Bacterial ApbC Protein Has Two Biochemical Activities That Are Required for *in Vivo* Function*

Jeffrey M. Boyd, Jamie L. Sondelski, and Diana M. Downs[1]

From the Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

The ApbC protein has been shown previously to bind and rapidly transfer iron-sulfur ([Fe-S]) clusters to an apoprotein (Boyd, J. M., Pierik, A. J., Netz, D. J., Lill, R., and Downs, D. M. (2008) *Biochemistry* 47, 8195– 8202. This study utilized both *in vivo* and *in vitro* assays to examine the function of variant ApbC proteins. The *in vivo* assays assessed the ability of ApbC proteins to function in pathways with low and high demand for [Fe-S] cluster proteins. Variant ApbC proteins were purified and assayed for the ability to hydrolyze ATP, bind [Fe-S] cluster, and transfer [Fe-S] cluster. This study details the first kinetic analysis of ATP hydrolysis for a member of the ParA subfamily of "deviant" Walker A proteins. Moreover, this study details the first functional analysis of mutant variants of the ever expanding family of ApbC/Nbp35 [Fe-S] cluster biosynthetic proteins. The results herein show that ApbC protein needs ATPase activity and the ability to bind and rapidly transfer [Fe-S] clusters for *in vivo* function.

Proteins containing iron-sulfur ([Fe-S]) clusters are employed in a wide array of metabolic functions (reviewed in Ref. 1). Research addressing the biosynthesis of the iron-molybdenum cofactor of nitrogenase in *Azotobacter vinelandii* led to the discovery of an operon (*iscA*[nif]*nif*USV*cys*E1) involved in the biosynthesis of [Fe-S] clusters (reviewed in Ref. 2). Subsequent experiments led to the finding of two more systems involved in the *de novo* biosynthesis of [Fe-S] clusters, the *isc* and the *suf* systems (3, 4). Like *Escherichia coli*, the genome of *Salmonella enterica* serovar Typhimurium encodes for the *isc* and *suf* [Fe-S] cluster biosynthesis machinery.

Recent studies have identified a number of additional or non-*isc*/-*suf*-encoded proteins that are involved in bacterial [Fe-S] cluster biosynthesis and repair. Examples include the following: CyaY, an iron-binding protein believed to be involved in iron trafficking and iron delivery (5–7); YggX, an Fe^{2+}-binding protein that protects the cell from oxidative stress (8, 9); ErpA, an alternate A-type [Fe-S] cluster scaffolding protein (10); NfuA, a proposed intermediate [Fe-S] delivery protein (11–13); YtfE, a protein proposed to be involved in [Fe-S] cluster repair (14, 15); and CsdA-CsdE, an alternative cysteine desulferase (16).

Analysis of the metabolic network anchored to thiamine biosynthesis in *S. enterica* identified lesions in three non-*isc* or -*suf* loci that compromise Fe-S metabolism as follows: *apbC*, *apbE*, and *rseC* (17–21). This metabolic system was subsequently used to dissect a role for *cyaY* and *gshA* in [Fe-S] cluster metabolism (6, 22, 23). Of these, the *apbC* (*mrp* in *E. coli*) locus was identified as the predominant site of lesions that altered thiamine synthesis by disrupting [Fe-S] cluster metabolism (17, 18).

ApbC is a member of the ParA subfamily of proteins that have a wide array of functions, including electron transfer (24), initiation of cell division (25), and DNA segregation (26, 27). Importantly, ATP hydrolysis is required for function of all well characterized members of this subfamily, and all members contain a "deviant" Walker A motif, which contains two lysine residues instead of one (GK*XXX*GK(S/T)) (28). ApbC has been shown to hydrolyze ATP (17).

Recently, five proteins with a high degree of identity to ApbC have been shown to be involved in [Fe-S] cluster metabolism in eukaryotes. The sequence alignments of the central portion of these proteins and bacterial ApbC are shown in Fig. 1. HCF101 was demonstrated to be involved in chloroplast [Fe-S] cluster metabolism (29, 30). The CFD1, Npb35, and huNbp35 (formally Nubp1) proteins were demonstrated to be involved in cytoplasmic [Fe-S] cluster metabolism (31, 32). Ind1 was demonstrated to be involved in the maturation of [Fe-S] clusters in the mitochondrial enzyme NADH:ubiquinone oxidoreductase (33). There is currently no report of any of these proteins hydrolyzing ATP.

Biochemical analysis of ApbC indicated that it could bind and transfer [Fe-S] clusters to *Saccharomyces cerevisiae* apo-isopropylmalate isomerase (34). Additional genetic studies indicated that ApbC has a degree of functional redundancy with IscU, a known [Fe-S] cluster scaffolding protein (35, 36).

In this study we investigate the correlation between the biochemical properties of ApbC (*i.e.* ATPase activity, [Fe-S] cluster binding, and [Fe-S] cluster transfer rates) and the *in vivo* function of this protein. This is the first detailed kinetic analysis of ATP hydrolysis for a member of the ParA subfamily of deviant Walker A proteins and the first functional analysis of a member of the ever expanding family of ApbC/Nbp35 proteins. Data presented indicate that noncomplementing variants have distinct biochemical properties that place them in three distinct classes.

EXPERIMENTAL PROCEDURES

Materials—FeCl$_3$ (ACS grade), thiamine (>99%), Li$_2$S (>98%), Fe(NH$_4$)$_2$(SO$_4$)$_2$ (>99%), 3-(2-pyridyl)-5,6-di(2-fu-

TABLE 1

Strains and plasmids

Unless indicated otherwise, strains were constructed for this study. The *yggX*::Gm allele has been described (77). Tn *10*d refers to the transposition-defective mini-Tn*10* (Tn*10*D-16 D-17) (78).

