93 with Phe, a smaller planar and nonpolar amino acid, results in a ScCobA variant with behavior similar to that of the WT enzyme in Co(II)ncinoid and Co(I)ncinoid in vitro assays.34 Indeed, the MCD spectrum of Co(II)Cbi+ in the presence of the ScCobAW93F/ATP complex is very similar to that obtained with ScCobAWT, though the prominent low-energy δ-band at ~12300 cm−1 is marginally broadened, suggesting that the Phe residue provides more conformational freedom to the bound corrinoid (Figure 6, traces A and D). A quantitative analysis of this spectrum reveals that a larger fraction of 4c Co(II)Cbi+ species is generated in the variant than in the ScCobAWT/ATP complex (Table 4D). In contrast, in the MCD spectrum of Co(II)Cbl with the ScCobAW93F/ATP complex, the intensity of the δ-band is decreased 4-fold from that observed in the presence of ScCobAWT. The MCD features at 15000 and 20000 cm−1 associated with the remaining 5c Co(II)Cbl species are very similar to those observed for the analogous species in ScCobAWT and distinct from those displayed by free Co(II)Cbl (Figure 7, traces A and D). This result indicates that the 5c fraction of Co(II)Cbl is also bound to the ScCobAW93F/ATP complex and adopts a conformation similar to that of the 5c Co(II)Cbl species in ScCobAWT. Thus, the decreased yield of 4c Co(II)Cbl species in ScCobAW93F can be attributed to the smaller size of the introduced Phe residue relative to the native Trp, which leads to a decreased level of steric crowding when Co(II)Cbl binds to the enzyme active site in the DMB-on form.30 Because of the weaker interaction of the H2O ligand with the Co(II) ion in Co(II)Cbl, the decreased level of steric crowding in the active site of ScCobAW93F has a smaller effect on the 4c→5c equilibrium when this species is used as the substrate.

W93H. Compared to the results obtained with ScCobAW93F, the MCD spectrum of Co(II)Cbi+ in the presence of the ScCobAW93H/ATP complex displays a broadening of the δ- and σ-bands and a decrease in their intensities. These changes signify a 2-fold decrease in the yield of 4c Co(II)Cbi+, consistent with a less constrained active site upon introduction of the smaller imidazole side chain (Figure 6 and Table 4, E).

As expected from the lower yield of 4c Co(II)Cbi+ in ScCobAW93H, increased contributions from 5c (i.e., water-bound) Co(II)Cbi+ are observed in the 16000–20000 cm−1 region of the MCD spectrum; however, these features are not identical to those seen in the corresponding MCD spectrum in the absence of ATP. In particular, the feature corresponding to the lowest-energy LF transition of Co(II)Cbi+ is red-shifted by ~300 cm−1 in the spectrum of the sample containing the ScCobAW93H/ATP complex (Figure 6E, band a), indicating that the 5c Co(II)Cbi+ fraction features an elongated Co—O(H2) bond.24,58 Thus, while a smaller fraction of 4c Co(II)Cbi+ species is generated in ScCobAW93H than in the wild-type enzyme, the remaining 5c Co(II)Cbi+ fraction is significantly perturbed upon binding to ScCobAW93H. The lack of features in the 14000–16000 cm−1 region of the MCD spectrum of Co(II)Cbi+ in the presence of ScCobAW93H indicates that the imidazole side chain of H93 does not serve as a ligand to the Co(II) ion, ruling out the possibility that His binding precludes the formation of a 4c species (Figure 6D; see Figure 9 for a comparison to a His-on species). As expected, the W93H substitution has a more dramatic effect on the 4c→5c equilibrium when Co(II)Cbl instead of Co(II)Cbi+ is used as the substrate. In fact, the MCD spectrum of Co(II)Cbl in the
ATP, the δATP complex. As in the case of Co(II)Cbi+, the intensity of the band in the MCD spectrum of Co(II)Cbl in the presence of the SeCobAW93F/ATP complex exhibits a very weak and broad δ-band, from which we can estimate that the yield of 4c Co(II)Cbl species is reduced more than 10-fold from that achieved by SeCobAWT and 20-fold from that of SeCobAW91W (Figure 6F). The remaining 5c Co(II)Cbl fraction is also bound to the active site of the double variant, but with a perturbed Co–O(H2) interaction, as indicated by the ~300 cm⁻¹ red-shift of the lowest-energy LF transition near ~1600 cm⁻¹ from its position observed for free Co(II)Cbl (Figure 6H). As expected from the low yield of 4c Co(II)Cbl in the SeCobAW93F/ATP complex, the MCD spectrum of Co(II)Cbl in the presence of this variant lacks the δ-band, indicating that no 4c Co(II)Cbl is generated under the experimental conditions used (Figure 7F). Further inspection of this spectrum indicates that the 5c SeCobAW93F/ATP complex is comparable to that observed with yields approaching those achieved by SeCobAWT.

