2005

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 49, pp. 40948–40956, December 9, 2005

Computer-assisted Docking of Flavodoxin with the ATP:Co(I)rrinoid Adenosyltransferase (CobA) Enzyme Reveals Residues Critical for Protein-Protein Interactions but Not for Catalysis*

Received for publication, June 21, 2005, and in revised form, October 3, 2005 Published, JBC Papers in Press, October 5, 2005, DOI 10.1074/jbc.M506713200

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The activity of the housekeeping ATP:co(I)rrinoid adenosyltransferase (CobA) enzyme of *Salmonella enterica* sv. Typhimurium is required to adenosylate *de novo* biosynthetic intermediates of adenosylcobalamin and to salvage incomplete and complete corrinoids from the environment of this bacterium. *In vitro*, reduced flavodoxin (FldA) provides an electron to generate the co(I)rrinoid substrate in the CobA active site. To understand how CobA and FldA interact, a computer model of a CobA·FldA complex was generated. This model was used to guide the introduction of mutations into CobA using site-directed mutagenesis and the synthesis of a peptide mimic of FldA. Residues Arg-9 and Arg-165 of CobA were critical for FldA-dependent adenosylation but were catalytically as competent as the wild-type protein when cob(I)alamin was provided as substrate. These results indicate that Arg-9 and Arg-165 are important for CobA·FldA docking but not to catalysis. A truncation of the 9-amino acid N-terminal helix of CobA reduced its FldA-dependent cobalamin adenosyltransferase activity by 97.4%. The same protein, however, had a 4-fold higher specific activity than the native enzyme when cob(I)alamin was generated chemically *in situ*.

The conversion of vitamin B_{12} (also known as cyanocobalamin, CNCbl) to its coenzymic form requires replacing the cyano Co(β) upper ligand with an adenosyl moiety. The resulting coenzyme B_{12} (also known as adenosylcobalamin (AdoCbl))[2] is essential for the function of AdoCbl-dependent enzymes (1–3). *De novo* corrin ring biosynthesis occurs via adenosylated intermediates, and adenosylation is also required for salvaging complete and incomplete corrinoids from the environment (4–6). Corrinoid adenosylation has been studied in several organisms (7–12), but the best understood system is the one in *Salmonella enterica* (13–16). In this bacterium the last step of the corrinoid adenosylation pathway, *i.e.* the transfer of the adenosyl group from ATP to the corrin ring, is catalyzed by the ATP:co(I)rrinoid adenosyltransferase (CobA, EC 2.5.1.17) enzyme (16). In addition to CobA, the *S. enterica* genome encodes two other ATP:cob(I)alamin adenosyltransferases encoded by the *eutT* and *pduO* genes (17–19). However, unlike the EutT and PudO enzymes, CobA has broad specificity for its corrinoid substrate, allowing it to adenosylate *de novo* corrin ring intermediates as well as complete (*e.g.* cobalamin (Cbl)) and incomplete corrinoids lacking the nucleotide loop (*e.g.* cobinamide (Cbi)) (4, 16).

The Co-C bond in coenzyme B_{12} is the result of a nucleophilic attack by the Co(I) ion on the C5′ carbon of ATP. *In vitro* the Co(I) ion is generated by the transfer of an electron from reduced flavodoxin A (FldA) to Co(II), an event that is currently thought to occur in the active site of CobA (14).

FldA is an electron transfer protein essential to cell survival (20). In addition to its involvement in the corrinoid adenosylation pathway (14), FldA provides reducing equivalents for the reactivation of MetH, the B_{12}-dependent methionine synthase (21). Elegant NMR spectroscopy studies identified the interacting surfaces of a fragment of MetH with FldA (22).

One unanswered question regarding the mechanism of catalysis by CobA is how the redox potential of the Co(II) to Co(I) transition (−610 mV) (23) is increased enough so the electron transfer from reduced FldA (−450 mV; semiquinonine/hydroquinone) (21, 24) can occur. As mentioned above, we previously hypothesized that FldA reduces Co(II) to Co(I) in the CobA active site, triggering the attack of the Co(I) nucleophile on the 5′ carbon of the ribosyl moiety of ATP (14). Stich *et al.* (25) recently reported that binding of the corrinoid substrate to the CobA/MgATP complex increases the Co(II)/Co(I) redox potential to within the range for FldA reduction (25), leading to the reduction of Co(II) to Co(I) in the active site of CobA. At present, the interactions of FldA with CobA are not understood. Here we report results of studies aimed at advancing our understanding of CobA·FldA interactions highlighting the use of computer modeling to generate experimentally testable models.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—TABLE ONE lists all strains and plasmids used in this work. TABLE TWO lists all the mutagenic PCR oligonucleotides used to generate the plasmids used in these studies.

Computer Modeling—The crystal structures of CobA (mmdbId: 15042 code 1G64) (12) and FldA (mmdbId:6851 code 1AHN) (21) were loaded into Deep View Version 3.7 (26) and aligned to bring CobA residues Arg-9, Arg-98, and Arg-165 within close proximity to FldA residues Asp-11, Glu-61, Asp-68, and Asp-93. No structural optimization was performed. The figures were generated using PyMOL (www.pymol.org).

PCR Methods—An Eppendorf Mastercycler® thermocycler was used to amplify templates listed in TABLE TWO using primers listed in TABLE TWO. Amplification products were cloned in vector pET15b (Novagen) to fuse an N-terminal hexahistidine tag to the gene product.

* This work was supported in part by National Institutes of Health Grant GM40313 (to J. C. E.-S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: AdoCbl, adenosylcobalamin; Cbi, cobinamide; CobA, ATP:co(I)rrinoid adenosyltransferase; FldA, flavodoxin; FMN, flavin mononucleotide; Fpr, ferredoxin (flavodoxin):$NADP^+$ reductase; Fre, flavin reductase; WT, wild type; Fre, flavin reductase.

TABLE ONE

Strains used in this study

Unless otherwise stated, all strains and plasmids were constructed during the course of this work.

