Residue Phe112 of the Human-Type Corrinoid Adenosyltransferase (PduO) Enzyme of Lactobacillus reuteri Is Critical to the Formation of the Four-Coordinate Co(II) Corrinoid Substrate and to the Activity of the Enzyme†, ‡

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ABSTRACT: ATP:Corrinoid adenosyltransferases (ACAs) catalyze the transfer of the adenosyl moiety from ATP to cob(I)alamin via a four-coordinate cob(II)alamin intermediate. At present, it is unknown how ACAs promote the formation of the four-coordinate corrinoid species needed for activity. The published high-resolution crystal structure of the ACA from Lactobacillus reuteri (LrPduO) in complex with ATP and cob(II)alamin shows that the environment around the α face of the corrin ring consists of bulky hydrophobic residues. To understand how these residues promote the generation of the four-coordinate cob(II)alamin variants of the human-type ACA enzyme from L. reuteri (LrPduO) were kinetically and structurally characterized. These studies revealed that residue Phe112 is critical in the displacement of 5,6-dimethylbenzimidazole (DMB) from its coordination bond with the Co ion of the ring, resulting in the formation of the four-coordinate species. An F112A substitution resulted in a 80% drop in the catalytic efficiency of the enzyme. The explanation for this loss of activity was obtained from the crystal structure of the mutant protein, which showed cob(II)alamin bound in the active site with DMB coordinated to the cobalt ion. The crystal structure of an LrPduOΔF112H variant showed a DMB-off/His-on interaction between the corrinoid and the enzyme, whose catalytic efficiency was 4 orders of magnitude lower than that of the wild-type protein. The analysis of the kinetic parameters of LrPduOΔF112H suggests that the F112H substitution negatively impacts product release. Substitutions of other hydrophobic residues in the Cbl binding pocket did not result in significant defects in catalytic efficiency in vitro; however, none of the variant enzymes analyzed in this work supported AdoCbl biosynthesis in vivo.

The chemistry of B12-dependent reactions and the biosynthesis of this complex coenzyme have been an area of intense investigation for decades. Coenzyme B12 [adenosylcobalamin (AdoCbl)]1 and methylcobalamin (MeCbl) are the two biologically active forms of B12. AdoCbl participates in radical-based intramolecular rearrangements (1–3), deaminations (4), dehydrogenations (5), reductions (6, 7), and reductive dehalogenations (8), while Cbl serves as a transient methyl carrier in methylation reactions (9–11). Despite the range of chemical reactions carried out by these forms of B12, MeCbl and, in some cases, AdoCbl bind to enzymes in very similar conformations. For example, the crystal structures of methylmalonyl-CoA mutase (MMCM, AdoCbl-dependent) and methionine synthase (MeCbl-dependent) revealed Cbl bound in the active site in a base-off—His-on conformation (12, 13). In contrast, the active sites of the bacterial and human ATP:Corrinoid adenosyltransferases do not have a histidyl residue in the proximity of the cobalt ion, suggesting that catalysis does not occur via a DMB-off/His-on intermediate.

One fascinating aspect of the corrinoid adenosylation reaction is the reduction of the Co(II) corrinoid substrate. While the environment inside the cell is sufficiently reductive to drive the reduction of Co4+ to Co3+ (14), it is not low enough to drive the reduction of Co3+ to Co2+ (15, 16). ACA enzymes bind cob(II)alamin and facilitate its reduction by generating a four-coordinate cob(II)alamin species in the active site (17–20). The four-coordinate species lacks axial ligands, the absence of which stabilizes the 3d1 orbital and raises the Co2+ to Co3+ reduction midpoint potential to within the range of reducing agents inside the cell (17). By binding...
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cob(II)alamin in the active site, ACA enzymes generate a Co$^+$ “super-nucleophile” and prevent its quenching by deleterious side reaction. At present, however, there is no direct evidence that ACA enzymes deficient in their ability to generate the four-coordinate cob(II)alamin are impaired in catalyzing the adenosylation reaction.