**Substitutions of Residue F91.** F91W. In the MCD spectrum of Co(II)Cbl in the presence of SeCobAW91W and ATP, the δ- and β-bands are as intense as in the spectrum obtained with the SeCobAWT/ATP complex (Figure 6B). Interestingly, the λ- and σ-bands are red-shifted by ~100 cm⁻¹ from their positions in the spectra of other SeCobA variants capable of generating 4c Co(II)Cbl. As both of these bands arise from mixed CT and LF transitions of 4c Co(II)Cbl that are sensitive to perturbations of the frontier MOs of the corrin ring, these shifts indicate that the conformation of the corrin ring is uniquely perturbed in the active site of the SeCobAW91W/ATP complex. As in the case of Co(II)Cbi⁺, the intensity of the δ-band in the MCD spectrum of Co(II)Cbl in the presence of the SeCobAW91W/ATP complex is comparable to that observed in the spectrum with the SeCobAWT/ATP complex (Figure 7B). These results conclusively demonstrate that SeCobAW91W is capable of generating 4c Co(II)Cbl and Co(II)Cbi⁺ species with yields approaching those achieved by SeCobAWT. However, kinetic studies of SeCobAW91W with Co(II)Cbl showed catalytic activity diminished relative to that of SeCobAWT, while the activity with Co(I)Cbl was largely retained, suggesting that the Co(II)/Co(I) reduction step is detrimentally affected by the F91W substitution. These seemingly conflicting results can be reconciled by recognizing that the larger size of the indole group of residue 91 in SeCobAW91W relative to the phenyl group in SeCobAWT introduces new steric constraints into the active site that affect the orientation of the bound 4c Co(II)Cbl, as evidenced by the shift in the λ- and σ-bands observed spectroscopically. These structural changes could suppress the rate of electron transfer to the Co(II) ion and/or lead to uncontrolled side reactions of the transiently generated Co(I)Cbl “super-nucleophile”. While our spectroscopic results suggest that this improper orientation of the Co(II)Cbl substrate occurs only in the SeCobAW91W/ATP complex, thus favoring the latter scenario, further experiments are needed to pinpoint the origin of the decreased activity observed for this variant.

**W93F/F91W.** The MCD spectrum of Co(II)Cbi⁺ in the presence of the SeCobAW93F/F91W/ATP complex exhibits a very weak and broad δ-band, which can contain any discernible contribution from the δ-band transition, while the features at higher energies are analogous to those observed in the presence of SeCobAWT and SeCobAW93F. These findings indicate that the side chain of residue W93 is particularly important for promoting the dissociation of the DMB moiety from the Co(II) ion, as replacement of the native heterocyclic nine-membered indole ring by smaller phenyl and imidazole groups progressively shifts the equilibrium toward 5c SeCobA Cbl species, inhibiting the formation of 4c Co(II)Cbl in SeCobAW93F.