Strain ID	Genotype	Plasmid	Allele	Protein encoded	Source
E. coli strains					
JE3892	BL21(λ*DE3*)				Laboratory collection
Derivatives of DH5α					
JE4638		pFRE3	*fre*$^+$	H_6Fre	Laboratory collection
JE5302		pFPR1	*fpr*$^+$	Fpr	Laboratory collection
JE7779		pFLDA4	*fldA*$^+$	$FldAH_6^{WT}$	
JE7928		pFLDA6		$FldAH_6^{D68R}$	
JE7929		pFLDA7		$FldAH_6^{D93R}$	
JE7930		pFLDA8		$FldAH_6^{D68R/D93R}$	
JE3544		pET15b			Laboratory collection
JE4263		pCOBA17	*cobA1316*	H_6CobA^{WT}	Laboratory collection
JE7187		pCOBA58	*cobA1317*	H_6CobA^{R9A}	
		pCOBA59	*cobA1318*	H_6CobA^{R9E}	
JE7190		pCOBA61	*cobA1319*	H_6CobA^{R98A}	
JE7191		pCOBA62	*cobA1320*	H_6CobA^{R98E}	
JE7473		pCOBA64	*cobA1321*	H_6CobA^{R165A}	
JE7474		pCOBA65	*cobA1322*	H_6CobA^{R165E}	
JE7475		pCOBA66	*cobA1323*	$H_6CobA^{R9E/R98E}$	
JE7476		pCOBA67	*cobA1324*	$H_6CobA^{R9E/R165E}$	
JE7477		pCOBA68	*cobA1325*	$H_6CobA^{R98E/R165E}$	
JE7478		pCOBA69	*cobA1326*	$H_6CobA^{R9E/R98E/R165E}$	
JE7947		pCOBA71	*cobA1327*	H_6CobA^{A134L}	
JE2879		pCOBA16	*cobA*$^+$	$CobA^{WT}$	Laboratory collection
JE6177		pCOBA23	*cobA1328*	$CobA^{\Delta N2-26}$	Laboratory collection
S. enterica strains					
JE2886	*metE205 ara-9* Δ902(*cobA-trp*)/pGP1-2 T7 *rpo*$^+$ *kan*$^+$	pCOBA16	cobA$^+$	$CobA^{WT}$	Laboratory collection
Derivatives of JE4182					
JE4182	*metE205 ara-9*/pGP1-2 T7 *rpo*$^+$ *kan*$^+$				Laboratory collection
JE8079		pFLDA4		$FldAH_6^{WT}$	
JE8080		pFLDA6		$FldAH_6^{D68R}$	
JE8081		pFLDA7		$FldAH_6^{D93R}$	
JE8082		pFLDA8		$FldAH_6^{D68R/D93R}$	
Derivatives of JE7180					
JE7180	*metE205 ara-9 cobA366*::Tn*10*d(*cat*$^+$) Δ*eut1141*(Δ*eutT*)				
JE7584		pET15b			Laboratory collection
JE7455		pCOBA17	*cobA1316*	H_6CobA^{WT}	
JE7572		pCOBA58	*cobA1317*	H_6CobA^{R9A}	
JE7573		pCOBA59	*cobA1318*	H_6CobA^{R9E}	
JE7575		pCOBA61	*cobA1319*	H_6CobA^{R98A}	
JE7576		pCOBA62	*cobA1320*	H_6CobA^{R98E}	
JE7578		pCOBA64	*cobA1321*	H_6CobA^{R165A}	
JE7579		pCOBA65	*cobA1322*	H_6CobA^{R165E}	
JE7580		pCOBA66	*cobA1323*	$H_6CobA^{R9E/R98E}$	
JE7581		pCOBA67	*cobA1324*	$H_6CobA^{R9E/R165E}$	
JE7582		pCOBA68	*cobA1325*	$H_6CobA^{R98E/R165E}$	
JE7583		pCOBA69	*cobA1326*	$H_6CobA^{R9E/R98E/R165E}$	
JE8123		pCOBA71	*cobA1327*	H_6CobA^{A134L}	
JE7564		pCOBA23	*cobA1328*	$CobA^{\Delta N2-26}$	Laboratory collection

Site-directed Mutagenesis—The Stratagene QuikChange® site-directed mutagenesis kit was used to create point mutants in plasmids pCOBA17 and pFLDA4 (TABLE TWO). Nonradioactive BigDye® (Amersham Biosciences) protocols for DNA sequencing were used to verify the presence of mutations. Plasmids were reconstructed and then sequenced at the DNA sequencing facility at the University of Wisconsin-Madison Biotechnology Center.

Purification of His-tagged CobA Proteins—Plasmids were transformed into *Escherichia coli* strain BL21(λDE3) for overexpression (JE3892). Fresh transformants were used to inoculate 2 ml of fresh

TABLE TWO

Primers used for mutagenesis

Unless otherwise stated, all CobA proteins contained N-terminal H_6 tags for purification. FldA proteins contained C-terminal H_6 tags. Fwd, forward; rev, reverse.

Protein, mutation	Template	5′ Primer	3′ Primer	Resulting plasmid
$CobA^{WT}$ (untagged)	Genomic	Laboratory collection		pCOBA16
$CobA^{\Delta N2-26}$ (untagged)	pCOBA16	Laboratory collection		pCOBA23
$CobA^{WT}$	Genomic	Se cobA NdeI fwd 5′-CCAAGGAATCGCCATATGAGTGATGAACGTTATCAGC	Se cobA BamHI rev 5′-AAGCGAACCGGATCCGTCGGGCGGTAAAA	pCOBA17
$CobA^{R9A}$	pCOBA17	CobA R9A fwd 5′-CGTTATCAGCAGGCCCAGCAGAAGGTAAAAGATCG	CobA R9A rev 5′-CGATCTTTTACCTTCTGCTGGGCCTGCTGATAACG	pCOBA58
$CobA^{R9E}$	pCOBA17	CobA R9E fwd 5′-CGTTATCAGCAGGAGCAGCAGAAGGTAAAAG	CobA R9E rev 5′-CGATCTTTTACCTTCTGCTGCTCCTGCTGATAAC	pCOBA59
$CobA^{R98A}$	pCOBA17	CobA R98A fwd 5′-TGGGAGACGCAAAATGCCGAGGCAGACACCGCAGC	CobA R98A rev 5′-GCTGCGGTGTCTGCCTCGGCATTTTGCGTCTCCCA	pCOBA61
$CobA^{R98E}$	pCOBA17	CobA R98E fwd 5′-TGGGAGACGCAAAATGAGGAGGCAGACACCGCAGC	CobA R98E rev 5′-GCTGCGGTGTCTGCCTCCTCATTTTGCGTCTCCCA	pCOBA62
$CobA^{R165A}$	pCOBA17	CobA R165A EcoRV fwd 5′-GGTCGCGGCTGTCACGCGGATATCCTTGATCTTGCGG ATACCGTCAGCGAACTGCGTCCGG	CobA R165A EcoRV rev 5′-CCGGACGCAGTTCGCTGACGGTATCCGCAAGATCA GCCGCGACC	pCOBA64
$CobA^{R165E}$	pCOBA17	CobA R165E EcoRV fwd 5′-GGTCGCGGCTGTCACGAGGATATCCTTGATCTTGCGG ATACCGTCAGCGAACTGCGTCCGG	CobA R165E EcoRV rev 5′-CCGGACGCAGTTCGCTGACGGTATCCGCAAGATCA AGGATATCCTCGTGACAGCCGCGACC	pCOBA65
$CobA^{R9E/R98E}$	pCOBA59	CobA R98E fwd	CobA R98E rev	pCOBA66
$CobA^{R9E/R165E}$	pCOBA59	CobA R165E EcoRV fwd	CobA R165E EcoRV rev	pCOBA67
$CobA^{R98E/R165E}$	pCOBA62	CobA R165E EcoRV fwd	CobA R165E EcoRV rev	pCOBA68
$CobA^{R9E/R98E/R165E}$	pCOBA66	CobA R165E EcoRV fwd	CobA R165E EcoRV rev	pCOBA69
$CobA^{A134L}$	pCOBA17	CobA A134L fwd 5′-ATGGTGCTGTATGACTATTTACCGCTGGAA	CobA A134L rev 5′-GTCATACAGCACCATATAGGTCAGCTCATCC	pCOBA71
$FldA^{WT}$	Genomic	Se FldA NcoI NdeI fwd 5′-TTTTTTTTTCCATGGTACATATGGCTATCACTGGCATC TTTTTC	SeFldA XhoI stop XbaI rev 5′-TTTTTTTTTTCTAGATCACTCGAGGGCATTGAGAAT TTCGTCGAGATG	pFLDA4
$FldA^{D68R}$	pFLDA4	Se FldA D68R3 fwd 5′-GATCGTTTCTTTCCAACTCTCGAAGAGATTGATTTCAAC	Se FldA D68R3 rev 5′-GAAACGATCCCAGTCACACTGCGCTTC	pFLDA6
$FldA^{D93R}$	pFLDA4	Se FldA D93R3 fwd 5′-GATCGTTTCTTTCCAACTCTCGAAGAGATTGATTTCAAC	Se FldA D93R3 rev 5′-ATATCGTTCCTGATCACCACAACCAAACAGCGCAA	pFLDA7
$FldA^{D68R/D93R}$	pFLDA7	Se FldA D93R3 fwd	Se FldA D93R3 rev	pFLDA8