The crystal structure of the Lactobacillus reuteri PduO enzyme in complex with ATP and cob(II)alamin confirmed the existence of the four-coordinate cob(II)alamin intermediate (21). The protein environment around the vacant α-axial ligand region consists of several bulky and hydrophobic residues positioned in the proximity of the cob(II)alamin substrate (21). The role of these residues in the generation of the four-coordinate intermediate remains unclear. Kinetic and structural analyses reported here provide insights into the role of hydrophobic residues in the generation of the four-coordinate cob(II)alamin intermediate. We also report the structure and kinetic behavior of LrPduO with Cbl bound in the base-off--His-on conformation and discuss possible reasons why ACA enzymes do not bind Cbl in the His-on form.

**EXPERIMENTAL PROCEDURES**

**Protein Production and Purification.** Variants of LrPduO were generated using the QuickChange XL site-directed mutagenesis kit (Stratagene). The pTEV3 plasmid (22) carrying the wild-type Lr pduO$^+$ allele (23) was used as a template for polymerase chain reaction (PCR)-based site-directed mutagenesis as per the manufacturer’s instructions. The presence and nature of the mutations were verified by template for polymerase chain reaction (PCR)-based site-directed mutagenesis solutions was 12, 15, and 11 mg/mL for the LrPduO$^{F12H}$, LrPduO$^{F12H}$, and LrPduO$^{A183}$ variants, respectively. The models include residues 2–188 of the four-coordinate intermediate. We also report the structure and kinetic behavior of LrPduO with Cbl bound in the base-off--His-on conformation and discuss possible reasons why ACA enzymes do not bind Cbl in the His-on form.

Crystallization and Data Collection. LrPduO variant proteins were produced and purified using described protocols (23). All crystals of tag-less LrPduO proteins were grown using the vapor diffusion method in an anoxic chamber at 25 °C. The protein concentration in the crystal solution {glycerol [20% (v/v)], PEG 8000 [12% (w/v)], MES (100 mM, pH 6)}, or, for LrPduO$^{F12H}$, HEPPS (100 mM, pH 8.6), KCl (200 mM), or, for LrPduO$^{F12H}$, HEPPS (100 mM, pH 8.6), KCl (200 mM), or, for LrPduO$^{F12H}$, HEPPS (100 mM, pH 8.6), KCl (200 mM), or, for LrPduO$^{A183}$, ATP (2 mM), and MgCl$_2$ (2.5 mM) and, in an anoxic chamber, incrementally transferred in five steps to an anoxic cryoprotectant solution {glycerol [20% (v/v)], PEG 8000 [12% (w/v)], MES (100 mM, pH 8.6), KCl (200 mM), or, for LrPduO$^{F12H}$, HEPPS (100 mM, pH 8.6), KCl (200 mM), or, for LrPduO$^{F12H}$, HEPPS (100 mM, pH 8.6), KCl (200 mM), or, for LrPduO$^{A183}$, ATP (2 mM), and MgCl$_2$ (2.5 mM)}. The crystals were briefly exposed to oxygen (≤5 s) while they were flash-frozen in liquid nitrogen.

All crystals belong to space group R3 with one subunit in the asymmetric unit. Data sets were collected at the Advanced Photon Source in Argonne, IL, on beamline 19BM. Diffraction data were integrated and scaled with HKL2000 (27). Data collection statistics are summarized in Table 1.

Structure Determination and Refinement. The structures were determined by molecular replacement with SOLVE (28) starting from the model of the wild-type LrPduO protein in complex with ATP (PDB entry 2NT8). Final refinement was carried out with REFMAC (29). Heteroatoms, water molecules, and multiple conformations were built using COOT (30). The final LrPduO$^{F12A}$–ATP:cof(I)alamin, LrPduO$^{F12H}$–ATP:cof(I)alamin, and LrPduO$^{A183}$–ATP:cof(I)alamin models were refined to 1.5, 1.2, and 1.4, respectively. The models include residues 2–182, 1–188, and 2–181 for LrPduO$^{F12A}$, LrPduO$^{F12H}$, and LrPduO$^{A183}$, respectively. Ramachandran plots for all models show that >96% of residues are in the most favored region with no residues falling in the disallowed region. Refinement statistics are listed in Table 1.

