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Dihydroflavin-driven Adenosylation of 4-Coordinate Co(II) Corrinoids

*ARE COBALAMIN REDUCTASES ENZYMES OR ELECTRON TRANSFER PROTEINS?**

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The identity of the source of the biological reductant needed to convert cobalamin to its biologically active form adenosylcobalamin has remained elusive. Here we show that free or protein-bound dihydroflavins can serve as the reductant of Co^{2+}Cbl bound in the active site of PduO-type ATP-dependent corrinoid adenosyltransferase enzymes. Free dihydroflavins (dihydroriboflavin, $FMNH_2$, and $FADH_2$) effectively drove the adenosylation of Co^{2+}Cbl by the human and bacterial PduO-type enzymes at very low concentrations (1 μM). These data show that adenosyltransferase enzymes lower the thermodynamic barrier of the $Co^{2+} \rightarrow Co^{+}$ reduction needed for the formation of the unique organometalic Co–C bond of adenosylcobalamin. Collectively, our *in vivo* and *in vitro* data suggest that cobalamin reductases identified thus far are most likely electron transfer proteins, not enzymes.

Cobalamin (Cbl)[3] is an essential nutrient for animals, lower eukaryotes, and prokaryotes (1). Only some archaea and bacteria synthesize AdoCbl *de novo*, a process that involves at least 25 proteins (1). Enzymes that use AdoCbl (AdoCbl, coenzyme B_{12}) catalyze intramolecular rearrangements (2, 3), deaminations (4), dehydrations (5), reductions (6, 7), or reductive dehalogenations (8). In humans, AdoCbl is required for the metabolism of branched chain amino acids, short chain fatty acids, and cholesterol (9).

The cobalt-carbon bond of AdoCbl lies at the heart of the reactivity of this coenzyme. This is a unique organometallic bond that serves as a radical initiator (10). ATP:cob(I)alamin adenosyltransferase (ACA) enzymes catalyze the transfer of the adenosyl moiety from ATP to cob(I)alamin, resulting in the formation of this unique cobalt-carbon bond. Three types of ACA enzymes have been described thus far, CobA, PduO, and EutT (11–13). Although ACA enzymes carry out the same reaction, they do not share sequence homology at the nucleotide or amino acid level.

For adenosylation to occur, the cobalt ion of corrinoids must be reduced in two consecutive one-electron reductions from $Co^{3+} \rightarrow Co^{2+} \rightarrow Co^{+}$ (14). We previously reported that the reducing environment of the cell drives the $Co^{3+} \rightarrow Co^{2+}$ reduction (14, 15). The $Co^{2+} \rightarrow Co^{+}$ reduction, however, is more challenging because the reduction potential of $Co^{2+/+}$ couple in solution is −610 mV (16), which is lower than any known physiological reductant in the cell.

To solve this problem, the cell uses ACA enzymes to facilitate the reduction of cob(II)alamin before adenosylation can occur (Fig. 1). In this process, ACA enzymes bind 5-coordinate cob(II)alamin and displace the lower ligand 5,6-dimethylbenzimidazole, resulting in a 4-coordinate cob(II)alamin intermediate that lacks axial ligands (17–22). In this 4-coordinate cob(II)alamin intermediate, the $3d_z^2$ orbital of the cobalt ion is stabilized, raising the reduction potential ~250 mV and bringing the reduction potential of $Co^{2+/+}$Cbl to within physiological range (20). Recent structural and kinetic analyses revealed that a conserved phenylalanine in the active site of the PduO-type ACA enzyme is critical to the formation of the 4-coordinate cob(II)alamin intermediate (17, 21).

Even though we now have a better understanding of the mechanism of this unfavorable reduction, we still do not know the identity of the physiological reductant for the $Co^{2+} \rightarrow Co^{+}$ reduction, and how the electron is delivered. This has been a point of interest for several decades. Because the reduction potential of the $Co^{2+} \rightarrow Co^{+}$ reduction in solution is outside the physiological range, all scenarios investigated thus far invoke the participation of a reductase and/or an electron transfer protein.

In the case of the CobA ACA enzyme of *Salmonella enterica* (*Se*CobA), *in vitro* evidence for the involvement of a reduction system was reported (23). In this case, flavodoxin A (FldA) is reduced by the NADPH-dependent ferredoxin (flavodoxin) protein reductase (Fpr), and reduced FldA transfers one electron to cob(II)alamin present in the active site of *Se*CobA yielding cob(I)alamin. The latter is the nucleophile that attacks the 5′C of ATP yielding AdoCbl and triphosphate (23). The putative role of the Fpr/FldA system in cob(II)alamin reduction was supported by the results from studies of FldA/CobA interactions (24).

There are several reports of putative cobalamin reductases in the literature. For example, in *Brucella melitensis*, the flavoprotein CobR was reported to reduce the cobalt ion from $Co^{3+} \rightarrow$

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[3] The abbreviations used are: Cbl, cobalamin; AdoCbl, adenosylcobalamin; cob(III)alamin, Co^{3+}Cbl; cob(II)alamin, Co^{2+}Cbl; cob(I)alamin, Co^{+}Cbl; hATR, human adenosyltransferase; ACA, ATP:corrinoid adenosyltransferase; rTEV, recombinant tobacco etch virus; Fpr, ferredoxin (flavodoxin) protein reductase; FldA, flavodoxin A; $FMNH_2$, dihydroflavin mononucleotide; $FADH_2$, dihydroflavin adenine dinucleotide; Cbi, cobinamide.