Luria-Burtani broth (LB)/ampicillin medium. A starter culture (1 ml) was used to inoculate 100-ml cultures of LB/ampicillin medium. After overnight growth at 37 °C with shaking, cells were harvested by centrifugation at 15,000 × *g* at 4 °C in a Beckman/Coulter Avanti J-25I centrifuge equipped with a JLA-16.250 rotor. Cell paste was frozen at −80 °C until use. To purify proteins 5 ml of lysis buffer (1× Bug Buster reagent (Novagen) in 1× binding buffer, 0.1 mM phenylmethanesulfonyl fluoride) was used to resuspend the cell pellet. Cells were lysed at room temperature for 20 min and centrifuged for 30 min at 43,667 × *g*. Cell-free extract was passed through a 0.45-μm syringe filter before loading onto HisBind 900 cartridges (Novagen). Protein was purified as per the manufacturer's instructions. Protein was eluted into 1 mM EDTA. Fractions containing highly purified protein were pooled and dialyzed once against 50 mM Tris-Cl buffer, pH 8, (at 4 °C) containing 10 mM EDTA. Subsequent dialyses were performed against buffer without EDTA. In some cases protein was concentrated using a YM10 Centricon unit (molecular weight cutoff = 10,000; Amicon). Glycerol was added to 10% (v/v) and dithiothreitol to 1 mM; 100-μl drops of protein solution were flash-frozen using liquid N_2, and pellets were stored at −80 °C until used.

Native Wild Type (WT) and CobA$^{\Delta N2-26}$ Protein Purification—Native WT CobA protein was overexpressed on strain JE2886 and purified as described (15). Allele *cobA1328* encoding the mutant CobA protein with the N-terminal 2–26 amino acids deleted (CobA$^{\Delta N2-26}$) was cloned into plasmid pT7-7 (27) and overproduced in *E. coli* strain BL21(λDE3); all chromatographic steps were identical to those used to isolate native WT CobA protein.

FldA Overexpression and Purification—Plasmids encoding WT or mutant His-tagged FldA proteins were freshly transformed into strain JE4182. Four 2-liter flasks containing 750 ml of LB medium containing kanamycin (50 μl/ml) and ampicillin (100 μl/ml) were each inoculated with 10 ml of an overnight starter culture grown in the same medium. After the culture reached mid-log phase at 30 °C and 160 rpm shaking, riboflavin was added to a final concentration of 10 μM, and cultures were shifted to 42 °C for 1 h and incubated at 37 °C overnight. Cells were harvested at 4 °C and 15,000 × *g* for 10 min; cell paste was frozen at −80 °C until used.

Frozen cell paste was resuspended in 20 mM sodium phosphate buffer, pH 7.5, (at 4 °C) containing 0.5 M NaCl and 1 mM phenylmethanesulfonyl fluoride. The suspension was passed through a French press twice, 10 mg of DNase was added, and the lysate was incubated on ice for 10 min. The lysate was centrifuged at 4 °C at 43,667 × *g* for 30 min, and soluble protein was passed through a 0.45-μm syringe filter. Proteins were isolated from crude cell extracts using a ÄKTA fast protein liquid chromatograph equipped with a 5-ml HisTrap column (Amersham Biosciences). After column equilibration, cell-free extract was loaded onto the column, which was washed with 5 column volumes (CV) of 20 mM sodium phosphate buffer, pH 7.5, (at 4 °C) containing 0.5 M NaCl. Mutant FldA proteins were eluted with a 0–50% linear gradient of 20 mM sodium phosphate, pH 7.5, (at 4 °C), 0.5 M NaCl, 0.5 M imidazole over 15 column volumes. Fractions containing pure protein were pooled, concentrated in an YM10 Centricon unit, and dialyzed against 50 mM Tris-Cl, pH 7.5, (at 4 °C) containing 1 mM EDTA. Protein concentration was determined by A_{466} to measure flavin mononucleotide (FMN) bound to FldA (ϵ_{466} = 8250 M^{-1}) (28). Glycerol was added to 10% (v/v), and dithiothreitol was added to 1 mM before protein was flash-frozen and stored at −80 °C until used. Fpr protein (ferredoxin (flavodoxin):$NADP^+$ reductase) was purified as described (14).

Fre Protein Overexpression and Purification—H_6Fre (flavin reductase; EC 1.5.1.29) protein was overexpressed using *E. coli* BL21(λDE3)/pFRE3 (13). The latter was re-constructed before overexpression by transforming strain JE3892 with plasmid pFRE3. A single colony was used to inoculate 5 ml of fresh LB/ampicillin medium and incubated to stationary phase at 37 °C. 2.5 ml of the starter culture was used to inoculate four 1-liter flasks containing 250 ml of LB/ampicillin medium. All cultures were incubated at 37 °C to late log phase and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside with shaking for an additional 4 h. Cells were harvested at 15,000 × *g* for 10 min; the cell pellet was frozen at −80 °C until used. To purify H_6Fre (flavin reductase) protein, 20 ml of lysis buffer (1× Bug Buster reagent (Novagen) in 1× binding buffer, 1 mM phenylmethanesulfonyl fluoride) was used to resuspend the cell pellet. Cells were lysed at room temperature for 20 min and centrifuged for 30 min at 43,667 × *g*. Cell-free extract was filtered through a 0.45-μm and then a 0.2-μm syringe filter before loading onto HisBind 900® cartridges (Novagen). H_6-Fre Protein was purified as per the manufacturer's instructions. Protein was eluted directly into 5 mM EDTA. Fractions containing homogeneous protein were pooled, concentrated in an YM10 Centricon unit, and dialyzed once against 50 mM Tris-Cl buffer, pH 7.5, (at 4 °C) containing 10 mM EDTA. Subsequent dialyses were against buffer without EDTA. Glycerol was added to 10% (v/v) and dithiothreitol to 1 mM before aliquots were flash-frozen and stored at −80 °C.