In Vitro Adenosylation Activity Assays. The Co$^+$ assay was performed using the continuous spectrophotometric method described previously, without modifications (23). In this assay, the cobalt ion of Cbl is chemically reduced in solution to cob(I)alamin by Ti(III)citrate. The Co$^+$ assay was performed under anoxic conditions, and the adenosylation reaction was initiated by the addition of LrPduO. The reaction mixture included 2-aminobutyric acid/methionine-1,3-diol hydrogen chloride (Tris-HCl, 0.2 M; pH 8 at 37 °C), MgCl$_2$ (1.5 mM), HOCbl (0.1–20 μM), and ATP (1 μM to 1 mM). The Co$^{2+}$ assay was modified from a previously used end-point assay (26) to allow the appearance of product (AdoCbl) to be continuously monitored in the presence of a protein reducing system. The Co$^{2+}$ assays were performed under anoxic conditions at 37 °C. Empty quartz cuvettes were
flushed with oxygen-free N₂ for 5 min. Under a stream of O₂-free N₂, 2-amino-2-hydroxymethylpropane-1,3-diol hydrochloride (Tris-HCl, 0.2 M; pH 8 at 37 °C), KCl (0.1 M), MgCl₂ (1.5 mM), HOCbl (4 µM to 0.2 mM), Fpr (73 µg/mL), FldA (0.6 mg/mL), NADPH (1 mM), and ATP (1 µM to 1 mM) were added to the cuvette in the order stated. To ensure that all cob(III)alamin was reduced to cob(II)alamin, the reaction mixtures were incubated at 37 °C and MgCl₂ (1.5 mM), HOCbl (4 µM to 1 mM) were added to the cuvette in the order stated. To ensure that all cob(III)alamin was reduced to cob(II)alamin, the function of PduO was assessed in vivo. For this purpose, we used precursor of AdoCbl (ferase. CobA is needed for salvaging cobinamide (Cbi), a precursor of AdoCbl 

### RESULTS AND DISCUSSION

Spectroscopic and structural analyses have shown that PduO-type ACA enzymes facilitate the thermodynamically unfavorable Co²⁺ to Co⁺ reduction of the cobalt ion in corrinoids by generating a four-coordinate Co(II) corrinoid species with no axial (α and β) ligands (17, 18). To gain insights into the molecular basis of the formation of the four-coordinate Co(II) corrinoid species in PduO-type ACA enzymes, we performed structural and kinetic analysis of LrPduO enzyme variants.

We used two assays to distinguish between the two functions of LrPduO, namely, assistance in the reduction of Co⁵⁺ to Co⁺ and Co(I) corrinoid adenosylation. In one assay, we used the NADPH-dependent flavodoxin protein reductase (Fpr)/flavodoxin (FldA) system to reduce Co²⁺ to Co⁺; hereafter, this assay is termed the Co²⁺ assay. In the Co²⁺ assay, the PduO enzyme must bind cob(II)alamin and facilitate the generation of cob(I)alamin in its active site. Conversely, in the second assay, termed the Co⁺ assay, we used Ti(III)citrate to reduce Co²⁺ to Co⁺ in solution, allowing the cob(I)alamin adenosylation reaction to be measured directly. Cobalamin was used as the corrinoid substrate in both assays. The Co⁺ assay allowed us to indirectly assess the formation of the four-coordinate cob(II)alamin species, whereas the Co⁺ assay allowed us to assess the catalytic competency of the variants. We used both assays in combination with high-resolution crystal structures to identify residues involved in the formation of the four-coordinate cob(II)alamin species.