**Figure 7.** MCD spectra at 4.5 K and 7 T of Co(II)Cbl obtained in the presence of SeCobAWT and various variants. Solid lines show the spectra in the presence of ATP, while dotted lines are the corresponding traces in the absence of ATP. Panels A–G are labeled according to the amino acid substitution(s) introduced into SeCobA. The feature due to 4c Co(II)Cbl species (band δ) is highlighted by a vertical line. The relevant MCD features of free Co(II)Cbl (H) are labeled with lowercase Latin letters, and their positions are also highlighted by vertical lines.
F91H. The MCD spectrum of Co(II)Cbi⁺ in the presence of ATP-free SeCobA⁹¹¹H lacks any contributions from 4c Co(II)-corrinoid species and instead is very similar to the MCD spectrum of free Co(II)Cbl (Figure 9B). As the nucleotide loop and terminal DMB group are absent in Co(II)Cbi⁺, the characteristic spectroscopic features of nitrogen ligation observed in the MCD spectrum of Co(II)Cbi⁺ in the presence of SeCobA⁹¹¹H indicate that the newly introduced H91 residue can coordinate to the Co(II) ion. The corresponding EPR spectrum reveals that 50% of the Co(II)Cbi⁺ substrate is present in the His-on state (Figure 8B), while the remaining fraction retains the water ligand (Table 2, B). Upon addition of ATP to this complex, the positively signed MCD features at 17500 and 18700 cm⁻¹ and the negative feature at 17300 cm⁻¹ are absent in Co(II)Cbi⁺ (Figure 9C). The EPR spectrum obtained for this species indicates that ~100% of Co(II)Cbi⁺ is now present with nitrogen ligation from H91 (Figure 8C), as evidenced by the observed superhyperfine splittings due to ¹⁴N (I = 1) and the absence of features reminiscent of Co(II)Cbi⁺ (Figure 8C). The observed g values decrease significantly in response to His binding (Table 3, A and C), consistent with a further destabilization of the singly occupied Co 3d⁻¹-based MO caused by the increase in the extent of axial σ-antibonding interaction upon H₂O → His ligand substitution. Interestingly, the Aₛ(⁴⁰⁰Co) value for the His-bound Co(II)Cbi⁺ species in SeCobA⁹¹¹H is 130 MHz smaller than for free Co(II)Cbl, which may be due to the covalency of the Co–N(His) bond being larger than that of the Co–N(DMB) bond, or a tilting of the His ligand relative to the corrin ring (Figure 8 and Table 3, A and C).³⁸ The hyperfine structure is much better resolved in the EPR spectrum of the His-on Co(II)Cbi⁺ species than in the Co(II)Cbl spectrum (Figure 9C,D), indicating a markedly decreased conformational heterogeneity of the axial ligand. These findings suggest that the protein scaffold imposes a particular conformation on the His residue, possibly via H-bonding or via offset π-stacking interactions with nearby amino acids.

F91Y. The MCD spectrum of Co(II)Cbi⁺ in the presence of the ScCobA⁹¹¹/ATP complex is strikingly similar to that obtained with the ScCobA⁹¹¹/ATP complex, though the δ, β−, λ−, and σ-bands associated with the 4c Co(II)Cbi⁺ fraction are considerably more intense. Additionally, the features at ~5000 cm⁻¹ due to 5c Co(II)Cbi⁺ are notably absent in the variant spectrum, indicating that introduction of a Tyr residue at position 91 results in a nearly complete conversion of enzyme-bound Co(II)Cbi⁺ to a 4c species (Table 4, C). Similarly, the MCD spectrum of Co(II)Cbl in the presence of the ScCobA⁹¹¹/ATP complex reveals a 5-fold increase in the yield of 4c species relative to that achieved by the ScCobA⁹¹¹ enzyme (Table 4, C and Figure 7C). Thus, even though the relative yield of 4c Co(II)corrinoid species generated by ScCobA⁹¹¹ remains ~50% lower when Co(II)Cbl instead of Co(II)Cbi⁺ is used as the substrate, this variant is much more effective at generating 4c Co(II)corrinoid species than ScCobA⁹¹¹. Consistent with these results, ScCobA⁹¹¹ was found to have a 3-fold larger kcat and a 6-fold lower KM compared to those of ScCobA⁹¹¹ when Co(II)Cbl was used as the substrate.³⁴