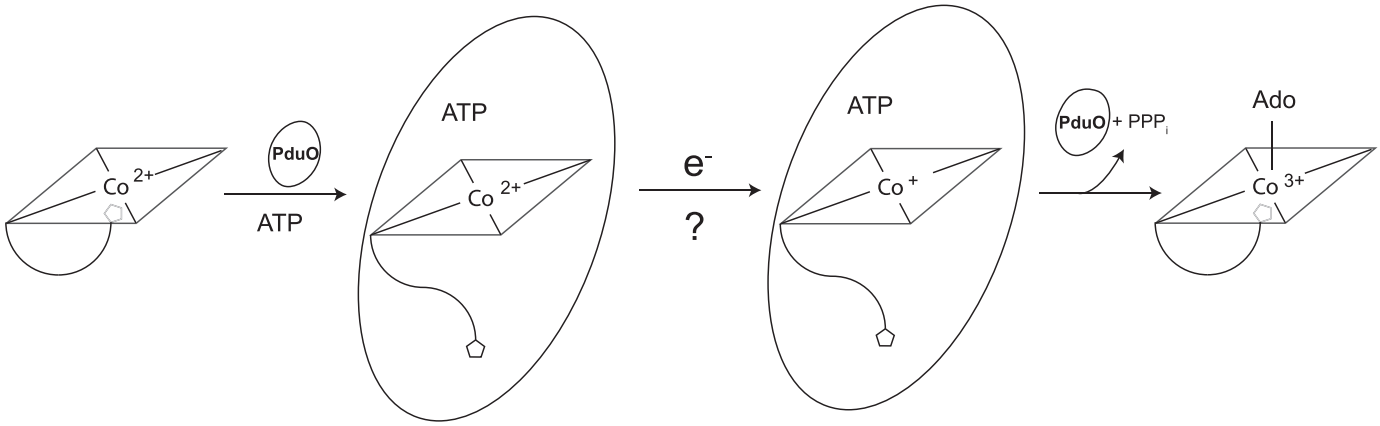

FIGURE 1. **Proposed mechanism for the reduction and adenosylation of cobalamin.** PduO-type ACA enzymes (*oval*) bind 5-coordinate cob(II)alamin and facilitate its reduction to cob(I)alamin by displacing the lower ligand. The latter event generates a 4-coordinate cob(II)alamin intermediate whose redox potential is raised enough so it can accept one electron from dihydroflavin. The resulting cob(I)alamin nucleophile attacks ATP to generate coenzyme B_{12} (*AdoCbl*) and triphosphate. In these studies, we investigated the source of the electron for the reduction of cob(II)alamin to cob(I)alamin.

$Co^{2+} \rightarrow Co^{+}$ in several corrinoids, including intermediates of the corrin ring biosynthetic pathway and cobalamin (25).

In the case of the PduO-type human adenosyltransferase (hATR), the source of the electron involved in the reduction of cob(II)alamin is unknown, and initial searches for the source of this electron putatively identified methionine synthase reductase (26) as a candidate reductant driving the reaction. This hypothesis was based on results of *in vitro* studies, which showed that, in the presence of hATR, methionine synthase reductase reduced cob(II)alamin to cob(I)alamin (27). An important caveat to the idea that methionine synthase reductase is the physiological reductant of hATR is that methionine synthase reductase is found in the cytosol, where methionine is synthesized (28), and not in the mitochondrion where AdoCbl synthesis occurs, leaving unanswered the questions about the origin of the electron involved in the hATR reaction.

The reductant needed for the synthesis of AdoCbl in bacteria that use PduO-type ACA enzymes is also unknown. Sampson *et al.* (29) reported results of *in vitro* experiments, which suggested that, in *S. enterica*, the PduS protein catalyzes two consecutive one-electron reductions needed to convert cob(III)alamin to cob(I)alamin. To our knowledge, there is no *in vivo* evidence to support any of the abovementioned results.

In this study, we report the dihydroflavin-dependent reduction of cob(II)alamin to cob(I)alamin, but only when cob(II)alamin is bound to the active site of PduO-type ACA enzymes. We show that flavoproteins, whose functions are unrelated to AdoCbl biosynthesis, can drive PduO-bound cob(II)alamin reduction.

EXPERIMENTAL PROCEDURES

Cloning of Genes into Overexpression Vectors—The *MMAB* human gene (encodes the PduO-type corrinoid adenosyltransferase; hATR) was synthesized by GenScript with *Escherichia coli* codon usage and NheI and NotI restriction sites at the 5′ and 3′ ends, respectively. To generate a recombinant protein of hATR with an rTEV protease-cleavable N-terminal hexahistidine (His_6) tag, the GenScript construct was cut with NheI and NotI enzymes, and the fragment was ligated into vector pTEV3 (30) cut with the same enzymes; the resulting plasmid was named PDU46.

The *S. enterica pduS* gene was PCR-amplified using strain TR6583 genomic DNA as template and primers that included NdeI and BamHI restriction sites at the 5′ and 3′ ends, respectively. The amplified fragment was ligated into plasmid pET15b (EMB Biosciences) yielding plasmid pPDU47, which directed the synthesis of His_6 N-terminally tagged PduS (H_6-PduS). DNA sequences were confirmed at the DNA sequencing facility of the University of Wisconsin, Madison.