**Residue Phe112 Displaces the Lower Ligand.** The crystal structure of LrPduO in complex with cob(II)alamin and ATP revealed cob(II)alamin bound in a conformation with the lower axial ligand (DMB) excluded from the active site (21) (Figure 1A). Residue Phe112 is positioned 3.8 Å from the cobalt ion, suggesting that this residue plays an important role in the formation of the four-coordinate cob(II)alamin intermediate (21, 34, 35). To further investigate the role of Phe112, we changed the coding sequence of the Lr pduO gene to encode variant protein LrPduO⁷¹²A. The latter was crystallized under anoxic conditions, and its X-ray structure was determined at 1.5 Å resolution. The crystal structure of LrPduO⁷¹²A revealed cob(II)alamin bound to the active site as a five-coordinate...
species with DMB serving as a fifth axial ligand (Figure 1B). The \( Lr\text{PduO}^{F112A} \) variant displayed extremely low activity in the Co\(^{2+} \) assay. However, in the Co\(^{3+} \) assay [adenosylation of cob(I)alamin], \( Lr\text{PduO}^{F112A} \) was catalytically competent and displayed only a slight decrease in \( k_{\text{cat}} \) (~4-fold) (Table 2). This result is consistent with the idea that, in the absence of a four-coordinate cob(I)alamin species, the enzyme is inactive. The kinetic parameters of the \( Lr\text{PduO}^{F112A} \) enzyme support the idea that the steric bulk presented by the phenyl moiety of the side chain of Phe112 is critical for the formation of the four-coordinate species and hence to the reactivity of the enzyme.

**Effects of an \( F112H \) Substitution.** Several Cbl-dependent enzymes share the Cbl-binding motif “DXHXXG”, in which the histidinyl residue coordinates to the cobalt ion (36) generating a DMB-off/His-on conformation of the cofactor (12, 37); the DXHXXG motif is not present in any ACA enzyme. The proximity of residue Phe112 to the cobalt ion of Cbl offered an opportunity to generate a DMB-off/His-on ACA enzyme by site-directed mutagenesis, allowing us to assess the effect of a DMB-off/His-on complex on the activity of the enzyme. The \( Lr\text{PduO}^{F112H} \) variant was constructed, and its X-ray crystal structure was determined to 1.5 Å resolution. The structure of \( Lr\text{PduO}^{F112H} \) confirmed that cob(I)alamin was bound in a DMB-off/His-on conformation (Figure 2). The F112H substitution resulted in a significant drop in \( k_{\text{cat}} \) (~60-fold) in assays Co\(^{2+} \) and Co\(^{3+} \) (Tables 2 and 3), suggesting that \( Lr\text{PduO}^{F112H} \) was impaired