**DISCUSSION**

More than 25 enzymes are required for the complete biosynthesis of AdoCbl by prokaryotes. A critical step in this _de novo_ pathway involves the attachment of a 5′-deoxyadenosyl (Ado) group to the cobalt ion on the Coβ face, carried out by the ScCobA ACAT in _S. enterica_.³⁴ While no eukaryotes are known to synthesize AdoCbl _de novo_, they retain genes encoding ACAT enzymes in their genomes. For example, the human ACAT, hATR, converts Co(II)Cbl to AdoCbl and delivers it to the methylmalonyl-CoA mutase (MMCM) enzyme to restore catalytic activity following cofactor deactivation.²¹,³²,⁵⁹ Intriguingly, the three distinct families of known ACATs seem to employ the same general catalytic mechanism, even though they share little primary sequence homology and differ with respect to the morphology of the corrinoid binding site.¹¹,²⁴,²⁵,²⁹ While a recent report has highlighted the molecular interactions that are critical for the catalytic activity of the _LrPduO ACAT_,⁶⁰ a homologue of hATR, the roles of individual active-site residues in the remaining ACAT families have not yet been elucidated. To enhance our current understanding of the mechanism by which ScCobA and related ACATs catalyze the Ado group transfer to ATP from Co(II)corrinoid substrates, we have employed MCD spectroscopy to monitor the effects of active-site amino acids.
Table 2. EPR Parameters for Co(II)Cbl in the Absence and Presence of the ScCobAp9H/ATP Complex

| species                  | g values          | A(14N) (MHz) | A(59Co) (MHz) |
|--------------------------|-------------------|--------------|---------------|
| (A) Co(II)Cbl           | 2.002 2.345 2.335 | 410 240 240 | n/a b         |
| (B) Co(II)Cbl/ScCobAp9H | 2.002 2.345 2.335 | 410 240 240 | n/a b         |
| base-off                 | 2.002 2.365 2.335 | 305 40 30   | 60 10 10      |
| (C) Co(II)Cbl/ScCobAp9H/ATP | 2.002 2.335 2.280 | 280 40 30   | 52 10 10      |
| (D) Co(II)Cbl           | 2.002 2.335 2.275 | 305 40 30   | 60 10 10      |

aValues for free Co(II)Cbl are also shown for comparison. bNot applicable.

Figure 9. MCD spectra collected at 4.5 K and 7 T of (A) free Co(II)Cbl, (B) Co(II)Cbl in the presence of ScCobA, and (C) Co(II)Cbl in the presence of ScCobA and ATP. For reference, the spectrum of free Co(II)Cbl is shown in panel D. The most intense features of free Co(II)Cbl are highlighted by dashed vertical lines. The lower-energy region of the spectra of protein-bound species is scaled by a factor of 3 to highlight unique features in this region.

Effects of Amino Acid Substitutions on the 5c → 4c Co(II)Corrinoid Conversion Yield and Co–C(Ado) Bond

Formation. The KM values established from recent kinetic studies of ScCobAWT can be compared to the relative yields of formation of 4c Co(II)Cbl established by our MCD experiments (see the Supporting Information for details) to determine whether a given amino acid substitution at the active site of ScCobAWT mainly affects the Co(II)/Co(I) reduction step or the subsequent nucleophilic attack of the transiently generated Co(I) species on cosubstrate ATP. Because 4c Co(II)corrinoid formation is a prerequisite for generating the Co(I) "supernucleophile", changes to the 5c–4c Co(II)corrinoid equilibrium should correlate with enzymatic activity provided that the 5c → 4c Co(II)corrinoid conversion contributes to the rate-limiting step. As summarized in Table 4 (right columns), a correlation indeed exists between changes in enzymatic activity caused by amino acid substitutions and the relative yield of 4c Co(II)corrinoid, indicating that the reduced activity of the variants is largely due to perturbations to the 5c–4c Co(II)corrinoid equilibrium.

Table 3. Kinetic Parameters for the Adenosylation of Co(II)Cbl and Co(II)Cbl by ScCobAWT

| species                  | KM (μM) | kcat (s⁻¹) | kcat/KM (M⁻¹ s⁻¹) |
|--------------------------|---------|------------|--------------------|
| Co(II)Cbl                | 16.3 ± 3.5 | (7.7 ± 0.4) × 10⁻³ | (4.7 ± 0.6) × 10⁷ |
| Co(II)Cbl                | 25 ± 5 | (6.0 ± 0.9) × 10⁻³ | (2.0 ± 0.4) × 10⁷ |
| Co(II)/Co(I) Cbl         | 25.4 ± 9.0 | (6.7 ± 0.7) × 10⁻³ | (2.6 ± 0.4) × 10⁷ |
| Co(II)/Co(I) Cbl         | 66 ± 18 | (5.0 ± 0.7) × 10⁻³ | (0.8 ± 0.2) × 10⁷ |

aFrom ref 34.
The change in solvent accessible surface area (ΔSA) for each pair of different Pairs of Amino Acid Residues in the Active Site of ScCobAWT and Several Variants in the “Closed” Conformations and Estimated Free Energy Changes for the Equilibrium between the 4c and 5c States of the Co(II)Cbl and Co(II)Cbi+ Substrates (ΔΔGk) Based on the 4c → 4c Co(II)rrinoid Conversion Yields from Table 4