Growth Behavior Analysis—Ten μl of overnight cultures of all strains was used to inoculate 190 μl of fresh medium in each well of a 96-well microtiter dish; all strains were cultured in triplicate. Growth media contained 1× no-carbon E medium (29), 1 mM Mg^{+2}, 0.5 mM methionine, 1× trace minerals (30), 50 mM NH_4Cl, 50 mM ethanolamine, pH 7.0. When added, ampicillin was used at 100 μg/ml, kanamycin was used at 50 μg/ml, and AdoCbl, hydroxycobalamin, or dicyano-cobinamide was used at 200 nM. For growth on glycerol as the sole carbon and energy source, ethanolamine and methionine were omitted, and glycerol was added to 30 mM. Cultures were incubated with shaking for up to 96 h at 37 °C in a SpectraMAX Plus automatic plate reader (Molecular Devices). Western blots were performed to determine whether complementation defects were due to protein expression or stability issues. However, because of the sensitivity of the growth assay, even undetectable levels of CobA^{WT} allowed full complementation.

Cobalamin Adenosyltransferase Assays—Fig. 1 shows three different ways to generate the co(I)rrinoid substrate for CobA. Reactions contained 4 mg of potassium borohydride (KBH_4), 50 μM hydroxycobalamin, 0.8 mM Mn^{2+}, 0.4 mM ATP, 3 μM, 0.5 μM CobA. AdoCbl formation was determined by measuring the decrease in A_{525} after 15 min at 37 °C and after 5 min of photolysis. When FldA was used as the reductant instead of KBH_4, assays contained 50 μM hydroxycobalamin, 0.8 mM Mn^{2+}, 0.5 mM NADPH, 0.4 mM ATP, 3 μM Fpr, 2 μM FldA, 2 μM CobA and were incubated for 30 min at 37 °C. Peptide inhibition assays contained concentrations of FldA ranging from 0.25 to 2 μM. For mutant FldA proteins, Fpr protein was omitted and replaced by 0.5 μM Fre protein and 50 μM FMN. FldA peptides were synthesized by the Peptide Sequencing Facility at the University of Wisconsin-Madison Biotechnology Center.

RESULTS

Modeling CobA·FldA Interactions—We artificially docked CobA with FldA using ExPasy Deep View (www.expasy.org/spdbv) software (Fig. 2). Without making any structural modifications, three positively charged surface residues on CobA (Arg-9, Arg-98, Arg-165) were aligned with three negatively charged residues on FldA (Glu-61, Asp-68, Asp-93). These FldA residues were previously shown (28) to be involved in its interactions with the Cbl-dependent methionine synthase (MetH)

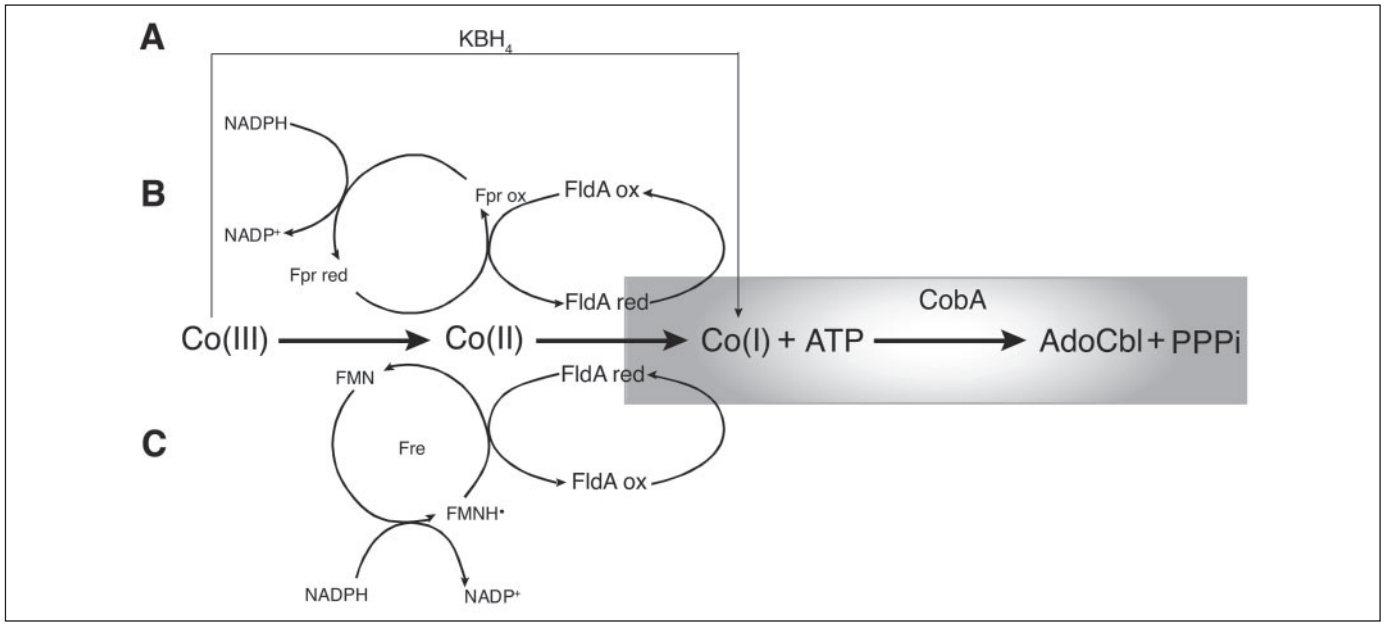

FIGURE 1. **Assays used to assess the production of AdoCbl by CobA.** *A*, KBH_4 is used to reduce Co(III)Cbl to Co(I)Cbl directly. *B*, the Fpr/FldA system converts cob(III)alamin to cob(II)alamin. CobA (represented by the *shaded box*) binds cob(II)alamin and Mg/ATP. Reduced FldA then provides the electron to reduce cob(II)alamin in the CobA active site. *C*, in these studies we attempted to bypass Fpr by providing reduced flavins using Fre and NADPH.

enzyme. In this hypothetical CobA·FldA complex, the FMN cofactor of FldA was within 10 Å of the Cbl substrate in the CobA active site, and hydrophobic patches on both proteins were brought together (Fig. 2*D*).