### Table 2: Kinetic Parameters of \( Lr\text{PduO} \) Variants Using the Co\(^{2+} \) Assay

<table>
<thead>
<tr>
<th>enzyme</th>
<th>( K_m ) (( \mu \text{M} ))</th>
<th>( k_{\text{cat}} ) (s(^{-1} ))</th>
<th>( k_{\text{cat}}/k_{\text{m}} ) (M(^{-1} ) s(^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>( 2.2 \pm 0.1 )</td>
<td>( 2.6 \pm 0.1 ) \times 10(^{-2} )</td>
<td>( 1.2 \pm 0.1 ) \times 10(^4 )</td>
</tr>
<tr>
<td>F112A</td>
<td>( 34.6 \pm 8.8 )</td>
<td>( 5.6 \pm 0.0 ) \times 10(^{-3} )</td>
<td>( 1.7 \pm 0.4 ) \times 10(^3 )</td>
</tr>
<tr>
<td>F112H</td>
<td>( 2.6 \pm 0.0 )</td>
<td>( 3.8 \pm 0.1 ) \times 10(^{-4} )</td>
<td>( 1.4 \pm 0.1 ) \times 10(^3 )</td>
</tr>
<tr>
<td>F112Y</td>
<td>( 1.2 \pm 0.1 )</td>
<td>( 1.6 \pm 0.0 ) \times 10(^{-3} )</td>
<td>( 1.4 \pm 0.1 ) \times 10(^3 )</td>
</tr>
<tr>
<td>F112W</td>
<td>( 2.0 \pm 0.6 )</td>
<td>( 1.8 \pm 0.2 ) \times 10(^{-3} )</td>
<td>( 9.8 \pm 3.4 ) \times 10(^3 )</td>
</tr>
<tr>
<td>F163A</td>
<td>( 15.9 \pm 6.8 )</td>
<td>( 1.5 \pm 0.1 ) \times 10(^{-2} )</td>
<td>( 1.8 \pm 0.4 ) \times 10(^3 )</td>
</tr>
<tr>
<td>F187A</td>
<td>( 1.2 \pm 0.4 )</td>
<td>( 2.1 \pm 0.4 ) \times 10(^{-2} )</td>
<td>( 1.7 \pm 0.6 ) \times 10(^4 )</td>
</tr>
<tr>
<td>ΔS183</td>
<td>( 3.1 \pm 0.2 )</td>
<td>( 2.3 \pm 0.6 ) \times 10(^{-2} )</td>
<td>( 7.5 \pm 2.2 ) \times 10(^3 )</td>
</tr>
</tbody>
</table>

\( k_{\text{cat}} \) (s\(^{-1} \)) | \( k_{\text{cat}}/k_{\text{m}} \) (M\(^{-1} \) s\(^{-1} \)) |
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<tbody>
<tr>
<td>( 0.13 \pm 0.01 )</td>
<td>( 2.4 \pm 0.1 ) \times 10(^{-2} )</td>
</tr>
<tr>
<td>( 1.9 \pm 0.5 )</td>
<td>( 6.6 \pm 0.5 ) \times 10(^{-3} )</td>
</tr>
<tr>
<td>( 2.8 \pm 0.4 )</td>
<td>( 4.2 \pm 0.1 ) \times 10(^{-4} )</td>
</tr>
<tr>
<td>( 0.55 \pm 0.05 )</td>
<td>( 1.7 \pm 0.0 ) \times 10(^{-3} )</td>
</tr>
<tr>
<td>( 0.77 \pm 0.12 )</td>
<td>( 2.0 \pm 0.2 ) \times 10(^{-3} )</td>
</tr>
<tr>
<td>( 0.19 \pm 0.03 )</td>
<td>( 1.5 \pm 0.3 ) \times 10(^{-2} )</td>
</tr>
<tr>
<td>( 0.11 \pm 0.01 )</td>
<td>( 1.8 \pm 0.1 ) \times 10(^{-2} )</td>
</tr>
<tr>
<td>( 0.07 \pm 0.06 )</td>
<td>( 2.0 \pm 0.2 ) \times 10(^{-2} )</td>
</tr>
</tbody>
</table>

\(^{a}\)The substrate cob(I)alamin was generated chemically using Tit(III)citrate. \(^{b}\)Significant error due to absorbance near the detection limit.
in its ability to catalyze the adenosylation of cob(I)alamin and/or release the AdoCbl product. Notably, the specific activity of \( LrPduO^{F112H} \) for the reduction of cob(I)alamin to cob(I)alamin was only 19-fold lower than that of the wild-type \( LrPduO \) protein (1.5 and 28 mM Co\(^{1+} \) min\(^{-1} \) mg\(^{-1} \), respectively).

We also measured an ~5 min lag in the reduction of cob(I)alamin (Co\(^{1+} \) assay), but not in the adenosylation reaction (Co\(^{4+} \) assay); the observed lag did not depend on the concentration of the reagents or substrates (data not shown). Overlays of spectral scans (from 300 to 700 nm) obtained at different times showed a lag in the generation of product (i.e., AdoCbl; 525 nm), but not in the reduction of the substrate (Figure 3). Since the Co\(^{2+} \) assay is essentially a coupled assay (i.e., reduction followed by adenosylation), the observed lag is consistent with cob(I)alamin adenosylation being the rate-limiting step in the reaction catalyzed by \( LrPduO^{F112H} \). Reaction lags are typically observed in coupled assays where the second step in the reaction is rate-limiting (38).