Table 5. DFT-Computed Relative Dispersion Energies (ΔE03) for Different Pairs of Amino Acid Residues in the Active Site of ScCobAWT and Several Variants in the “Closed” Conformation

| ScCobA substitution | active-site model | ΔE03 (kJ/mol) | ΔSA (Å²) | ΔΔGk (kJ/mol) |
|---------------------|------------------|---------------|---------|---------------|
| W93A                | Phe, Ala         | 115           | −56.7   | >89           |
| W93H                | Phe, His         | 64            | −34.7   | 0.74          | 8.98         |
| W93F                | Phe, Phe         | 40            | −17.9   | −4.2          | 7.7          |
| W93F/F91W           | Trp, Phe         | 17            | 27.2    | 5.7           | >9           |
| WT                  | Phe, Trp         | 0             | 0.0     | 0.00          | 4.60         |
| F91Y                | Trp, Trp         | −13           | 8.5     | −5.79         | 0.82         |
| F91W                | Trp, Trp         | −39           | 48.5    | −3.37         | 5.09         |

The change in solvent accessible surface area (ΔSA) for each pair of residues in the absence of the protein is provided as a measure of the bulkiness of each pair. All values are shown in relation to Co(II)Cbi+ bound to the ScCobAWT/ATP complex (bold). Cases in which the ΔSA values are >27 Å² are highlighted in italics.

Our MCD spectra also provide insight into the geometry adopted by the Co(II)Cbl substrate in the “open” conformation of ScCobA.

Table 4. Positions of the δ-Band in the MCD Spectra of 4c Co(II)Cbi+ and Co(II)Cbl Generated in the Active Sites of ScCobAWT and Several Variants

| ScCobA substitution | Co(II)Cbi+ | Co(II)Cbl |
|---------------------|------------|-----------|
|                      | ν(δ) (cm⁻¹) | Δν(δ) (cm⁻¹) | 4c yield (%) | ν(δ) (cm⁻¹) | Δν(δ) (cm⁻¹) | 4c yield (%) | expected yield (%) |
| (A) none (WT)       | 12270      | 0          | 50          | 12350      | 80          | 8           | 25           |
| (B) F91W            | 12230      | −40        | 85          | 12260      | −10         | 7           | 4            |
| (C) F91Y            | 12300      | 30         | >95         | 12320      | 50          | 40          | 45           |
| (D) W93F            | 12240      | −30        | 89          | 12220      | −50         | 2           | 68           |
| (E) W93H            | 12180      | −90        | 41          | 12200      | 10          | 19          |
| (F) W93F/F91W       | 12210      | −60        | 5           | n/a        |             |             |              |
| (G) W93A            | n/a        | n/a        | n/d         | n/a        | n/a         | n/a         |              |

“Shifts in the δ-band are shown in relation to the position of this feature in the spectrum of Co(II)Cbi+ in the presence of the ScCobAWT/ATP complex. Also shown are the relative yields of 4c species estimated from the δ-band intensities. The expected yields based on kinetic results obtained with Co(II)Cbl are also shown (see the Supporting Information for details).”