Strain	Genotype	
DM5104	DM1 wild type	
DM5986	*apbC55*:Tn*10*d(Tc) *yggX*::Gm[a]	
DM9450	*purF2085 apbC55*::Tn*10*d(Tc)	

Plasmid	Vector and insert	Insert
pET20b	pET20b(Ap)	None
pES1	pET20b(Ap)	*apbC*[b]
pES2	pET20b(Ap)	*apbC* (K121A)[b]
pJB1	pET20b(Ap)	*apbC* (S122A)
pJB2	pET20b(Ap)	*apbC* (C70A)
pJB3	pET20b(Ap)	*apbC* (C283A)
pJB4	pET20b(Ap)	*apbC* (C286A)
pJB5	pET20b(Ap)	*apbC* (S182A)
pJB6	pET20b(Ap)	*apbC* (S116A)
pJB7	pET20b(Ap)	*apbC* (C283A,C286A)

[a] Resistances are indicated as follows: Tc, tetracycline; Ap, ampicillin; Gm, gentamycin.

[b] Plasmids were constructed for a previous study (17).

ryl)-1,2,4-triazine-5′,5″-disulfonic acid (>99%), ascorbic acid (>99%), $(C_2H_3O_2)_2Zn\cdot2H_2O$ (reagent grade), *N,N*-dimethyl-*p*-phenylenediamine sulfate (98%), and L-cysteine (>98%) were purchased from Sigma. DL-*threo*-3-Isopropylmalic acid (96%) was purchased from Wako Pure Chemical Co Osaka, Japan. All other chemicals were of the highest purity available. The BCA protein assay kit and bovine serum albumin were purchased from Pierce. *E. coli* BL21 (AI*) cells were purchased from Novagen.

Bacterial Strains, Culture Media, and Growth Conditions—Bacterial strains were derivatives of *S. enterica* serovar Typhimurium strain LT2 (specifically DM1) whose genotypes are shown in Table 1. Plasmids carrying the desired allele of *apbC* fused to a C-terminal His tag in pET20b were constructed as described (17) using XhoI instead of NcoI. All constructs used were verified by sequencing at the University of Wisconsin Biotechnology Center. Plasmids were moved between strains via electroporation. Overnight cultures were grown in Difco nutrient broth with appropriate antibiotic for strains harboring a plasmid. Test cultures were grown at 37 °C shaking at an approximate 45° angle to increase aeration. The absorbance of cultures was taken at 650 nm on a Bausch and Lomb Spectronic 20.

Nutritional Analysis—Complementation analyses were performed in strains where plasmids encoded the only copy of *apbC* present in the cell. Complementation was assessed using 5-ml cultures of no carbon E (NCE) medium supplemented with 1 mM $MgSO_4$ and trace minerals. Cultures were started with a 1:100 inoculum. When present in the media, the following were used at the specified final concentration: adenine, 0.4 mM; thiamine, 100 nM; glucose, 11 mM; tricarballylate, 20 mM; gluconate, 11 mM; and ampicillin, 30 µg/ml.

Three growth assays, using two genetic backgrounds, were used to examine the ability of ApbC proteins to function *in vivo*. The two genetic backgrounds used were *apbC yggX* and *purF apbC*. *In vivo* function was assayed by growth on tricarballylate as a carbon and energy source or growth in the absence of exogenous thiamine on either glucose or gluconate, respectively.

The requirement for ApbC in these growth conditions has been described previously (17, 18, 23, 35, 37).

Anaerobic Work—Anaerobic work was performed using a Coy anaerobic glove box (Grass Lake, MI) or vacuum manifold. Before placement inside the anaerobic chamber, solutions were made anoxic by repeated evacuation and flushing with N_2 gas passed over a heated copper column for removal of O_2. Outside of the glove box, all solutions were added to anaerobic cuvettes using gas-tight Hamilton syringes.

ApbC and Leu-1 Protein Purification—Purification of ApbC and Leu-1 has been described previously (34).

Protein Concentration Determination—Protein concentration was determined using a colorimetric assay or an empirically determined extinction coefficient (apo-ApbC-His$_{280}$ = 43.1 mM^{-1} cm^{-1}). The colorimetric assay was copper-based and used a reagent containing bicinchoninic acid to detect of the cupreous ion (Pierce). Bovine serum albumin (2 mg/ml) was used as a standard.

[Fe-S] Cluster Reconstitution—Iron-sulfur cluster reconstitution was described previously (34). Briefly, ApbC protein (2.1 mg/ml; 52 µM) suspended in buffer A (50 mM Tris, pH 8.0, 150 mM NaCl) was incubated for 1 h in an anoxic environment in the presence of 5 mM DTT.[2] A 5-fold excess of $FeCl_3$ was added to the protein followed by a 5-fold excess Li_2S. Proteins were incubated for 1 h, and excess S^{2-}, Fe^{3+}, and DTT was removed by passing the reaction mixture over a PD-10 column (GE Healthcare). One mM DTT was added to protein post desalting.

Leu-1 Activation Assays—Leu-1 activation assays have been described previously (34). Briefly, apo-isopropylmalate isomerase (apo-Leu-1) (25 µM) was pre-reduced with 5 mM DTT in the anaerobic glove box for at least 1 h prior to assay initiation. The Leu-1 activation assays contained 3.5 µM Leu-1, 5 mM DTT, 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl in a total volume of 500 µl. Assays were initiated by the addition of 4 µM ApbC protein. Ten-µl aliquots of the assay mixture were removed at time points, and Leu-1 was assayed for the ability to convert 3-isopropylmalate to dimethylcitraconate acid spectrophotometrically (dimethylcitraconate ϵ_{235} = 4.35 mM^{-1} cm^{-1}) (38).