**The Severity of the Effect of Different \( \alpha \) Ligands on \( LrPduO \) Activity Varies Substantially.** Even though \( LrPduO^{F112A} \) and \( LrPduO^{F112H} \) proteins bound cob(I)alamin as a five-coordinate species (Figures 1B and 2), their enzymatic activity was vastly different. The critical distinction between these two variants lies in the nature of the \( \alpha \) ligand. In \( LrPduO^{F112H} \), the \( \alpha \) ligand is a histidyl side chain of the enzyme, whereas in \( LrPduO^{F112A} \), the \( \alpha \) ligand is DMB. The explanation for the kinetic differences is not immediately obvious since the Co—N coordination bond in both variants is very similar in the crystal structures. The apparent similarity, however, may be imposed by the packing in the crystals. Indeed, recent spectroscopic analysis of these variants revealed the coordination bond between Co and N(His\(^{112} \)) to be longer than the bond between Co and N(DMB) (K. Park et al., manuscript in preparation). A longer Co—N(His\(^{112} \)) coordination bond in \( LrPduO^{F112H} \) would result in a faster displacement of the lower ligand, and therefore a higher \( k_{cat} \) in the Co\(^{2+} \) assay.

To explain the data shown in Figure 3, we propose that the reduced \( k_{cat} \) of \( LrPduO^{F112H} \) may be the result of a slower nucleophilic attack by the Co ion of cob(I)alamin on the 5‘-carbon of ATP, and/or a slow product release. Considering that the Co—C bond in AdoCbl is formed with cob(I)alamin (a four-coordinate Cbl species), wild-type \( LrPduO \) may first generate an AdoCbl/DMB-off product, a cob(II)alamin species that is energetically unstable in the absence of an \( \alpha \) axial ligand. Consequently, a DMB-on conformation would be favored prior to, or concomitant with, product release. In \( LrPduO^{F112H} \), the coordination bond with N(His) may sta-

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**Table 3: Kinetic Parameters of Wild-Type \( LrPduO \) and Variants Using the Co\(^{2+} \) Assay**