Our MCD spectra also provide insight into the geometry adopted by the Co(II)Cbl substrate in the “open” conformation of ScCobA.
of ScCobA, prior to removal of the DMB moiety. A comparison of the MCD features exhibited by free Co(II)Cbl and of the 5c Co(II)Cbl species in the presence of the different ScCobA/ATP complexes investigated reveals small but noticeable differences. In particular, the decrease in the intensity of the negatively signed band at \( \sim 15 \) 000 cm\(^{-1}\), along with the minor red-shift of the positive feature at \( \sim 17000 \) cm\(^{-1}\) observed for ScCobAWT and all variants except for ScCobAW93A (Figure 7), indicates that the 5c Co(II)Cbl fraction is enzyme-bound, with a perturbed Co–N(DMB) bonding interaction. This finding is consistent with the crystal structure of ScCobAWT, which revealed that in the “open” conformation, residue W93 is spatially very close to the ribose moiety that makes up part of the nucleotide loop of Co(II)Cbl. This steric clash may contribute to the elongated Co–N(DMB) bond of the 5c Co(II)Cbl species observed in the X-ray crystal structure of ScCobAWT (see Table 1). As the nearby F91 side chain is properly positioned to participate in a \( \pi \)-stacking interaction with residue W93 in the “open” conformation, substitution of F91 would be expected to introduce further perturbations into the Co–N(DMB) bond of enzyme-bound Co(II)Cbl, consistent with our MCD data. Thus, we conclude that in the “open” conformation of ScCobAWT, the F91 and W93 residues are positioned so as to weaken the Co–N(DMB) bond via steric interactions with the bulky DMB moiety.

**Mechanism of 4c Co(II)Corrinoid Formation.** Because MCD spectroscopy provides a uniquely sensitive tool for discriminating between 4c and 5c Co(II)corrinoids, it is possible to use our data as the basis for estimating the change in free energy for the formation of 4c species in response to active-site amino acid substitutions, \( \Delta G_{4c} \) (see the Supporting Information for details). Using Co(II)Cbi\(^{+}\) bound to the ScCobAWT/ATP complex as the reference point, our analysis yields a range of \( \Delta G_{4c} \) values between \(-6\) and 9 kJ/mol based on the detection limit of 4c Co(II)corrinoid species by our MCD instrument (\(-1\)% relative to the entire population of Co(II)corrinoids). On average, \( \Delta G_{4c} \) increases by \(-8\) kJ/mol when Co(II)Cbl instead of Co(II)Cbi\(^{+}\) is used as the substrate (Table 4, right columns), which can be attributed to the increased strength of the Co–N(DMB) bond relative to that of the Co–O(H\(_2\)) bond. The increase in Co–N(DMB) bond strength estimated from our results is consistent with the 40-fold decrease in \( k_m \) values previously observed for the binding of AdoCbl to MCMC (which binds AdoCbl in a base-off, His-on fashion\(^3\) relative to hATR (which excludes the binding of axial ligands to the Co\(\alpha\) face of the corrin ring where the DMB group would usually be found).\(^{21,37,62}\) However, this difference is not constant across the entire series of ScCobA variants investigated, because specific, species-dependent intermolecular interactions between the protein side chains and the DMB moiety are likely important for promoting Co–N(DMB) bond dissociation (vide supra). The fact that ScCobAWT achieves a higher 5c \( \rightarrow \) 4c conversion yield with Co(II)Cbi\(^{+}\), as the substrate is consistent with its main role in the adenosylation of incomplete corrinoids that generally lack the nucleotide loop and terminal DMB base.