The second-order rate constant for ApbC dependent Leu-1 activation was determined by linearization of the data and fitting the data using linear regression. The ApbC-dependent activation of Leu-1 is summarized by Reaction 1,

$$\text{holo-ApbC} + \text{apo-Leu-1} \rightarrow \text{holo-Leu-1}$$
REACTION 1

The amount of holo-ApbC protein present (4 µM initial) in the assay mixture at a given time point was determined using Equation 1,

$$[\text{holo-ApbC}] = 4\text{-}(SA_x/SA_{40}) \cdot 4 \qquad \text{(Eq. 1)}$$

where SA_x is the specific activity of Leu-1 at a fixed time point, and SA_{40} is the specific activity of Leu-1 after the reaction had proceeded for 40 min. The second-order rate constant of cluster transfer was determined by fitting the 1/[holo-ApbC protein] *versus* time post the addition of holo-ApbC protein to

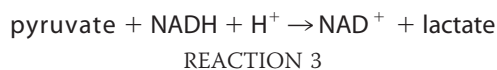[2] The abbreviation used is: DTT, dithiothreitol.

apo-Leu-1 data using linear regression, where k is the slope of the fit.

Spectroscopic Techniques—The UV-visible absorption spectra were recorded with a Lambda Bio 40 spectrophotometer (PerkinElmer Life Sciences) using 1.5-ml Sterna Cell cuvettes that can be anaerobically sealed (Atascadero, CA).

Metal Analysis—Inductively coupled plasma-mass spectrometry was conducted by the Soil and Plant Analysis Laboratory at the University of Wisconsin-Madison. The concentration of non-heme iron and acid-labile sulfide was also determined as described elsewhere (39).

Assay to Monitor Inorganic Phosphate—The direct colorimetric malachite green/molybdate/polyvinyl alcohol assay as described by Chan *et al.* (40) was used to monitor inorganic phosphate (P_i) release from nucleotide hydrolysis. Assays contained 0–0.34 mg of purified wild-type ApbC, 0 or 5 mM $MgCl_2$, 0 or 200 mM KCl, 0 or 5 mM nucleotide triphosphate, and 50 mM Tris, pH 8.0. The assays were initiated by addition of ApbC. The mixture was allowed to react at room temperature for 30 min before 20 μl of the reaction mixture was removed, diluted to 250 μl with buffer A, and quickly added to 1 ml of the malachite green/molybdate/ polyvinyl alcohol assay mixture. The mixture was incubated for 15 min at room temperature before sample absorbance was monitored at 630 nm in a Molecular Devices SpectraMAX Plus microplate reader. Because of daily variation of absorbance, a standard curve containing 0–15 nmol of P_i was constructed for each set of assays.

Coupled Spectrophotometric Assay for ApbC ATPase Activity—A continuous spectrophotometric assay was developed that couples ADP production with the oxidation of NADH to NAD^+ (41) as shown in Reactions 2 and 3.

$$ADP + phosphoenolpyruvate \rightarrow ATP + pyruvate$$
<div align="center">REACTION 2</div>

$$pyruvate + NADH + H^+ \rightarrow NAD^+ + lactate$$
<div align="center">REACTION 3</div>

The assays were initiated when purified ApbC protein (10–350 μg) was added to an assay mixture containing 0.2 mM NADH, 4 mM phosphoenolpyruvate, 0–12 mM $MgCl_2$, 200 mM KCl, 20 units of lactate dehydrogenase, 20 units of pyruvate kinase, 0.01–10 mM ATP (stocks prepared in 1 M Tris, pH 8.0), and 50 mM Tris, pH 8.0. The assay volume was 1 ml, and assays were conducted anaerobically in 1.5 ml of Sterna Cell cuvettes (Atascadero, CA) that had a screw cap top and rubber septum. After the addition of ApbC, the oxidation of NADH was monitored by following the absorbance change at A_{340} from 2 to 4 min (linear region).

The linear steady-state portion of the A_{340} *versus* time plot was used to determine the Hill coefficient by fitting initial velocity data to Equation 2 ($R^2 \geq 0.99$),

$$v = V_{max} \cdot S^h/(K_m^h + S^h) \tag{Eq. 2}$$

where v is velocity; S is the concentration of varied substrate; V_{max} is the velocity at saturating substrate; K_m is the Michaelis constant for the varied substrate, and h is the Hill coefficient

(labeled n_H in Table 4). The kinetic parameters V_{max} and K_m were determined by fitting the v *versus* [ATP] initial velocity data to Equation 3 ($R^2 \geq 0.99$),

$$v = V_{max}^1 \cdot S/(K_m^1 + S) + V_{max}^2 \cdot S/(K_m^2 + S) \tag{Eq. 3}$$

The velocity data as plotted in the double-reciprocal form was fit by Equation 4,

$$y = (a + bx + cx^2)/(1 + ex) \tag{Eq. 4}$$

where the initial slope is defined as $b − ae$; the slope of the asymptote is c/e; the y intercept is a, and the asymptote intercept is $(be − c)/e^2$.

Computational Analysis—Nonlinear and linear regression analyses and curve fitting to first-order rate laws were performed using the software SigmaPlot (version 9.0).

Bioinformatic Analysis—Protein sequences were acquired from the NCBI website in FASTA format. The accession numbers for sequences used are as follows: Ind1 EAK91699; Nbp35 CAA96797.1; Cfd1, AAS56623; Hcf101, AAR97892.1, ApbC (mrp), NP_461098; and Nubp1, NP_002475.2.

Sequence alignments were performed using the Clustal_W function of the Lasergene software by DNAstar, Madison, WI. Default settings were used, including the Gonnet Series for Protein Weight Matrix, gap penalty of 10, and a gap length penalty of 0.2. Selected regions of sequences were used to give the best alignment of the similar regions. The protein sequences for the proteins used in Fig. 1 were acquired in FASTA format from the NCBI Protein Sequence Data Base.