Competitive Inhibition of FldA-dependent AdoCbl Synthesis by a FldA Peptide—Based on our computer model, residues TWYYGEAQCDWDD[68] of FldA were predicted to be part of the surface interacting with CobA. We hypothesized that a peptide mimic of this region should block CobA·FldA interactions. Because the same region of FldA is known to also interact with Fpr, the latter was present in the reaction mixture in large excess and was preincubated with FldA before peptide, ATP, and CobA were added to start the cobalamin adenosyltransferase assay reaction. The control peptide TWYYG**A**AQCDWD**A**[68] contained E61A and D68A mutations to determine whether, as predicted, these two charged residues contributed to any interactions of FldA with CobA. A third FldA peptide of similar size and hydrophobicity (WPTAGYHFEASKG[132]) was used as negative control. The control peptide did not contain any residues known to interact with MetH (Fig. 3) and, hence, were predicted to have no effect of the interactions of FldA with CobA.

The effect of all three peptides on FldA-dependent AdoCbl synthesis was assayed *in vitro*. In these assays, AdoCbl formation relied on productive CobA·FldA interactions for the generation of the cob(I)alamin nucleophile. To optimize the assay conditions, various concentrations of FldA were used in assays where substrates and CobA were held constant. The K_m for FldA was 5.65 μM and V_{max} = 31.8 nmol min^{-1} mg^{-1} (Fig. 4). For Lineweaver-Burk analysis, subsaturating levels of FldA were used such that the reaction proceeded at a rate between 4 and 50% V_{max} when no peptides were added. Each of the three peptides was then varied from 2–6 μM for each concentration of FldA.

Peptide TWYYGEAQCDWDD[68] inhibited AdoCbl formation by ~30% when present at a 1.5:1 peptide:FldA ratio. At 59% V_{max}, the specific activities were 19 and 13, respectively. This level of inhibition was significant considering the small size of the peptide. According to Lineweaver-Burk analysis, this inhibition was competitive (K_m peptide = 29 μM and K_i = 1.3 mM). A 13-amino acid peptide mimic of FldA had only a 5-fold decrease in CobA binding efficiency. This decreased affinity might be due to several reasons. The peptide could have greater conformational flexibility than the FldA protein or lack a secondary structure needed for interactions with CobA. In addition, the peptide may not span all the residues involved in the CobA·FldA interaction.

In contrast, the mutagenized and control peptides (TWYYGAAQCDWDA[68] and WPTAGYHFEASKG[132], respectively) failed to inhibit AdoCbl production even when present at 10-fold increase over FldA. These results suggested that the TWYYGEAQCDWDD[68] peptide did bind to CobA and interfered with the FldA docking. Results with the mutagenized peptide indicated that this inhibition depended on residues Glu-61 and Asp-68.

In Vivo Assessment of CobA Activity—To test the effect of mutations in residues Arg-9, Arg-98, and Arg-165, we site-directed mutagenized each one of these residues to Ala or Glu. Permutations of mutated residues were also constructed, and their effects were tested *in vitro* and *in vivo*.

Plasmids containing mutant *cobA* alleles were transformed into a *metE cobA* strain of *S. enterica* (JE7180) to assess their level of function *in vivo*. Strains were grown under conditions that demanded low or high levels of cobalamin synthesis. When grown on glycerol as sole carbon source, a *metE cobA* strain cannot convert dicyano-cobinamide into Cbl because enzymes that assemble the nucleotide loop of Cbl require AdoCbi (4, 31). Therefore, in the absence of Cbl, the Cbl-dependent methionine synthase (MetH) cannot methylate homocysteine to produce methionine. Hence, the *metE cobA* strain is an AdoCbi, Cbl, or methionine auxotroph. When grown on ethanolamine as the carbon and energy source, AdoCbl synthesized by CobA is needed to activate transcription of the ethanolamine utilization (*eut*) operon and to provide AdoCbl for ethanolamine ammonia-lyase function (18, 32).

Of the CobA mutant proteins tested, only $CobA^{R98A}$, $CobA^{R98E}$, and $CobA^{R165A}$ supported growth on ethanolamine as carbon and energy source, albeit to a reduced level relative to the wild-type strain (Fig. 5*A*). It was surprising to learn, however, that protein $CobA^{R98A}$ with <20% of the $CobA^{WT}$ activity (TABLE THREE, column C) supported growth on ethanolamine at ~80% that of the growth rate observed with a strain synthesizing $CobA^{WT}$ enzyme (Fig. 5). Surprisingly, all *cobA* alleles