<table>
<thead>
<tr>
<th>enzyme</th>
<th>( K_m ) (( \mu )M)</th>
<th>( k_{cat} ) (s(^{-1} ))</th>
<th>( k_{cat}/K_m ) (M(^{-1} ) s(^{-1} ))</th>
<th>( K_m ) (( \mu )M)</th>
<th>( k_{cat} ) (s(^{-1} ))</th>
<th>( k_{cat}/K_m ) (M(^{-1} ) s(^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>5.5 ± 1.1</td>
<td>(2.9 ± 0.1) × 10(^{-2} )</td>
<td>(5.5 ± 1.1) × 10(^{1} )</td>
<td>7.8 ± 1.1</td>
<td>(3.8 ± 0.5) × 10(^{-2} )</td>
<td>(4.8 ± 0.9) × 10(^{1} )</td>
</tr>
<tr>
<td>F112A</td>
<td>UD(^{a} )</td>
<td></td>
<td></td>
<td>UD(^{a} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F112H</td>
<td>10.4 ± 0.8</td>
<td>(5.9 ± 0.2) × 10(^{-4} )</td>
<td>(5.7 ± 0.5) × 10</td>
<td>53.0 ± 8.6</td>
<td>(6.7 ± 0.2) × 10(^{-4} )</td>
<td>(1.3 ± 0.2) × 10(^{1} )</td>
</tr>
<tr>
<td>F112Y</td>
<td>9.4 ± 0.4</td>
<td>(2.3 ± 0.1) × 10(^{-3} )</td>
<td>(2.5 ± 0.2) × 10(^{2} )</td>
<td>34.2 ± 10.7</td>
<td>(2.7 ± 0.4) × 10(^{-3} )</td>
<td>(8.3 ± 2.8) × 10(^{1} )</td>
</tr>
<tr>
<td>F112W</td>
<td>7.3 ± 0.1</td>
<td>(2.8 ± 0.1) × 10(^{-3} )</td>
<td>(3.9 ± 0.2) × 10(^{-2} )</td>
<td>28.8 ± 5.7</td>
<td>(3.2 ± 0.1) × 10(^{-3} )</td>
<td>(1.1 ± 0.2) × 10(^{2} )</td>
</tr>
<tr>
<td>F163A</td>
<td>96.1 ± 7.1(^{b} )</td>
<td>(1.5 ± 0.1) × 10(^{-20} )</td>
<td>(1.6 ± 0.2) × 10(^{-20} )</td>
<td>134 ± 13</td>
<td>(2.7 ± 0.4) × 10(^{-2} )</td>
<td>(2.0 ± 0.3) × 10(^{2} )</td>
</tr>
<tr>
<td>F187A</td>
<td>7.9 ± 1.0</td>
<td>(1.4 ± 0.1) × 10(^{-2} )</td>
<td>(1.8 ± 0.3) × 10(^{3} )</td>
<td>15.8 ± 3</td>
<td>(1.5 ± 0.1) × 10(^{-2} )</td>
<td>(9.3 ± 1.9) × 10(^{2} )</td>
</tr>
<tr>
<td>AS183</td>
<td>9.9 ± 0.9</td>
<td>(1.7 ± 0.1) × 10(^{-2} )</td>
<td>(1.7 ± 0.2) × 10(^{3} )</td>
<td>16.3 ± 1.7</td>
<td>(1.8 ± 0.1) × 10(^{-2} )</td>
<td>(1.1 ± 0.1) × 10(^{3} )</td>
</tr>
</tbody>
</table>

\(^{a}\) The substrate cob(II)alamin was generated using a protein reducing system. The saturating level of ATP was at least 100-fold greater than the \( K_m \); however, the saturating level of cob(II)alamin was limited to being 3–10-fold greater than the \( K_m \) as a result of assay sensitivity. \(^{b}\) Unable to determine constants due to extremely low activity. \(^{c}\) Kinetic parameters obtained at subsaturating concentrations of cob(II)alamin.
that bind Cbl in its base-off/His-on form would not have functional groups that can interact with the cobalt ion of Cbl. (Table 3). The ialamin, albeit not as efficiently as the wild-type protein Lr bilize Co\(^{3+}\) of AdoCbl by \(LrPduO^{F112H}\). Arrows and increasing darkness in the spectra represent successive time points after reaction was initiated by the addition of ATP (light gray, 1 min; black, 11 min). \(LrPduO^{F112H}\) facilitates the reduction of cob(I- I)alamin (as evidenced by the decrease in absorbance at 473 nm); however, there is a lag in the generation of AdoCbl (as evidenced in initial lag in absorbance at 525 nm).

bilize Co\(^{3+}\) of AdoCbl, resulting in an AdoCbl/His-on species. For product to be released, AdoCbl/His-on must first undergo the thermodynamically unfavorable step that generates AdoCbl/DMB-off. This scenario would be consistent with a slow product release step and would explain the equivalent drop in the \(k_{cat}\) of \(LrPduO^{F112H}\) with cob(I)alamin and cob(II)alamin relative to that of the wild-type enzyme. Similarly, other Phe112 variants (i.e., \(LrPduO^{F112Y}\) and \(LrPduO^{F112W}\)) retained their ability to adenosylate cob(I-)alamin, albeit not as efficiently as the wild-type protein (Table 3). The \(k_{cat}\) values of variants \(LrPduO^{F112W}\) and \(LrPduO^{F112Y}\) decreased ~10-fold in both assays, suggesting a problem with product release like the one observed with \(LrPduO^{F112H}\). This is possible since Trp112 and Tyr112 have interviews with aromatic residues around the corrin ring. In hemproteins, interactions between heme and aromatic side chains have been proposed to stabilize the holoprotein fold and to contribute to the high affinity of the protein for heme (39). To determine whether additional aromatic residues play a role in PduO-mediated catalysis, two phenylalanine residues, Phe163 and Phe187, located inside the hydrophobic pocket were changed by site-directed mutagenesis.