The ApbC amino acid sequence was used as the input for the three-dimensional Jigsaw version 2.0 Comparative Modeling Server by Cancer Research UK using the automatic mode (42– 44). The resulting text file alignment was viewed using the PyMOL Viewer from Delano Scientific, LLC, for protein structures. Regions of the proteins that do not have significant similarity are excluded from the structures. The accession numbers of the structures used to thread the ApbC sequence are 2bej_A for Soj and 2afk_H for NifH and can be found on the NCBI website.

RESULTS

Protein Sequence Alignments Show Significant Conservation of Protein Sequence within the ApbC/Nbp35 Subfamily of ParA Homologs—The ApbC/Nbp35 subfamily proteins show a high degree of conservation in their central and C-terminal domains. Representative members of the ApbC/Nbp35 family are shown in Fig. 1. The N-terminal 100 amino acids of bacterial ApbC are sparsely conserved in the plant homolog HCF101, yeast Nbp35, human Nubp1, and mitochondrial Ind1 and therefore are not included in the alignments. The N-terminal extension is missing in yeast Cfd1. The N-terminal region of ApbC showed no similarity to characterized functional domains of the N-terminal extension found in ParA and ParA homologs (25). The central and C-terminal domains of these proteins contain two highly conserved domains as follows: the Walker A box for ATP binding/hydrolysis, and a C*XX*C motif in the C-terminal third of the protein. In Fig. 1 the Walker A box, conserved Cys residues, and conserved Ser are *boxed* for clarity. All six of these
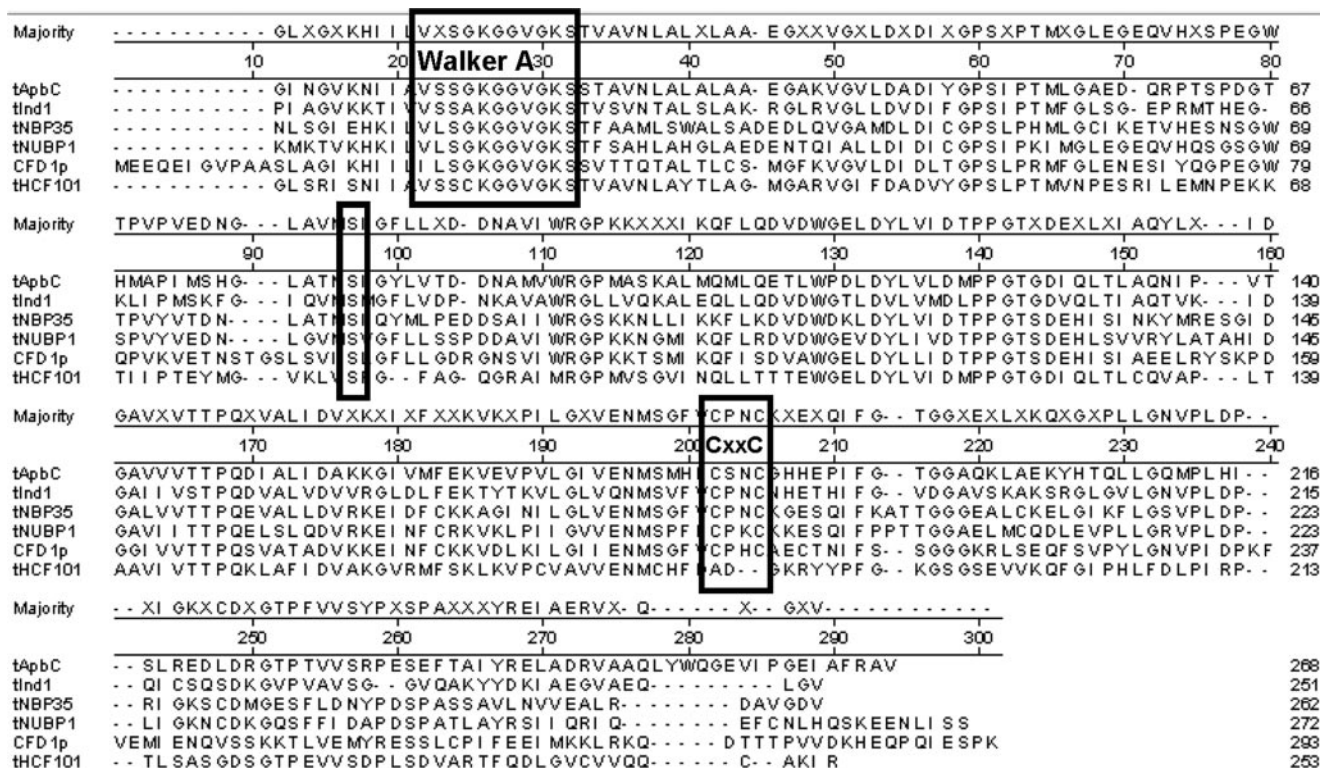
FIGURE 1. **Protein sequence alignments of members of the ApbC/Nbp35 subfamily of ParA family of proteins.** Protein alignments were assembled using the Clustal_W method in the Lasergene® software and show only the central portion of the proteins, which have the highest sequence conservation. The *three boxed areas* highlight the Walker A box, conserved Ser residue, and C*XX*C motif. Proteins listed are as follows: ApbC (*S. enterica* serovar Typhimurium LT2), CFD1 (*S. cerevisiae*), Nbp35 (*S. cerevisiae*), HCF101 (*Arabidopsis thaliana*), huNpb35 (formally Nubp1) (*Homo sapiens*), and Ind1 (*Candida albicans*).

proteins are thought to be involved in [Fe-S] cluster metabolism (29–35).