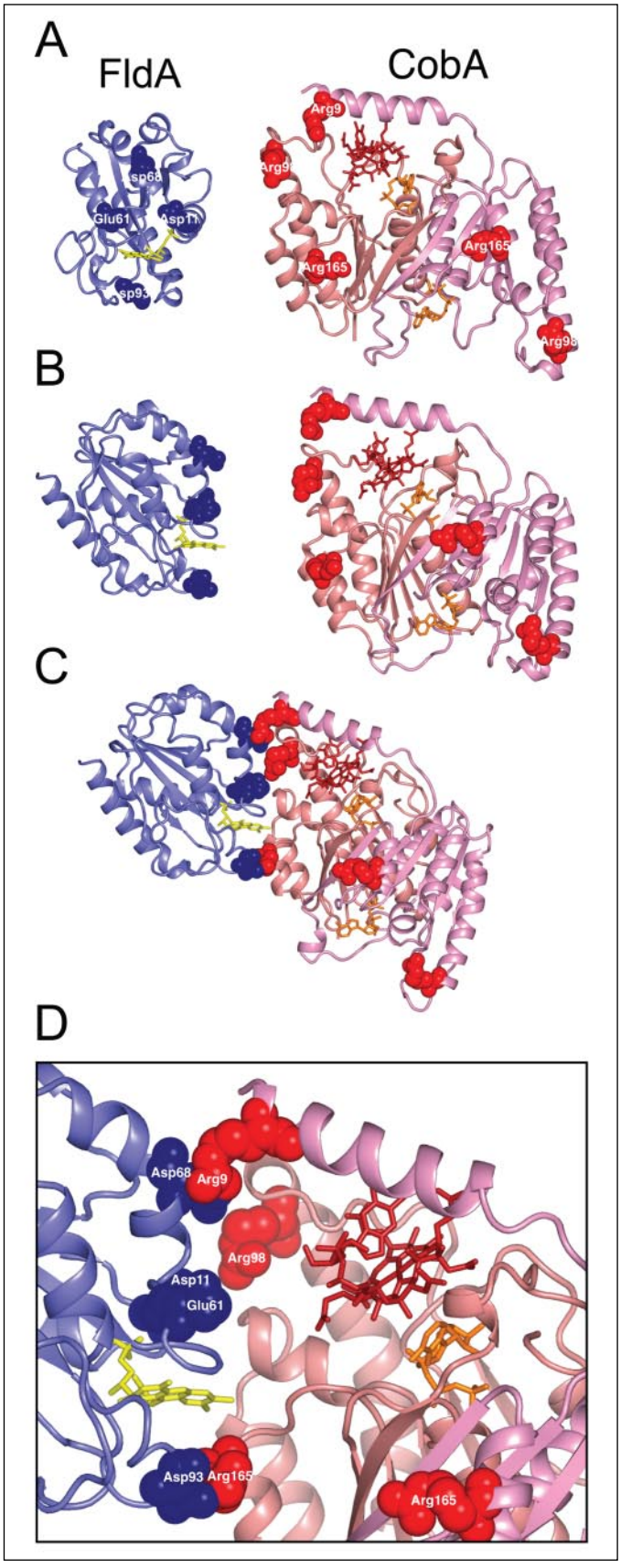

FIGURE 2. **Computer model of the CobA·FldA complex.** *A*, crystal structures of CobA (monomers are colored *salmon* and *pink*) and FldA (*light blue*) are shown. Selected basic surface residues on FldA are colored *blue*, whereas elected acidic side chains on CobA are colored *red*. *B*, the structures in *A* are rotated 90° toward each other. *C*, CobA and FldA were aligned so that negatively charged residues on FldA (*blue*) lined up with positively charged residues on CobA (*red*) to create the docked model. *D*, close-up of the CobA·FldA complex. At the docking interface FldA residue Asp-68 contacts residue Arg-9 of CobA, and FldA residue Asp-93 contacts CobA residue Arg-165. Residue Asp-11 of FldA is close to, but not contacting residue Arg-98 of CobA. Additionally, the FMN cofactor of FldA (*yellow*) is within 10 Å of bound Cbl (*maroon*) and ATP (*orange*) in the CobA active site. Surface hydrophobic patches on both proteins overlap (not shown).

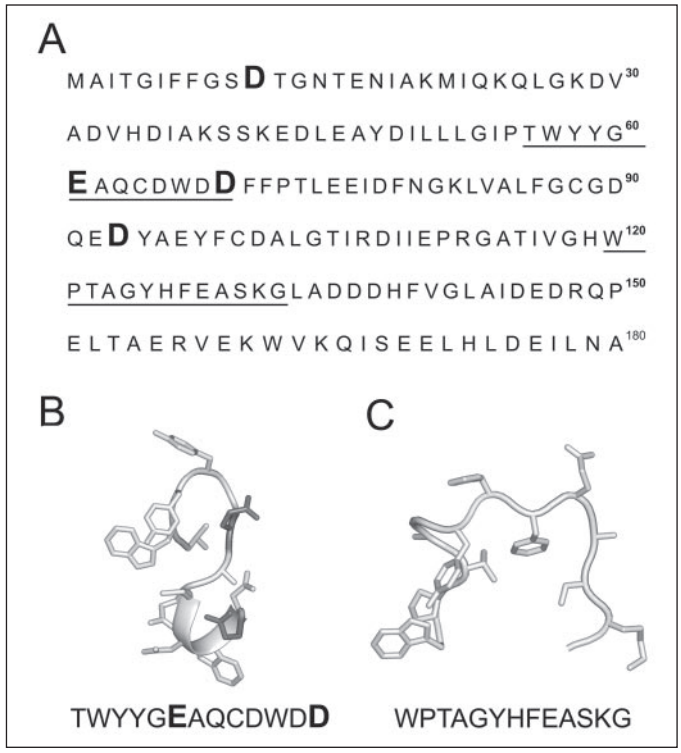

FIGURE 3. **Peptide inhibition of the CobA·FldA interaction.** *A*, the primary sequence of FldA is shown above. A subset of acidic residues known to interact with methionine synthase (MetH) is shown in *boldface*. Peptides designed to inhibit CobA·FldA interaction (TWYYGEAQCDWDD[68]) and a control peptide (WPTAGYHFEASKG[132]) are *underlined*. The crystal structure of FldA was used to model the structure of each peptide (*B* and *C*). FldA residues Glu-61 and Asp-68 are *shaded gray*. Peptide TWYYGAAQCDWDA[68] is identical to TWYYGEAQCDWDD[68], except that the *shaded residues* are mutated to Ala.

tested complemented the Cbl auxotrophy of the *cobA* mutant strain during growth on glycerol (data not shown). We interpret these data to mean that all mutant CobA proteins sufficiently interacted with FldA to produce enough Cbl to satisfy the methionine requirement of the cell. These results indicated that the wild-type CobA enzyme synthesizes an excess of AdoCbl. The inability of some CobA variants to complement a *cobA* strain during growth on ethanolamine (demands more AdoCbl) reflected the negative effects of the mutations on CobA·FldA interactions rather than being the result of expression problems or unstable CobA mutant proteins.

In Vitro Assessment of CobA Activity—Having two ways of generating the Co(I) nucleophile (Fig. 1, *A* and *B*) allowed us to determine whether the CobA residues identified by the model were relevant to docking, catalysis, or both.

Activity of CobA Variants When the Cob(I)alamin Substrate Was Generated Using a Chemical Reductant—To distinguish between docking defects and loss of catalytic activity, we measured the activity of mutant CobA enzymes independent of CobA·FldA docking. For these experiments, we reduced cob(III)alamin to cob(I)alamin with potassium borohydride (KBH_4) before adding CobA (Fig. 1*A*). Regardless of the change at residues Arg-9 or Arg-165 (*i.e.* R9A, R9E, R165A, or R165E), the specific activity of the resulting mutant CobA proteins was

equal or better than that of the wild-type enzyme (TABLE THREE, column B; lines 2, 3, 6, 7 *versus* line 1). Although it is a formal possibility that Arg-9 and Arg-165 mutations negatively affect binding of cob(II)-alamin but not cob(I)alamin to CobA, we believe such a defect would have to be pronounced since saturating levels of cob(II)alamin were used in the FldA-dependent assay.

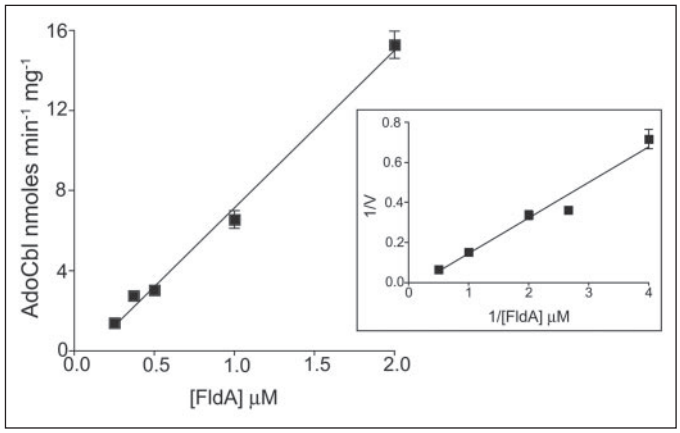

FIGURE 4. **Kinetics of FldA-dependent AdoCbl synthesis by CobA.** Substrates and CobA were held constant, whereas FldA concentrations were varied. AdoCbl formation was determined after 30 min by detecting a change in A_{525} after photolysis. The K_m for FldA is 5.7 μM, and the V_{max} is 31.8 nmol min^{-1} mg^{-1}. *Inset*, Lineweaver-Burk plot.