(i) Phe163. Residue Phe163 is conserved in all PduO-type enzymes, except in \(S.\) enterica PduO, where tyrosine occupies this position. The structurally equivalent residue in the human ACA enzyme was suggested to be involved in Cbl binding (34, 35). Notably, the \(K_m\) of \(LrPduO^{F163A}\) for cob(II)alamin increased significantly (17-fold), but the \(k_{cat}\) remained unchanged (Table 3). In contrast, the \(K_m\) and the \(k_{cat}\) of \(LrPduO^{F163A}\) for cob(I)alamin were very similar to those of the wild-type enzyme (Table 2). Together, these results suggest that Phe163 and DMB interact when Cbl binds to the active site of \(LrPduO\). The negative effect of the substitution is not observed with cob(I)alamin because this form of Cbl does not have the axial ligands.

(ii) Phe187. Although residue Phe187 is not conserved, it is part of the C-terminal loop (Ser183–Arg188) that becomes ordered upon binding of the corrinoid substrate (21). The kinetic parameters of \(LrPduO^{F187A}\) for cob(I)alamin and cob(II)alamin were not significantly different from the parameters of the wild-type enzyme (Tables 2 and 3).
To analyze the contribution of the C-terminal loop to the function of LrPduO, we deleted the last six amino acid residues of the protein (i.e., Ser183–Arg188). The crystal structure of the LrPduO \textsuperscript{A183–188} protein was determined to 1.4 Å resolution (Figure 4). The absence of the C-terminal residues resulted in greater conformational freedom for residue Phe112 in the active site, with the electron density suggesting two alternative conformations for the side chain of Phe12. The crystal structure of the LrPduO \textsuperscript{A183–188} protein also showed that a four-coordinate cob(II)alamin intermediate could still form in the active site of the enzyme (Figure 4). Consistent with this finding, the catalytic efficiency of the LrPduO \textsuperscript{A183–188} protein was only modestly reduced (3–4-fold) (Tables 2 and 3), suggesting that the C-terminus of the proteins can be modified or truncated without there being a profound impact on the catalytic efficiency of the enzyme.

In Vivo Functionality of LrPduO Variants. None of the variants, even those with measurable activity, supported AdoCbl biosynthesis in vivo (data not shown). The fact that the LrPduO \textsuperscript{A183–188} protein did not support in vivo biosynthesis of AdoCbl, in spite of its modest decrease in activity, suggests that this part of the protein may be important for protein–protein interactions. ATP corrinoid adenosyltransferases are thought to transport this valuable but limited coenzyme to the corresponding AdoCbl-dependent enzyme (40). In the human adenosyltransferase, a truncation of the final 16 residues has been shown to result in an early onset of methylmalonic aciduria (41).

CONCLUSIONS
Here we report structural and kinetic data to support the idea that the inability to generate the four-coordinate cob(I)alamin intermediate renders a human-type ACA enzyme inactive. The side chain of residue Phe112 in the active site of the enzyme is critical to the function of the enzyme for three reasons. (i) It is in the proximity of the Co ion of the ring. (ii) It is bulky. (iii) It cannot form a coordination bond with the Co ion. Hence, when cob(II)alamin binds to the enzyme, the lower ligand is displaced and the resulting four-coordinate cob(II)alamin species remains as such because the phenyl side chain cannot form a coordination bond with the Co ion.

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