Production and Purification of Proteins—hATR protein was produced by transforming the plasmid PDU46 into *E. coli* strain BL21(DE3) for overexpression. Strains were grown at 37 °C with shaking in 1.5 liter of lysogenic broth (LB) medium (31, 32) supplemented with ampicillin (100 μg/ml). After the culture reached an absorbance at 650 nm (A_{650}) of 0.6–0.7, synthesis of phage T7 RNA polymerase enzyme was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.5 mM. Cells were grown for an additional 5 h at 37 °C with shaking. Cells were harvested by centrifugation at 12,000 × *g* with a Beckman/Coulter Avanti J-25I centrifuge equipped with a JLA-16.250 rotor. The cell pellet was frozen at −80 °C until used. His_6-hATR protein was purified from cell pellets that were thawed and resuspended in 30 ml of Tris-HCl buffer (0.1 M, pH 8.0 at 4 °C) containing the protease inhibitor phenylmethanesulfonyl fluoride (0.8 mM), imidazole (20 mM), and NaCl (0.5 M). Cells were broken using a French pressure cell (1.03×10^7 kilopascals). Cell-free extracts were cleared by centrifugation at 4 °C for 1 h at 45,000 × *g*. The resulting supernatant was filtered (0.45 mm; Nalgene), and proteins were resolved on a 5-ml HisTrap FF column (Amersham Biosciences). Proteins were desorbed from the column with a linear gradient of Tris-HCl buffer (0.1 M, pH 8.0 at 4 °C) containing imidazole (0.5 M) and NaCl (0.5 M). Fractions containing hATR protein were pooled and incubated for 2 h at room temperature with rTEV protease (33–35) in a 2:50 rTEV:hATR molar ratio. The hATR, rTEV protein mixture was sequentially dialyzed at 4 °C against buffer A (0.1 M Tris-HCl, pH 8 at 4 °C) containing 0.5 M NaCl, 2 mM EDTA, buffer B (buffer A lacking EDTA), and buffer C (0.1 M Tris-HCl, pH 8 at 4 °C) containing 10 mM imidazole and 0.5 M NaCl. The rTEV, hATR protein mixture was loaded onto a 5-ml HisTrap FF column. The flow-through was

collected and dialyzed against Tris-HCl buffer (0.1 M, pH 8.0 at 4 °C) containing 0.5 M NaCl and 10% glycerol (v/v). Tag-less hATR used for kinetic analysis was stored at −80 °C until used.

The plasmid encoding the *Se*H_6-PduS protein was transformed into the *E. coli* strain BL21 (DE3) and was used for overexpression. Overproduction strains were grown at 28 °C with shaking in super broth medium supplemented with ampicillin (100 μg/ml). When the culture reached an $A_{650} = 0.6$, isopropyl β-D-thiogalactopyranoside (0.5 mM) was added, and the culture was shifted to 15 °C and grown overnight at that temperature. Cells were harvested by centrifugation and were broken by French pressing (as above), and the *Se*H_6-PduS protein was isolated as described for hATR.

The *Lr*PduO wild-type protein and the variant *Lr*PduO^{F112A} contained an rTEV protease-cleavable N-terminal His_6 tag. Tagged *Se*H_6-PduS protein was isolated using Ni^{2+} affinity chromatography as described elsewhere (36). The tag was from homogeneous *Se*H_6-PduS with rTEV protease prepared as described (37). FMN reductase, FldA, ferredoxin (flavodoxin): $NAD(P)^+$ reductase (Fpr), and *Se*CobA were produced and purified as described, without modifications (23, 38).

In Vitro Activity Assays—Adenosylation activity assays of Cbl were performed using the continuous spectrophotometric methods described elsewhere (21, 36). Cob(III)alamin was chemically reduced to cob(I)alamin with Ti(III)citrate (36). In assays that employed cob(II)alamin, a reduced electron transfer protein (FldA, Fpr, PduS, or YdjA) or free dihydroflavins were used to reduce cob(II)alamin to cob(I)alamin. Free dihydroflavins were generated inside an anoxic chamber by incubating FAD/FMN with NADH (1 mM) for 3 h. The flavin cofactor of Fpr was reduced to dihydroflavin by incubating the protein with NADH (1 mM). This sample of reduced Fpr was used to reduce the cofactor of FldA. The concentration of the electron transfer proteins was either one-to-one molar ratio or ≥10-fold in excess to the concentration of the adenosyltransferase.

Cob(II)alamin reduction was assessed using a continuous spectrophotometric assay that monitored changes in the absorbance at 374 nm (36). The reaction mixture contained FMN/FAD (0.1–10 μM), NADH (1 mM), ATP (1 mM), PduO-type enzyme (7–10 μg/ml), FMN reductase (10 μg/ml), KCl_2 (0.1 M), $MgCl_2$ (1.5 mM), Tris-Cl buffer (0.2 M, pH 8 at 37 °C). Because the reduction of cob(II)alamin was coupled to the adenosylation of cob(I)alamin, the reaction was started by the addition of the PduO-type adenosyltransferase. FMN reductase was used to accelerate the reduction of flavins and was not required for the reduction of cob(II)alamin when $FMNH_2$ or $FADH_2$ was added to reaction.

Adenosylation activity assays of Cbi were performed using an end point assay inside an anoxic chamber. The reactions were incubated in the dark at room temperature for 4 h. The reaction mixture contained the same reagents as in the reduction of cob(II)alamin assay (see above) except Co(II) cobinamide was the substrate. The presence of AdoCbi was assessed spectophotometrically and by bioassay using an AdoCbl/AdoCbi auxotrophic strain of *S. enterica* (JE7180). A 2.5-μl sample of the reaction products was spotted on non-carbon essential minimal medium (39) seeded with strain JE7180. The non-carbon essential medium was supplemented with 90 mM ethanolamine, 150 μM 5,6-dimethylbenzimidazole, 0.5 mM methionine, 0.5 mM glycerol, 1 mM $MgSO_4$, 30 mM NH_4Cl, and trace minerals.