In Vivo Function of ApbC Is Abolished by Mutations in the Walker A Motif, CXXC Motif, and a Conserved Serine—A number of mutant alleles of apbC were created using plasmid pES1, which encodes the C-terminal hexahistidine-tagged ApbC protein (17). Directed mutations substituted single residues to alanine in three regions of the protein (see Fig. 1) as follows: 1) deviant Walker A motif necessary for ATP binding/hydrolysis (ApbC$_{K116A}$, ApbC$_{K121A}$, and ApbC$_{S122A}$) (28); 2) conserved cysteine residues with spacing consistent with a metal-binding/thioredoxin motif (ApbC$_{C283A}$ and ApbC$_{C286A}$) (45); and 3) conserved serine (ApbC$_{S182A}$) and nonconserved cysteine (ApbC$_{C70A}$).

To probe the *in vivo* function of ApbC variants, complementation assays were conducted examining three growth defects using two genetic backgrounds. Complementation of two of the growth defects required restoration of the low flux thiamine biosynthesis pathway (the amount of thiamine pyrophosphate in wild-type *S. enterica* grown in minimal glucose medium is 41.4 pmol/mg dry weight (46)). Stains lacking *apbC* and *yggX* (or *purF*) are thiamine auxotrophs likely because of poor cluster occupancy of ThiH/ThiC (47, 48, 80). Complementation of a third growth defect required function of the high flux tricarballylate catabolic metabolic pathway (35, 37, 49, 50). Strains lacking *yggX* and *apbC* are unable to use tricarballylate as a carbon and energy source, and presumably, this is from poor [Fe-S] cluster occupancy of TcuB (35).

Plasmids carrying alleles of *apbC* were introduced into two genetic backgrounds (*apbC yggX* and *apbC purF*) and provided the only source of *apbC* in the resulting strains. Each of the seven mutant alleles was tested for the ability to complement growth in both the low and high flux demand situations. The data in Table 2 show that substitutions in the Walker A (ApbC$_{K116A}$, ApbC$_{K121A}$, and ApbC$_{S122A}$) and C*XX*C (ApbC$_{C283A}$ and ApbC$_{C286A}$) motifs resulted in proteins unable to complement any of the three growth conditions. Changing the nonconserved Cys-70 to Ala had no detectable effect on *in vivo* function of the protein. In all cases, the empty vector and wild-type control plasmids displayed the growth patterns anticipated by past experiments (17, 35).

The ApbC$_{S182A}$ variant qualitatively restored thiamine synthesis, although there was a 2–4-h lag before exponential growth initiated (data not shown). In addition, the doubling times of the *yggX apbC* or *purF apbC* strains carrying ApbC$_{S182A}$, on glucose or gluconate in the absence of thiamine, were ~1.6-fold higher than with the wild-type protein. The ApbC$_{S182A}$ variant was unique in its ability to restore growth under the low demand growth condition monitoring thiamine biosynthesis but not under the high demand condition monitoring tricarballylate utilization.

All ApbC variant proteins accumulated to qualitatively similar levels in *yggX apbC* cells when expressed off a plasmid and detected by Western blot analysis (data not shown). Thus, the phenotypes seen were not because of differential protein stability.

TABLE 2
Growth allowed by ApbC variants

Doubling times were calculated using the formulas $\mu = \ln(X/X_o)/T$, where μ is the growth rate; X and X_o are absorbance at 650 nm; T(h) is the time between absorbance readings X and X_o; and doubling time $(g) = (\ln 2)/\mu$ (79). Numbers represent the average of three independent cultures.

| | Relevant chromosomal genotype | | | | |
| | *apbC yggX* | | | *purF apbC* | |
ApbC variant	Glu[a]	Glu + Thi	TC + Thi	Glcn + Adn	Glcn + Adn + Thi
Vector	NG (0.33)[b]	2.5 ± 0.32	NG (0.21)	NG (0.16)	1.5 ± 0.07
Wild type	1.6 ± 0.03	1.7 ± 0.16	2.7 ± 0.26	2.4 ± 0.28	1.6 ± 0.08
ApbC(C70A)	1.7 ± 0.17	1.6 ± 0.06	3.1 ± 0.54	2.0 ± 0.02	1.4 ± 0.04
ApbC(K116A)	NG (0.25)	1.8 ± 0.12	NG (0.24)	NG (0.18)	1.7 ± 0.26
ApbC(K121A)	NG (0.31)	2.2 ± 0.10	NG (0.19)	NG (0.10)	2.1 ± 0.24
ApbC(S122A)	NG (0.27)	2.3 ± 0.16	NG (0.15)	NG (0.12)	1.9 ± 0.09
ApbC(S182A)	2.7 ± 0.09	1.7 ± 0.08	NG (0.28)	2.4 ± 0.15	1.5 ± 0.24
ApbC(C283A)	NG (0.27)	2.5 ± 0.12	NG (0.19)	NG (0.15)	1.6 ± 0.16
ApbC(C286A)	NG (0.23)	2.4 ± 0.22	NG (0.20)	NG (0.18)	1.6 ± 0.04

[a] Abbreviations used are as follows: Glu, glucose; Thi, thiamine; TC, tricarballylate; Glcn, gluconate; Adn, adenine.
[b] NG, no growth was determined when strains showed doubling times of greater than 6 h and/or a final A_{650} less than 0.35. Average final A_{650} is indicated in parentheses for strains determined as no growth.
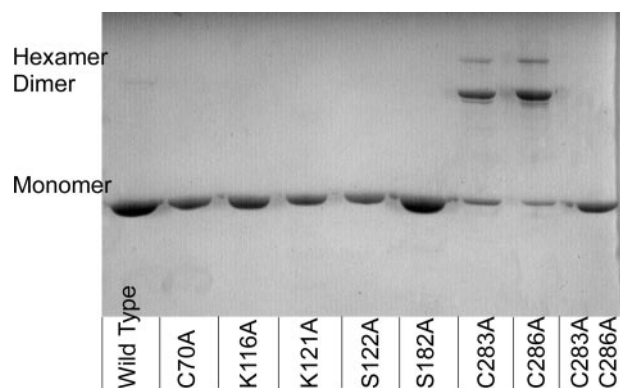

FIGURE 2. **Nonreducing SDS-PAGE analysis of purified ApbC proteins.** The ApbC protein in each lane is labeled at the *bottom* of the gel, and the migration of protein as a monomer, dimer, and hexamer is labeled on the *left-hand side* of the gel. Three μg of each ApbC protein was loaded onto the gel.