A comparison of the “open” and “closed” conformations observed in the crystal structure of ScCobAWT complexed with Co(II)Cbl and MgATP reveals only minor differences in the vicinity of the Co\(\beta\) face of the corrin ring that is oriented towards cosubstrate ATP. In contrast, large conformational differences exist near the Co\(\alpha\) face of the corrin ring, in particular with regard to residues F91 and W93 that move by \(-12.1\) and \(-7.5\) Å, respectively, relative to their solvent-exposed positions in the “open” conformation, to fill the space originally occupied by the DMB moiety. Given the size and hydrophobicity of these residues, as well as their positioning in an offset \( \pi \)-stacking configuration in the “closed” conformation, it is likely that dispersion interactions play a role in stabilizing the “closed” over the “open” conformation of ScCobAWT.\(^{53,64}\) To evaluate this possibility, the magnitude of dispersion interactions involving residues 91 and 93 was estimated by DFT computations (see Materials and Methods for details). Inspection of the computed relative dispersion energies, \( \Delta E_{D} \), for various combinations of residues reveals a correlation between these values and the relative population of 4c Co(II)Cbi\(^{+}\) generated in the different ScCobA variants investigated (Table 4). While the computed \( \Delta E_{D} \) values are generally much larger than the dispersion energies reported for related model systems,\(^{63,65}\) they properly reproduce the experimental trends and correlate well with the size of the interacting \( \pi \)-systems.\(^{66}\) Consistent with the inability of ScCobAW93A to convert Co(II)Cbl and Co(II)Cbi\(^{+}\) to 4c species and the lack of catalytic activity displayed by this variant, the computed \( E_D \) value for the Phe-Ala fragment is 115 kJ/mol smaller than that obtained for the Phe-Trp fragment present in ScCobAWT, highlighting the importance of the F91 residue in stabilizing the “closed” conformation of the protein. A largely reduced \( E_D \) value (by \(-64\) kJ/mol) is also predicted for the Phe-His pair, consistent with our spectroscopic data for ScCobAW93F, which indicate that this variant is relatively ineffective at converting Co(II)Cbi\(^{+}\) to a 4c species and fails to promote dissociation of the DMB group from Co(II)Cbl. Finally, replacement of Phe with Tyr, a polar aromatic residue, results in a modest (\(-13\) kJ/mol) increase in the computed \( E_D \) value, in qualitative agreement with the higher relative 4c Co(II)corrinoid yields observed experimentally for the ScCobAP91F variant.

The \( E_D \) values obtained for the remaining active-site models agree less well with the experimental trends, supporting our hypothesis that additional factors affect the 5c \( \rightarrow \) 4c Co(II)corrinoid conversion yield (vide supra). As our spectroscopic results reveal a uniquely perturbed conformation of the Co(II)corrinoid substrate in the ScCobA variants possessing the F91W substitution (ScCobAP91W and ScCobAP91W93F), it is worth noting that the introduction of the larger indole moiety dramatically increases the bulkiness of the active site, as indicated by the >27 Å\(^2\) increase in the solvent accessible surface area (\( \Delta S_A \)) of paired residues 91 and 93 in these variants (Table 5). Thus, it is likely that our simple models do not properly account for all of the changes in protein conformation that are needed to accommodate the larger residues. Similar factors may contribute to the poor agreement between the computed \( E_D \) value and the relative yield of 4c Co(II)corrinoid formation in the case of ScCobAW93F.

Despite certain exceptions to the general trend in the \( E_D \) values, our computational results provide strong evidence that favorable enthalpic contributions from offset \( \pi \)-stacking interactions between residues 91 and 93 in the “closed” conformation of ScCobA contribute to 4c Co(II)corrinoid formation. In addition, our results indicate that in the absence of a bulky, planar residue, as in the case of ScCobAW93F, steric interactions needed for axial ligand exclusion no longer exist, possibly allowing solvent molecules to interact with the corrin ring, as shown by the essentially unperturbed conformation of Co(II)corrinoids in the presence of this variant. Similarly, the
introduction of smaller aromatic residues (as in ScCobA<sup>W93F</sup>
and ScCobA<sup>W93F</sup>) likely diminishes the conformational rigidity
of the active site, consistent with the broadening of the δ-band
in the corresponding MCD spectra (Figure 6, traces D and E),
while introduction of larger residues results in significant steric
strain that may cause distortions of the corrin ring, as observed
for ScCobA<sup>F91W</sup>. Furthermore, our experimental and computa-
tional results indicate that the replacement of a hydrogen atom
in the F91 side chain with a hydroxyl group does not result in
large changes in the conformation of the active site. However,
its does increase the number of dispersion interactions with the
nearby W93 residue, thus stabilizing the “closed” conformation.
The calculated ∼13 kJ/mol increase in <i>E<sub>d</sub></i> is on the order of our
experimentally estimated difference in ∆∆<i>G<sub>4c</sub></i> of ∼8 kJ/mol for the
dissociation of the DMB moiety from Co(II)Cbl versus H<sub>2</sub>O
dissociation from Co(II)Cbi<sup>+</sup> (vide supra), indicating that
this contribution alone may be sufficient to account for the
dramatic increase in the 4c Co(II)Cbl yield achieved by
ScCobA<sup>F91Y</sup>.