RESULTS AND DISCUSSION

Dihydroflavins Drive the Conversion of Cob(II)alamin to AdoCbl by PduO-type ACA Enzymes—The search for the physiological reductant needed for the conversion of vitamin B_{12} into coenzyme B_{12} has been going on for decades, with claims of the existence of different reducing systems being reported (14, 40). In the last few years, however, ACA enzymes have been shown to critically contribute to this process by increasing the reduction potential of cob(II)alamin to within physiological range (18–20, 22). It remains unclear, however, whether the Co(II) → Co(I) reduction is driven by an enzyme or by an electron transfer protein.

We found that the *Lactobacillus reuteri* PduO (*Lr*PduO) enzyme can adenosylate cob(II)alamin in the presence of free dihydroflavins ($FMNH_2$ or $FADH_2$). This was surprising, because the idea that a reductase enzyme is required for the reduction of cob(II)alamin has been generally accepted. To determine whether free dihydroflavin-driven cob(II)alamin reduction worked with other PduO-type ACA enzymes, we substituted the human PduO-type adenosyltransferase for the bacterial enzyme in the reaction mixture. Our results showed that indeed dihydroflavins quantitatively drove the adenosylation of cob(II)alamin by the human adenosyltransferase enzyme (Fig. 2*A*).

The formation of AdoCbl was confirmed based on two parameters. First, spectral changes of the corrinoid in the reaction mixture (*i.e.* an increase in $A_{525\,nm}$ (AdoCbl), with a concomitant decrease at $A_{473\,nm}$), revealed the conversion of cob(II)alamin to AdoCbl. Second, exposure of the latter to light under anoxic conditions resulted in homolytic cleavage of the Co–C bond yielding cob(II)alamin (Fig. 2*B*). Together, these data supported the conclusion that AdoCbl was present in the mixture. AdoCbl was not detected in control experiments performed in the absence of dihydroflavins or the PduO-type enzymes.

Further evidence that AdoCbl was present in the reaction mixture was obtained by bioassay. For this purpose, we used an AdoCbl auxotrophic strain of *S. enterica* (JE7180, *cobA366*::Tn*10*d(*cat*$^+$) *eut1141*(Δ*eutT*)), which cannot grow on ethanolamine, unless AdoCbl is provided in the medium (41). Reaction products were spotted on a no-carbon essential/ethanolamine medium agar plate seeded with strain JE7180. Cell growth was assessed after overnight incubation in the dark at 37 °C. Only reaction mixtures that included PduO-type enzymes, ATP, and cob(II)alamin supported growth of JE7180; control reactions lacking ATP or PudO-type enzymes did not (Fig. 2*C*).

Formation of the 4-Coordinate Cob(II)alamin Intermediate Is Required for Dihydroflavin-driven AdoCbl Synthesis—PduO-type ACA enzymes facilitate the unfavorable reduction of cob(II)alamin by generating a 4-coordinate intermediate in the active site of the enzyme (17, 19, 21, 22). The conserved residue Phe-112, located in the active site of PduO, is critical for the displacement of the lower ligand base of cob(II)alamin (17, 21).