TABLE 3
Variant ApbC proteins bind and transfer iron-sulfur clusters

ApbC proteins were reconstituted as described under "Experimental Procedures." Iron and sulfide were quantified as described elsewhere (39). Metal binding data are the average of two independent reconstitutions.

ApbC variant	Iron	Sulfur	Rate of cluster transfer to Leu-1[a]
			$M^{-1} min^{-1}$
ApbC	2.2 ± 0.2	2.1 ± 0.1	39,000 ± 2,000
ApbC$_{K16A}$	2.0 ± 0.2	1.9 ± 0.0	25,000 ± 1,000
ApbC$_{K21A}$	1.9 ± 0.1	1.9 ± 0.1	27,000 ± 1,000
ApbC$_{S22A}$	1.9 ± 0.2	1.8 ± 0.1	43,000 ± 1,000
ApbC$_{S182A}$	1.7 ± 0.2	1.8 ± 0.0	45,000 ± 1,000
ApbC$_{C283A}$	1.8 ± 0.1	1.7 ± 0.2	45,000 ± 1,000
ApbC$_{C286A}$	1.7 ± 0.2	1.9 ± 0.1	44,000 ± 5,000
ApbC$_{C283A,C286A}$	0.9 ± 0.2	1.0 ± 0.0	ND[b]

[a] Leu-1 activation assays were conducted as described previously (34). The second-order rate constant of cluster transfer was determined by a linear transformation of the data and fitting the data using linear regression as described under "Experimental Procedures."
[b] ND means not determined.

Purification and Metal Analysis of ApbC Proteins—ApbC proteins were expressed in *E. coli* BL21 AI* using the pET20 system and purified as C-terminal hexahistidine-tagged fusion proteins via Ni^{2+}-affinity chromatography. The single column purification yielded proteins that were >95% pure. The behavior of all ApbC variants resembled that of the wild-type protein during purification except for ApbC$_{C286A}$ and ApbC$_{C283A}$, which had lower yields and precipitated upon long dialysis or high concentration (>10 mg/ml).

To examine the possibility that variant ApbC proteins could form intermolecular disulfide bonds, the electrophoretic mobility of purified ApbC proteins was examined using nonreducing SDS-PAGE. Fig. 2 shows the separation of ApbC proteins into monomers, dimers, and hexamers using a nonreducing SDS-polyacrylamide gel. Covalent linkage of ApbC monomers was seen with the ApbC$_{C283A}$ and ApbC$_{C286A}$ proteins. A small but reproducible amount of dimer was also seen with the wild-type protein. All other ApbC proteins migrated as monomers. Upon the addition of 1 mM β-mercaptoethanol to the sample buffers, all of the protein samples migrated exclusively as monomers (data not shown). These data suggest that ApbC$_{C283A}$ and ApbC$_{C286A}$ can form covalent disulfide linkages spanning the dimer interface.

Metal Binding by Variant ApbC Proteins—As was seen with the wild type, none of the ApbC proteins examined co-purified

with a detectable concentration of iron or sulfur atoms. ApbC did bind two iron and two sulfur atoms per monomer when chemically reconstituted with ferrous iron and sulfide under reducing conditions (34).

All the ApbC variant proteins, except ApbC$_{C283A,C286A}$, bound approximately two iron and two acid-labile sulfide atoms per ApbC monomer as has been reported previously with wild-type ApbC (Table 3) (34). The ApbC$_{C283A,C286A}$ variant protein bound approximately one atom of each iron and sulfide and was not further characterized here.

ApbC Variant Proteins Can Transfer [Fe-S] Clusters—The previously described model system using isopropylmalate isomerase protein Leu-1 from *S. cerevisiae* was used to assay the ability of variant ApbC proteins to transfer their respective [Fe-S] clusters (34, 51). Previous results showed that 2 mol of holo-ApbC are required for full activation of 1 mol of apo-Leu-1 (34). The assays described here had 3.5 μM Leu-1 protein and 4 μM ApbC protein; therefore, apo-Leu-1 was present in a 1.75-fold excess of holo-ApbC dimer.