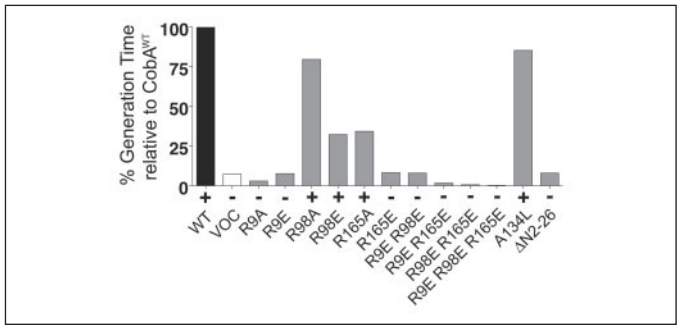

FIGURE 5. ***In vivo* and *in vitro* assessment of CobA function.** Plasmids encoding CobA^{WT} and variants of it were transformed into strain JE7180 (*metE205 ara-9 cobA366*::Tn*10*d(*cat*$^+$) Δ*eut1141*(Δ*eutT*)). When grown on ethanolamine as sole carbon and energy source, only CobA^{WT}, CobA^{R98A}, CobA^{R98E}, CobA^{R165A}, and CobA^{A134L} proteins produced enough AdoCbl to support growth. + and − signs indicate whether cultures grew to A_{650} >0.2 after 12 h on ethanolamine; the *bar graph* indicates the growth rate compared with strains carrying the WT *cobA* allele. *VOC*, vector-only control.

Activity of CobA Variants When the Cob(I)alamin Substrate Was Generated Using the Fpr/FldA Reduction System—To determine whether CobA residues Arg-9 and Arg-165 were involved in docking, the specific activity of each mutant CobA protein was determined in assays where CobA·FldA interaction was required for the generation of the cob(I)alamin substrate (Fig. 1*B*). The results from these assays (TABLE THREE, column C) were consistent with the results from *in vivo* experiments using these plasmids (Fig. 5). In reactions where CobA^{R9A}, CobA^{R9E}, and CobA^{R165E} were used, we measured very low levels of adenosyltransferase specific activity (TABLE THREE, column C; lines 2, 3, 7 *versus* line 1). In contrast, when CobA^{R98A}, CobA^{R98E}, and CobA^{R165A} proteins were used in the assay, the adenosyltransferase specific activity we measured was 7–40-fold higher (TABLE THREE, column C; lines 4, 5, 6 *versus* line 1). Combinations of the above mutations resulted in proteins with undetectable activity (TABLE THREE, column C; lines 8–11). On the basis of the data in Fig. 5 and TABLE THREE, we conclude that residues Arg-9 and Arg-165 are important to docking but not to catalysis.

The interpretation of the data regarding residue Arg-98 is less straightforward. In isolation, the effect of a mutation at Arg-98 depends on the nature of the substitution. A drastic change in charge and size of the side chain (R98A) results in a substantial loss of specific activity relative to the wild-type enzyme (143 *versus* 10; TABLE THREE, column B; lines 1 *versus* 4). A drastic change in charge but not in the size of the side chain (R98E) reduces the specific activity of the enzyme less than 2-fold relative to the wild type (143 *versus* 97; TABLE THREE, column B; lines 1 *versus* 5). CobA^{R98A} was more active *in vivo* than in either *in vitro* assay. Interestingly, the combination of R98E with R165E mimicked the effect of the R98A change (CobA$^{R98E/R165E}$ specific activity = 20; TABLE THREE, column B; lines 1 *versus* 10), an effect that was reversed by the introduction of the R9E mutation (CobA$^{R9E/R98E/R165E}$ specific activity = 108; TABLE THREE, column B; lines 1 *versus* 11). With the data in hand we suggest that Arg-98 is necessary for structural integrity of CobA.

Effect of Compensatory Mutations in FldA—Based on the model of the CobA·FldA complex (Fig. 2), we hypothesized that changing FldA residues Asp-68 and Asp-93 to Arg would compensate for the negative

TABLE THREE

Mean specific activities of CobA proteins as a function of the reductant used

Line no.	A CobA variant	B KBH_4 reductant		C FldA^{WT}/Fpr reductant		D Complementation[c]
		SA[a]	%SA[b]	SA[a]	%SA[b]	
1	H_6CobA^{WT}	143 ± 6.0	100	13 ± 0.3	100	+
2	H_6CobA^{R9A}	154 ± 1.4	108	0.8 ± 0.3	6	−
3	H_6CobA^{R9E}	151 ± 1.2	106	0.4 ± 0.1	3	−
4	H_6CobA^{R98A}	10 ± 5.6	7	2 ± 0.6	17	+
5	H_6CobA^{R98E}	97 ± 5.8	68	5 ± 0.5	41	+
6	H_6CobA^{R165A}	138 ± 5.8	97	2 ± 0.8	16	+
7	H_6CobA^{R165E}	143 ± 7.1	100	0.1 ± 0.1	1	−
8	H_6CobA$^{R9E/R98E}$	56 ± 5.5	40	ND[d]		−
9	H_6CobA$^{R9E/R165E}$	94 ± 6.4	66	ND		−
10	H_6CobA$^{R98E/R165E}$	20 ± 4.4	14	ND		−
11	H_6CobA$^{R9E/R98E/R165E}$	108 ± 1.5	75	0.3 ± 0.2	3	−
12	H_6CobA^{A134L}	150 ± 5.8	105	15 ± 1.2	115	+

[a] Specific activity in AdoCbl nmol min^{-1} mg^{-1}.
[b] SA *versus* H_6CobA^{WT} enzyme.
[c] Measured by cultures reaching an $A_{650\ nm}$ > 0.2 after 12 h of incubation at 37 °C during growth on ethanolamine as C and energy source.
[d] ND, not detectable.

effects of CobA mutations R9E and R165E on FldA-dependent synthesis of AdoCbl. A problem with these experiments arose when the Fpr enzyme failed to reduce $FldA^{D68R}$ and $FldA^{D93R}$ mutant proteins (data not shown). In an attempt to circumvent this problem, we used the NADH:FMN reductase (Fre) enzyme (13, 33) to reduce free FMN, which would indirectly reduce the flavin cofactor in mutant FldA enzymes (Fig. 1*C*). If the Fre system worked, $CobA^{R9E}$ was predicted to preferentially interact with either $FldA^{D68R}$ or $FldA^{D93R}$ proteins. Or else $CobA^{R9E}$ would interact equally well with the $FldA^{D68R}$ and $FldA^{D93R}$ proteins if the orientation for docking were not important. However, $CobA^{R9E}$ was not expected to interact with wild-type FldA or the $FldA^{D68R/D93R}$ proteins. The first observation we made was that when we used the Fre enzyme, the specific activity of $CobA^{R9E}$ increased 12-fold over the level measured when Fpr was used to reduce FldA (4.5 *versus* 0.4). $CobA^{R9E}$ was not as active when either $FldA^{D68R}$ (specific activity = 1.7) or $FldA^{D93R}$ (specific activity = 2) was used in the assay. When $FldA^{D68R/D93R}$ protein was used, the specific activity of $CobA^{R9E}$ was as high as when wild-type FldA was used (both 4.5). Results from assays using permutations of CobA and FldA mutant proteins did not reveal any additional insights into CobA·FldA interactions.