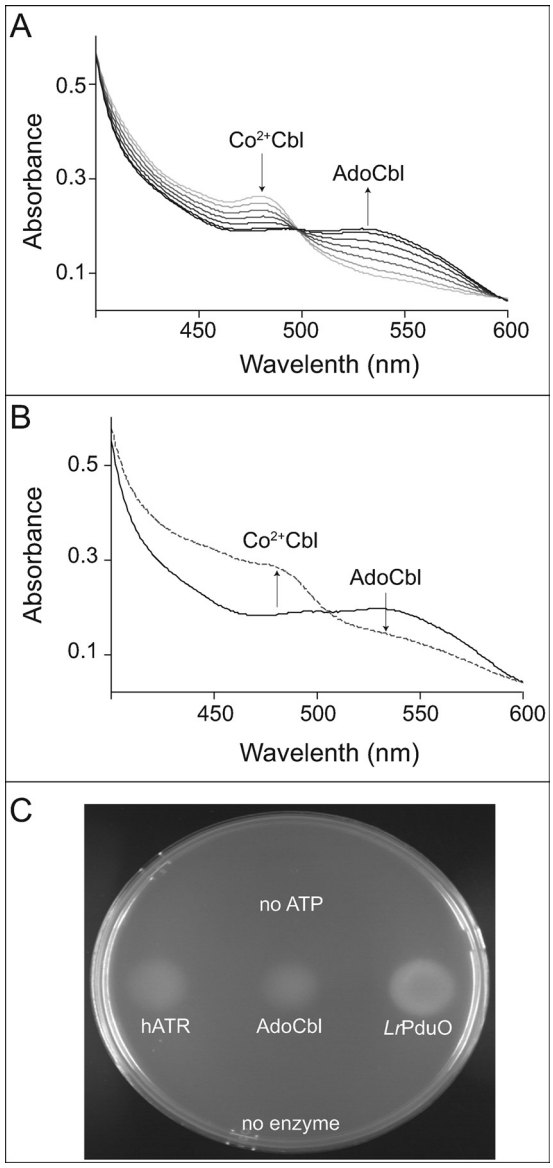

FIGURE 2. **Free dihydroflavins drive the conversion of 4-coordinate cob(II)alamin bound to PduO-type ACA enzymes to AdoCbl.** *A,* spectral changes associated with the enzymatic conversion of cob(II)alamin by hATR. *Arrows* and *increasing darkness* in the spectra represent successive time points after the reaction was initiated by addition of hATR (*light gray*, 1 min; *black*, 10 min). *B,* spectral changes associated with photolysis of AdoCbl. The presence of AdoCbl was confirmed by exposing the product of reaction mixture from *A* (*black*) under a tungsten lamp for 10 min in an anoxic cuvette (*dotted line*). *C,* presence of AdoCbl in reaction mixture *A* was confirmed by bioassay using an AdoCbl auxotroph strain of *S. enterica* (JE7180).

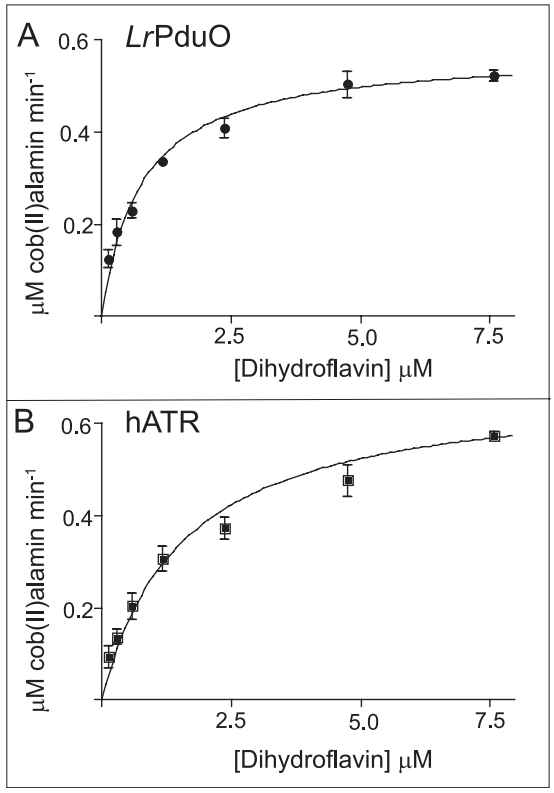

FIGURE 3. **Rate of cob(II)alamin reduction driven by $FMNH_2$.** Kinetics of the initial rate of reduction of cob(II)alamin using free dihydroflavins as the source of the electron in a coupled assay with *Lr*PduO (*A*) and hATR (*B*). The concentration of $FMNH_2$ in the reaction mixture needed to drive the reaction at half the maximal velocity was 0.8 ± 0.1 μM for *Lr*PduO and 1.6 ± 0.2 μM for hATR.

The *Lr*PduO^{F112A} variant enzyme cannot displace the lower ligand and is therefore inactive (36).

Control experiments showed that free dihydroflavins did not reduce cob(II)alamin in the absence of the bacterial or human PduO-type ACA enzyme, nor did they drive the reduction of cob(II)alamin bound to the active site of the *Lr*PduO^{F112A} variant (data not shown). The above results led us to conclude that dihydroflavins can reduce 4-coordinate but not 5-coordinate cob(II)alamin.

Reduction of Enzyme-bound 4-Coordinate Cob(II)alamin Occurs at Low Concentrations of Dihydroflavins—To address how much dihydroflavin was required to reduce 4-coordinate cob(II)alamin, we analyzed the rate of reduction by varying the concentration of dihydroflavin in the presence of *Lr*PduO or hATR (Fig. 3, *A* and *B*, respectively). Remarkably, the dihydroflavin concentrations resulting in half the maximal velocity of the reaction catalyzed by *Lr*PduO or hATR were 0.8 ± .1 and 1.6 ± 0.2 μM, respectively. The velocity of cob(II)alamin reduction was the same when dihydroriboflavin, reduced flavin mononucleotide ($FMNH_2$), or reduced flavin adenine dinucleotide ($FADH_2$) was used as the reducing agent of cob(II)alamin. Dihydroflavins reduced cob(II)alamin twice as fast in the presence of hATR compared with *Lr*PduO (k_{cat}, 4.1×10^{-2} s^{-1} *versus* 2.2×10^{-2} s^{-1}). Together, these results strongly suggest that once the PduO-type adenosyltransferase binds cob(II)alamin and the 4-coordinate intermediate is formed, the reduction potential of the cobalt ion is raised to levels where the Co(II) → Co(I) reduction can occur without the participation of an accessory protein.

Rate Comparisons between Dihydroflavin- and Reduced Flavoprotein-driven AdoCbl Synthesis—Although it was clear that free dihydroflavins (riboside, mononucleotide, and dinucleotide forms) drove the conversion of 4-coordinate cob(II)alamin to AdoCbl, it was unclear whether free dihydroflavins were the physiological reductant needed for this reaction. An alternative source of reducing power would be flavoproteins. To analyze how well dihydroflavins reduced 4-coordinate cob(II)alamin, we compared the rate of adenosylation by bacterial and human PduO-type ACA enzymes when