All ApbC variants were capable of activating apo-Leu-1, but the data show ApbC protein variants can be divided into two classes (Table 3). One class of ApbC proteins, consisting of wild type, ApbC$_{S122A}$, ApbC$_{S182A}$, ApbC$_{C283A}$, and ApbC$_{C286A}$, activated Leu-1 rapidly and effectively with a second-order rate constant of ~40,000 M^{-1} min^{-1}. The second class of ApbC variant proteins, consisting of ApbC$_{K116A}$ and ApbC$_{K121A}$, acti-
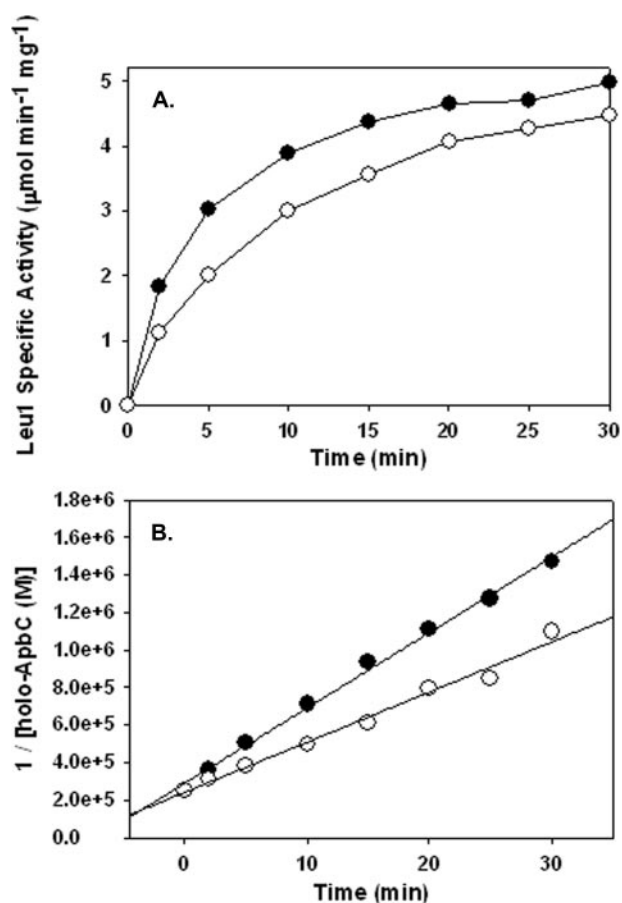
FIGURE 3. **ApbC proteins activate Leu-1 at variable rates.** *A,* time course of ApbC- (●) or ApbC$_{K121A}$ (○)-dependent Leu-1 activation. Leu-1 activity was monitored at fixed time points by the formation of isopropylmaleate, which was monitored at A_{235}. Assays contained 4 μM reconstituted ApbC and 3.5 μM apo-Leu-1. The reaction was started by adding ApbC to the reaction vessel. *B,* plot of 1/[holo-ApbC protein] *versus* time post ApbC protein addition. The second-order rate constant of cluster transfer was determined by fitting the data using linear regression. The concentration of holo-ApbC protein at a given time point was estimated using Equation 1.

TABLE 4

Nucleotide specificity of ApbC

Inorganic phosphate release was detected after a 30-min incubation using the malachite green/molybdate/polyvinyl alcohol assay as described under "Experimental Procedures." Assays were done in triplicate, and the error is listed as standard deviation. Unless indicated, assays contained 0–0.34 mg of purified ApbC, 6 mM MgCl$_2$, 200 mM KCl, 5 mM nucleotide mono-, di-, or triphosphate, and 50 mM Tris, pH 8.0.

Nucleotide (5 mM)	P$_i$ release
	nmol mg^{-1}
ATP	518 ± 18
ATP − Mg^{2+}	ND[a]
ATP − K$^+$	63 ± 27
ATP + AMP	429 ± 36
ATP + ADP	ND
ADP	ND
AMP	ND
dATP	261 ± 30
CTP	42 ± 21
GTP	75 ± 15
TTP	ND
UTP	ND

[a] No phosphate release was detected.

TABLE 5

Kinetic analysis of variant ApbC proteins

ATP hydrolysis was monitored using a spectrophotometric assay coupling the hydrolysis of ATP to the oxidation of NADH using the enzymes pyruvate kinase and lactic dehydrogenase. Assays contained the following: 0.2 mM NADH, 4 mM phosphoenolpyruvate, 20 units of each pyruvate kinase and lactic dehydrogenase, 0–12 mM MgCl$_2$, 200 mM KCl. K_m, V_{max}, and n_H (Hill coefficient) values were determined by fitting the *v versus* [S] curves to modified forms of either the Michaelis-Menten-Henri equation (Equation 3) or Hill equation (Equation 2). Assays were done in triplicate, and the error is listed as standard deviation.

	K_{m1}	K_{m2}	k_{cat}	n_H[a]
	μM	*μM*	*min^{-1}*	
Wild type	1.6 ± 1.1	300 ± 43	1.92	0.48 ± 0.05
K116A	5.7 ± 2.9	1260 ± 170	3.40	0.49 ± 0.06
K121A	6.4 ± 2.7	930 ± 330	1.05	0.35 ± 0.04
C283A	15.0 ± 6.0	3670 ± 1300	0.49	0.29 ± 0.05
C286A	11.0 ± 8.0	1380 ± 600	0.84	0.32 ± 0.06

[a] Abbreviation used is as follows: n_H, Hill coefficient.

vated Leu-1 at ~60% of the rate of the first class. The data distinguishing the two classes in cluster transfer are shown in Fig. 3.

Kinetic Analysis of ApbC ATP Hydrolysis Activity—A discontinuous direct colorimetric assay designed to detect and quantify inorganic phosphate (P$_i$) production, indicative of nucleotide hydrolysis, was used to examine the requirements for ApbC ATPase activity and nucleotide specificity. As shown in Table 4, P$_i$ release was dependent upon the presence of both nucleotide and Mg^{2+}. An 8-fold increase in P$_i$ release was seen upon the addition of K$^+$ to the reaction mix. Although ApbC was unable to hydrolyze the α-β phosphodiester bond of ADP, the inclusion of equal amounts of ADP and ATP in the reaction mix resulted in complete inhibition of ApbC ATPase activity. A 1.2-fold decrease in the amount of P$_i$ release was seen with the inclusion of both AMP and ATP. ApbC was capable of hydrolyzing dATP, CTP, and GTP, but the amount of P$_i$ production was significantly lower than the amount of P$_i$ production from ATP hydrolysis.

A continuous spectrophotometric assay coupling ATP hydrolysis to NADH oxidation using the enzymes pyruvate

kinase and lactic dehydrogenase (Reactions 2 and 3) was used to define the kinetic parameters for ATPase activity of ApbC proteins. High protein concentrations and anaerobic reducing conditions (to prevent NADH oxidation) were used to compensate for the slow rate of ATP hydrolysis catalyzed by ApbC.