Implications for the Mechanism of Co(II)rrinoid
Reduction and Adenosylation in Vivo. Crystallographic
studies revealed that binding of ATP to ScCobA<sup>WT</sup> causes the
active site to adopt a conformation in which it can engage in
dipolar interactions with the acetamide and propionamide side
chains on the corrin ring and thus facilitate the binding of the
Co(II)rrinoid substrate. In this “open” conformation of
ScCobA<sup>WT</sup>, the lower axial ligand (either DMB or H<sub>2</sub>O)
remains associated with the Co(II) ion but likely interacts with
the nearby W93 residue. Further structural changes to the
active-site structure occur upon formation of the “closed”
conformation, in particular to the N-terminal region of the
adjacent subunit as well as the segment between residues M87
and C105, which adopt helical structures. As a result, the
Co(II)rrinoid substrate shifts ∼0.3 Å closer to the S′-carbon of
ATP while the F91 and W93 residues move below the Co(II)rrinoid
species, the proximity of the two substrates is critical for
avoiding undesired side reactions. The positioning of the
corrinoid relative to the cosubstrate ATP is likely controlled,
and by <i>p</i>-stacking interactions between residues F91 and W93.

In the X-ray crystal structure of ScCobA<sup>WT</sup>, the distance
between the Co(II) ion of the 4c fraction of Co(II)Cbl and the
S′-carbon of cosubstrate ATP is ∼3.0 Å, which is only ∼1.0 Å
larger than the Co–C(Ado) bond distance in AdoCbl<sup>+</sup>. As this
4c fraction of Co(II)Cbl is activated for one-electron reduction
via the flavin to produce a “supernucleophilic” Co(I)rrinoid
species, the proximity of the two substrates is critical for
avoiding undesired side reactions. The positioning of the
coordinoid relative to the cosubstrate ATP is likely controlled,
atast in part, by the conformation of the M87–C105 loop. In
support of this hypothesis, ScCobA<sup>F91W</sup> displays a drastically
diminished catalytic activity because of the misalignment of the
coordinoid substrate, even though it achieves wild-type-like
relative yields of formation of 4c Co(II)Cbl and Co(II)Cbi<sup>+</sup>. These
findings highlight the delicate balance of interactions that
must be present in the active site of ScCobA for the formation
of 4c Co(II)rrinoids. Our findings also provide clues about why
Ralstonia species express CobA with Tyr at position 91 in lieu
of the Phe present in ScCobA.<sup>34</sup> While in vivo ScCobA is
responsible for the adenosylation of a variety of corrinoid
substrates,<sup>35</sup> our results indicate that Co(II)Cbl provides a
significant challenge for this enzyme, as only ∼8% of Co(II)Cbl
is converted to a 4c species. Substitution of F91 with a Tyr
residue increases the amount of 4c species generated from
Co(II)Cbl 5-fold. It is possible that these homologous CobAs
with active-site tyrosines have been selected for in vivo
conditions that demand a very high rate of turnover of AdoCbl,
or perhaps these organisms are “fed” cobalamin by mutualistic
strains and no longer need to process cobinamide. Another
possibility is that the active-site tyrosine is more effective at
generating 4c species of cobamidates that possess Coβ ligands
other than DMB, which could include one of several types of
purines, phenolics, or DMB derivatives. The in vitro
effectiveness of ScCobA<sup>WT</sup> to adenosylate alternative cobamides
has yet to be determined.

## ASSOCIATED CONTENT

## Supporting Information

Additional details regarding the preparation of samples, absorption
data, and equations and strategy used to evaluate ∆∆<i>G<sub>4c</sub></i>
values and relative yields of 4c Co(II)rrinoids. 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.

### ADDITIONAL NOTE

“θ(LA)” is the angle between the plane containing the <i>N<sub>A</sub></i>, C<sub>4</sub>−
and N<sub>3</sub> atoms and the plane containing the N<sub>6</sub>, C<sub>14</sub>−C<sub>10</sub>−
and N<sub>0</sub> atoms, while ϕ(SA) corresponds to the angle between
the planes containing the N<sub>9</sub>, C<sub>19</sub>, C<sub>14</sub> and N<sub>3</sub> atoms and the
N<sub>6</sub>, C<sub>14</sub>−C<sub>10</sub> and N<sub>0</sub> atoms (see Figure S8 of the Supporting
Information for the atom numbering scheme used).

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