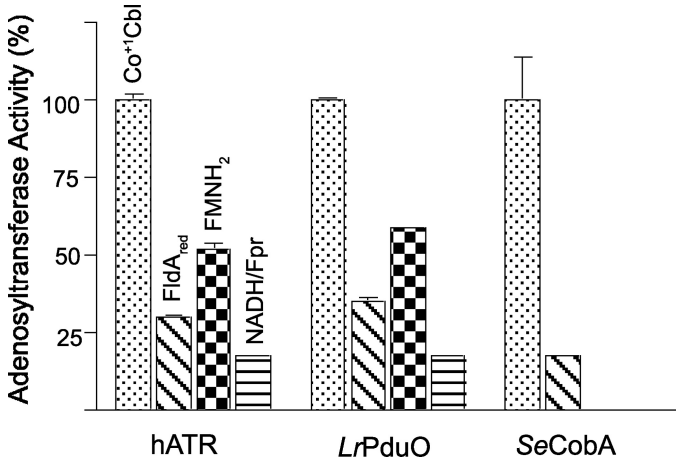

FIGURE 4. **Rate of adenosylation by dihydroflavins.** Cob(I)alamin was generated using Ti(III)citrate as reducing agent. The rate of adenosylation by the ACA enzyme with cob(I)alamin (*dotted*) was considered to be 100%. Adenosylation by PduO-type ACA enzymes using free dihydroflavins (*black* and *white squares*) was at least half the rate compared with the adenosylation rate measured with cob(I)alamin. Reduced FldA ($FldA_{red}$, *diagonal lines*) and Fpr (Fpr_{red}, *horizontal lines*) also drove the reduction of cob(II)alamin, although not as fast as free $FMNH_2$ or $FADH_2$. *Se*CobA protein was used as control to confirm that CobA-type ACA enzymes can only use $FldA_{red}$ as reductant but not Fpr. The concentration of flavoproteins was at least 10-fold in excess compared with concentrations of ACA enzymes.

free dihydroflavins or reduced flavoproteins were used as reductant.

To obtain a point of reference, we determined the catalytic competence of the PduO-type ACA enzyme tested. To do this, we used Ti(III)citrate to reduce cob(II)alamin to cob(I)alamin. The activity obtained with chemically reduced cob(II)alamin indicated the limit of the rate of formation of AdoCbl occurred once the Co(I) nucleophile was formed; this rate was set as 100% (Fig. 4).

Surprisingly, the rate of AdoCbl production with chemically reduced cob(I)alamin was only 2-fold faster than the rate obtained when free $FADH_2$ or $FMNH_2$ was used to reduce 4-coordinate cob(II)alamin bound to hATR or *Lr*PduO (Fig. 4). When an excess of Fpr or FldA was used in lieu of $FMNH_2$ or $FADH_2$, the rates of AdoCbl formation were half as fast as those observed with free dihydroflavins (Fig. 4). Most likely, the slower rate of AdoCbl formation when flavoproteins were used as the reductant was due to the less frequent collision rate between the dihydroflavin in the reductase and the 4-coordinate cob(II)alamin in the active site of PduO-type ACA enzymes.

What Is the Physiological Reductant for the Reduction of 4-Coordinate Cob(II)alamin in the Active Site of PduO-type ACA Enzymes?—Based on the above data, and the fact that the concentration of free dihydroflavins inside the cell is not known, we hypothesized that reduced flavoproteins whose flavin cofactor was close to the surface of the protein would reduce the 4-coordinate cob(II)alamin in the active site of PduO-type ACA enzymes.

To test this hypothesis, we analyzed three unrelated flavoproteins. We note that in *S. enterica* none of the chosen flavoproteins interact with the PduO enzyme, because the latter localizes to the lumen of the compartment known as the PduO metabolosome (42). Hence, adenosylation of cob(II)alamin resulting from the use of reduced flavoproteins could not be explained by expected interactions between PduO and the flavoprotein. The first two proteins we used were flavodoxin

TABLE 1

Specific activities of hATR and *Lr*PduO using different electron sources for the adenosylation of cob(II)alamin

The concentrations of Fpr and PduS were at a one-to-one molar ratio to the concentrations of PduO-type ACA enzymes.

Reductant	Specific activity	
	hATR	*Lr*PduO
	nmol min^{-1} μg^{-1} *of enzyme*	
Ti(III)citrate	$(4.9 \pm 0.2) \times 10^{-1}$	$(2.5 \pm 0.1) \times 10^{-1}$
Dihydroflavins	$(2.5 \pm 0.1) \times 10^{-1}$	$(1.4 \pm 0.1) \times 10^{-1}$
Fpr	$(7.2 \pm 0.9) \times 10^{-2}$	$(4.5 \pm 0.8) \times 10^{-2}$
PduS	$(2.6 \pm 0.2) \times 10^{-2}$	$(2.3 \pm 0.3) \times 10^{-2}$

(FldA, FMN) and ferredoxin (flavodoxin):NADPH reductase (Fpr, FAD), which were previously shown to function together as reductants in the adenosylation of cob(II)alamin by the *S. enterica* CobA ACA enzyme (23). Reduced flavodoxin ($FldA_{red}$) served as the electron donor for the adenosylation of cob(II)alamin by PduO- and CobA-type ACA enzymes (Fig. 4). In contrast, Fpr alone reduced cob(II)alamin bound to PduO-type ACA enzymes but did not reduce cob(II)alamin bound to *Se*CobA (Fig. 4). The rate of adenosylation, when Fpr served as the cob(II)alamin reductant (in one-to-one molar ratio with hATR), was 4-fold slower than when free dihydroflavins served as the reductant (Table 1). The rate of Fpr-dependent adenosylation of cob(II)alamin was very similar to the reported adenosylation rate using methionine synthase reductase as the reductant (0.07 *versus* 0.09 nmol min^{-1} μg^{-1} of hATR), even though the rates for methionine synthase reductase were determined using methionine synthase reductase concentrations 10-fold higher than the concentrations of hATR (27).