Effect of Changes in the Hydrophobic Topology of the CobA Docking Surface—The hydrophobic patch of CobA that is proposed to be involved in CobA·FldA docking has a concave surface. To investigate the contributions of hydrophobic packing to the interactions of CobA with FldA, residue Ala-134 was mutated to Leu. Ala-134 was chosen for mutagenesis because it is the only Ala in the hydrophobic patch. Hence, a change to Leu would be tolerated by the polypeptide. In contrast, mutating other residues in the patch (Phe or Trp) to a less bulky side chain like Ala or Gly would be expected to affect the structural integrity of the protein. The $CobA^{A134L}$ protein supported growth on glycerol or on ethanolamine as well as did $CobA^{WT}$ (Fig. 5), and its specific activity under all assay conditions (KBH_4 or Fpr/FldA) was as high as that of the $CobA^{WT}$ protein (TABLE THREE columns B, C; lines 1 *versus* 12). Further mutagenesis of CobA is necessary to determine the contributions (if any) of the hydrophobic patch of CobA to the interactions of CobA with FldA.

Deletion of the CobA N-terminal Helix Increases the Specific Activity of the Enzyme but Decreases CobA·FldA Docking—A striking feature of the CobA crystal structure is an N-terminal helix of 26 amino acids that remains disordered unless Mg/ATP and the corrinoid substrate are bound to the active site (12). The N-terminal helix of CobA may have several functions. It may help secure the corrinoid substrate in the active site, it may exclude water from the active site, thereby helping to stabilize the co(I)rrinoid nucleophile, or it may be important for FldA recognition or a combination of the above. To gain insights into the role of the N-terminal helix, we deleted it and tested the ability of the resulting $CobA^{\Delta N2-26}$ variant to function *in vivo* and *in vitro*. Deletion of this helix did not affect the ability of the mutant CobA protein to support growth of a *metE cobA* strain on glycerol (data not shown). However, the $CobA^{\Delta N2-26}$ protein failed to support growth on ethanolamine as sole carbon source (Fig. 5).

Results from *in vitro* assays showed that the specific activity of the $CobA^{\Delta N2-26}$ enzyme was only 3% of the $CobA^{WT}$ enzyme when FldA was used as the reductant. The same protein has 4-fold higher specific activity than the wild-type when cob(I)alamin was generated with KBH_4. Eliminating the N-terminal helix resulted in a more efficient enzyme as long as cob(I)alamin is in excess. When the concentration of cob(I)alamin in the active site depends on interaction with FldA, the absence of the N-terminal helix of CobA affects AdoCbl production. Deletion of the N-terminal helix of CobA may affect electron transfer, catalysis, or both.

DISCUSSION

Computer Modeling as a Tool to Investigate Protein-Protein Interactions—At present, there is a great deal of interest in improving our molecular understanding of how protein complexes form. Fortunately, the accumulation of data in protein databases is rapidly increasing as a result of structural genomics initiatives. Here we report one example of how this valuable information can be used to generate experimentally testable models of protein docking. Sophisticated, user-friendly software for the manipulation of protein structure data is available to researchers interested in studying enzyme complexes. As shown here, a computer-assisted model facilitates the design of peptide mimics predicted to strongly inhibit the interactions between partner proteins. Structure models and peptide mimics can be used to investigate the contribution of specific amino acid residues to the formation of biologically relevant protein-protein contacts.

Insights into CobA·FldA Interactions—We have identified part of the regions of the CobA and FldA proteins that allow electron transfer from the flavin in FldA to the co(II)rrinoid in the active site of CobA. Four pieces of evidence support the conclusion that FldA residues TWYYGEAQCDWDD[68] are part of the FldA structure that interacts with CobA. First, a 30% inhibition of CobA activity when the TWYYGEAQCDWDD[68] peptide is present in the assay is significant given the small size of the peptide (13 amino acids). Second, there was a complete lack of an inhibitory effect by another FldA-derived peptide of similar size and hydrophobicity. Third, mutagenized peptide TWYYG**A**AQCDWD**A**[68] failed to inhibit adenosylation, demonstrating that residues Glu-61 and Asp-68 are key components of the FldA surface interacting with CobA. Fourth, this peptide is part of the surface of FldA that interacts with MetH and Fpr (28).

Residues of CobA Critical for Docking—We suggest that residue Arg-9 is essential for docking of CobA with FldA (TABLE THREE). Regardless of the nature of the change at this position, CobA cannot dock with FldA if Arg-9 is altered. A strong case for docking can also be made for residue Arg-165. However, there is more tolerance for change at this position. A $CobA^{R165A}$ variant still supported growth on ethanolamine, indicating that FldA-dependent reduction of the substrate was sufficient to meet the high demand of AdoCbl required to grow on ethanolamine. Only a drastic change in the charge of the side chain (*e.g.* Arg to Glu) prevented docking of CobA with FldA. The role of residues Arg-9 and Arg-165 appears to be limited to CobA·FldA interaction, since the catalytic competence of mutant enzymes unable to dock with FldA was equal to that of the wild-type protein (TABLE THREE, column B, lines 1, 2–3, 6–7).

It is not surprising that a truncation of the N-terminal helix results in poor interactions of CobA with FldA. $CobA^{\Delta N2-26}$ protein is missing residue Arg-9 as well as other residues that may be involved in docking. What is surprising, however, is the substantial increase in the specific activity of the enzyme (4-fold higher than $CobA^{WT}$ enzyme). We speculate that this increase in specific activity may be due to an increase in the rate of substrate binding, product release, or both.

Results from recent EPR and MCD studies by Stich *et al.* (25) have provided evidence for the existence of a four-coordinate co(II)rrinoid in the active site of the wild-type CobA enzyme. The effect that FldA docking to CobA may have on electron transfer or stability of the co(II)rrinoid substrate is under investigation. Use of the $CobA^{\Delta N2-26}$ protein might be especially useful in probing the role of residues in the

N-terminal helix in activation and stabilization of the corrinoid substrate in the CobA active site.

Differences in the Sensitivity of the in Vivo Assays for AdoCbl Reveal the Coenzyme B_{12} Requirement of S. enterica—We typically use low Cbl levels (1 nM) in the culture medium to satisfy the methionine requirement of *S. enterica*. In contrast, optimal growth rates on ethanolamine as carbon and energy source require a >2 orders of magnitude higher concentration of Cbl in the medium (150 nM (34)). It was surprising to learn that a mutant CobA protein that is one-tenth as active as the wild-type enzyme can meet the demand for AdoCbl during growth on ethanolamine. This result explains why it has been difficult to isolate *cobA* alleles that do not contain nonsense or missense mutations that severely destabilize the protein. The low requirement for endogenous AdoCbl when growing on ethanolamine means that *Salmonella* strains carrying *cobA* alleles encoding defective CobA variants appear indistinguishable from *cobA*$^+$ strains on solid media.

Acknowledgments—We thank Ivan Rayment for help with PyMOL and Perry Frey for critical review of the manuscript.

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