We tested a third flavoprotein, YdjA, whose function is unknown and unrelated to AdoCbl biosynthesis. YdjA is a putative FMN-dependent nitroreductase enzyme (43).

Even though the rate of reduction of cob(II)alamin bound to PduO-type ACA enzymes by YdjA was too slow to be accurately measured, reduced YdjA did drive the adenosylation of cob(II)alamin (data not shown). The presence of AdoCbl in reaction mixtures containing YdjA was confirmed using a bioassay and *in vitro* by homolytic cleavage of the Co–C bond (as in Fig. 2). Control reaction mixtures lacking YdjA did not yield AdoCbl (data not shown).

As with Fpr and FldA, it is unlikely that YdjA is the physiological partner for the PduO in *S. enterica*. A more likely explanation for these results is that dihydroflavin bound to YdjA nonspecifically transferred one electron to the 4-coordinate cob(II)alamin species bound to the active site of *Lr*PduO.

It appears that, for efficient transfer of the electron, the location of the dihydroflavin cofactor is important. The crystal structure of YdjA shows that FMN is close to the surface (RCSB Protein Data Bank codes 3bm1 and 3bm2) (43). Hence, it is possible that any flavoprotein with a surface-exposed cofactor can drive the adenosylation of 4-coordinate cob(II)alamin. The fact that dihydroflavins (free or bound to protein) can drive the adenosylation of cob(II)alamin would explain why human or bacterial cob(II)alamin reductases involved in the adenosylation of Cbl have not been identified.

Putative Bifunctional Cobalamin Reductase of S. enterica PduS Protein Is a Flavoprotein—Sampson *et al.* (29) proposed that, in *S. enterica*, the PduS (*Se*PduS) protein is the source of

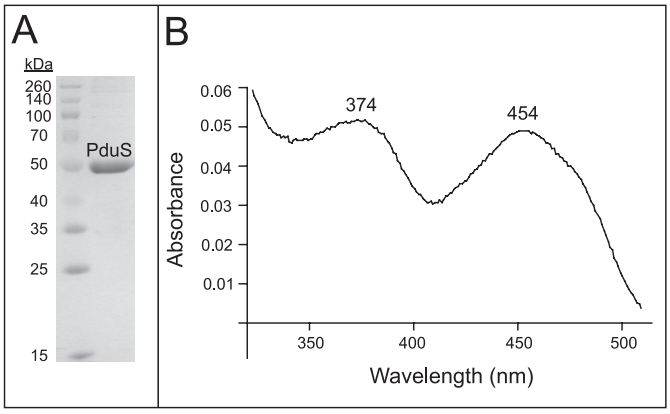

FIGURE 5. **UV-visible spectra of *Se*PduS and its flavin cofactor.** *A,* purified *Se*PduS separated by SDS-PAGE. Protein mass markers are shown on the *left*. *B,* spectral characteristics of flavin after heating *Se*PduS to isolate cofactor.

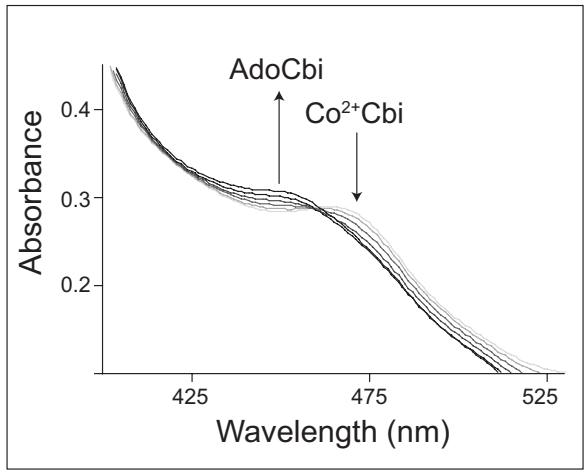

FIGURE 6. **Adenosylation of cobinamide.** Spectral changes are associated with the conversion of cob(II)inamide to AdoCbi by *Se*CobA using free dihydroflavins as reductants. *Arrows* and *increasing darkness* in the spectra represent successive time points after the reaction was initiated by addition of *Se*CobA.

electrons for the cob(III)alamin → cob(II)alamin and the cob(II)alamin → cob(I)alamin reductions. It is important to point out that these results were obtained *in vitro* using cell-free extracts and that supporting *in vivo* evidence was not reported.

To gain insights into the function of *Se*PduS, we isolated the protein under oxic and anoxic conditions (Fig. 5*A*). Notably, the fractions containing highly purified *Se*PduS were yellow, suggesting the presence of a flavin cofactor (Fig. 5*B*). Based on these results, we hypothesized that *Se*PduS reduced 4-coordinate cob(II)alamin bound to *Lr*PduO or hATR not necessarily because *Se*PduS had cob(II)alamin reductase activity but because it contained a flavin that could be reduced, and the resulting dihydroflavin could transfer one electron to 4-coordinate cob(II)alamin. *Se*PduS was a good reductant of 4-coordinate cob(II)alamin, regardless of whether it was purified in the presence or absence of oxygen. The rates of adenosylation of cob(II)alamin in the presence of *Se*PduS were within the same range as compared with Fpr or the previously reported methionine synthase reductase (Table 1).

Sampson *et al.* (29) also reported that the *Se*PduO enzyme was required to observe the proposed cob(II)alamin reductase activity of *Se*PduS. The explanation from the authors (29) for this requirement was that *Se*PduO served as the means to sequester and protect the reactive cob(I)alamin nucleophile from rapid, nonspecific quenching.

Based on our results, we suggest that the lack of the proposed cob(II)alamin reductase activity of *Se*PduS observed in the absence of *Se*PduO was due to the inability of dihydroflavins to reduce 5-coordinate cob(II)alamin species in solution, which would be the species present in a reaction mixture lacking *Se*PduO. This alternative interpretation of the data obtained by Sampson *et al.* (29) is supported by results reported here and by crystallographic data presented elsewhere (21), which indicate that the 4-coordinate cob(II)alamin is formed only upon binding to the active site of PduO-type ACA enzymes and that when 5-coordinate cob(II)alamin is bound to the active site, the enzyme is inactive.

Although we think it is plausible that *Se*PduS might be involved in the transfer of an electron to the Co(II) ion, we are unconvinced that *Se*PduS has cobalamin reductase activity. It is more likely that PduS is an electron transfer protein and not an enzyme.

Explanation of Previous Results—During the course of the structural analysis of *Lr*PduO in complex with cob(II)alamin and ATP, we observed the formation of AdoCbl in the absence of any electron transfer protein but in the presence of the FMN reductase enzyme, NADH, and FMN. The simplest explanation for this observation would be consistent with the data reported here, *i.e.* during crystallization, $FMNH_2$ drove the reduction of the 4-coordinate cob(II)alamin intermediate in the active site of *Lr*PduO. The availability of cob(I)alamin in the presence of ATP led to the formation of AdoCbl.

Adenosylation of Incomplete Corrinoids—In *S. enterica*, CobA is involved in the *de novo* biosynthesis of the corrin ring (44) and the synthesis of the nucleotide loop that tethers the lower ligand to the ring (45). Spectroscopic data showed that *Se*CobA generated the 4-coordinate corrinoid intermediate more readily with cobinamide (Cbi, Cbl precursor lacking the lower ligand) than with Cbl (18, 20).

In Fig. 6, we show that CobA can adenosylate cob(II)inamide (evident by the increase of AdoCbi at $A_{455\ nm}$ with the concomitant decrease of Co^{2+}Cbi at $A_{465\ nm}$) using free dihydroflavins as the reductant. The presence of AdoCbi was confirmed using bioassay conditions that demanded growth of strain JE7180 on ethanolamine (data not shown).

Previously, we showed that free dihydroflavins did not drive the reduction of cob(II)alamin bound to *Se*CobA (23). These results were intriguing, because spectroscopic data showed that the 4-coordinate cob(II)alamin species forms in the active site of *Se*CobA, albeit not as readily as the 4-coordinate of cob(II)inamide (20). Insufficient reaction time (30 min) was one plausible explanation for why dihydroflavins did not drive the reduction of cob(II)alamin in the active site of *Se*CobA (15). Thus, we extended the reaction time to 12 h inside an anoxic chamber in the dark. Under extended incubation conditions, *Se*CobA generated AdoCbl when free dihydroflavins were present. However, in contrast with PduO-type enzymes, $FldA_{red}$ was the only flavoprotein that drove the adenosylation of cob(II)alamin bound to *Se*CobA.

The *Lr*PduO^{F112A} variant, which cannot generate the 4-coordinate species of cob(II)alamin, did adenosylate cob(II)inamide when dihydroflavins were used as reductant. These results further support the importance of the role of the ACA enzyme in the generation of the 4-coordinate intermediate, which is needed to raise the redox potential so the reduction of Co(II) can occur. The hATR enzyme also used dihydroflavins to drive the adenosylation of cob(II)inamide. These results indicate that PduO-type ACA enzymes, regardless of their origin, have similar catalytic capabilities.

Criteria for Cobalamin Reductases—We suggest that the assignment of "cobalamin reductase" activity to any protein must be based on two criteria as follows: (i) evidence of binding of the corrinoid substrate to the protein; and (ii) for Co(II) corrinoid reductases, evidence of the formation of a 4-coordinate Co(II) corrinoid species in the site of the enzyme. To date, magnetic circular dichroism spectroscopy has proven to be a powerful means to establish the existence of 4-coordinate Co(II) corrinoid species. In the absence of this information, and in light of the data reported herein and structural data published elsewhere (21), we suggest that the proteins identified thus far as cobalamin reductases are not enzymes but are electron transfer proteins.

Conclusions—We draw two conclusions from the studies reported here. First, the redox potential of 4-coordinate Co(II) corrinoid species generated in the active site of ACA enzymes is within the physiological range but that of 5-coordinate cob(II)alamin is not. This is based on our results where PduO-type ACA enzymes can adenosylate cob(II)alamin in the presence of free dihydroflavins. Second, cobalamin reductases described thus far are most likely electron transfer proteins and not enzymes, and we propose the following criteria for the assignment of cobalamin reductase function to a protein. Because the $Co^{3+} \rightarrow Co^{2+}$ reduction can be driven in solution by dihydroflavins (15), evidence of binding of Co(III) corrinoids to the active site of the protein is central to the assignment of cob(III)alamin reductase function. As for cob(II)alamin reductases, the formation of a 4-coordinate cob(II)alamin species in the active site of the protein is proposed as the diagnostic feature of a *bona fide* cob(II)alamin reductase. In the absence of such information, putative cobalamin reductases should be considered as electron transfer proteins and not enzymes.

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