Kinetic parameters were determined from initial velocity data for the purified enzymes as described under "Experimental Procedures" (Table 5). The specific activity for wild-type ApbC was determined to be 45.7 nmol min^{-1} mg^{-1} (0.210 mg of ApbC, 5.1 mM MgCl$_2$, 5 mM ATP, and 200 mM KCl, 50 mM Tris, pH 8.0). Thus, the activity of ApbC was found to be 3-fold higher using the coupled assay than that calculated from the P$_i$ release data in Table 4. This difference may reflect inhibition of ApbC by the accumulating ADP in the discontinuous assay. The coupled assay has an ATP-regenerating system, and therefore very little ADP was present in the reaction mix upon initiation of the reaction by ApbC addition or during ApbC-catalyzed ATP hydrolysis.

The relative velocities at various ATP concentrations for ApbC are shown in Fig. 4. The *v versus* [ATP] plots (Fig. 4*A*) were best fit by modified forms of either the Hill equation (Equation 2) or the Michaelis-Menten-Henri equation (Equation 3) (Equation 3 as shown). The double-reciprocal plots (Fig. 4*B*) show data that are nonlinear and have a rising trend. This is
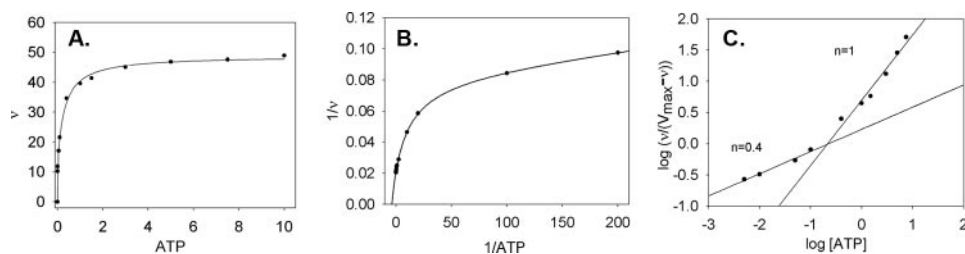
FIGURE 4. **Kinetic results demonstrate cooperative behavior for *S. enterica* ApbC protein when ATP is varied (wild-type ApbC shown).** The plots illustrate the velocity of ADP production while varying their concentration of ATP. Plots are as follows: *A*, *v versus* [substrate] fit by Equation 3; *B*, double-reciprocal plot of the data in plot 1 fit by Equation 4; *C*, Hill plot fit using a linear regression (*n* = slope). Datum points are the average of three assays. *v* is defined as nmol/min/mg.

TABLE 6

Noncomplementing ApbC variants can be divided into three classes

Growth results are as shown in Table 2.

Variant	Growth	ATP hydrolysis[a]	Rapid [Fe-S] cluster transfer	Class
ApbC	+	+	+	
ApbC$_{K116A}$	−	+	−	1
ApbC$_{K121A}$	−	+	−	1
ApbC$_{S122A}$	−	−	+	2
ApbC$_{S182A}$	−[b]	−	+	2
ApbC$_{C283A}$	−	+	+	3
ApbC$_{C286A}$	−	+	+	3

[a] Biochemical results are shown in Tables 3 and 5.
[b] The ApbC$_{S182A}$ variant could synthesize thiamine but could not utilize tricarballylate.

characteristic of enzymes displaying negative cooperativity (52). To verify negative cooperativity, Hill plots were constructed for the data. The Hill plot for the wild-type enzyme is shown in Fig. 4*C*. The plot shows data that have two separate linear regions on the graph, each having a distinct slope. One of the slopes (*n* = 0.4) corresponds to the Hill coefficient (n_H = 0.48; Table 5) derived from fitting the *v versus* [ATP] data to Equation 2.

The kinetic parameters for the wild-type and variant *S. enterica* enzymes are displayed in Table 5. Fitting the data to Equation 4 gives two K_m values; one K_m value for each hypothesized ATP-binding site. The wild-type enzyme had one low (1.6 μM) and one high (300 μM) K_m value for ATP, as would be expected of an enzyme that displays negative kinetic cooperativity for a substrate.

With the exception of ApbC$_{S122A}$ and ApbC$_{S182A}$, all of the variant ApbC proteins hydrolyzed ATP. The kinetic parameters vary slightly for each variant, and no unifying theme was noted. The kinetic differences observed for the variant proteins were manifest in turnover number, the second-site K_m, and Hill coefficient.

Effect of an [Fe-S] Cluster on ATPase Activity—The ATPase activity of holo- and apo-ApbC was examined. The presence of [Fe-S] cluster had no effect on the kinetic parameters of ATP hydrolysis (data not shown). In addition, it was previously shown that the addition of 10 mM Mg·ATP had no effect on the rate that ApbC activated Leu-1 (34).

DISCUSSION

ApbC is a cytosolic protein whose absence impacts [Fe-S] cluster metabolism in *S. enterica*. Previous work in our laboratory has described two biochemical activities for ApbC. ApbC protein can hydrolyze ATP and bind and transfer an [Fe-S]

cluster (17, 34). This study examined the two biochemical activities of a number of variant proteins that are not able to complement an *apbC* null mutation. The results herein show that both of these biochemical activities are required for *in vivo* function, and furthermore, they can be separated by mutation.

Previous data showed that the presence of ATP was not necessary the for [Fe-S] cluster transfer activity of ApbC (34). The data here confirm that the ability to hydrolyze ATP is not necessary for [Fe-S] cluster transfer. Based on these data we envision a general model in which ATP binding and hydrolysis are required to generate holo-ApbC *in vivo* and not for the subsequent transfer of the [Fe-S] cluster.

In this study we targeted residues in three locations of the protein for directed mutagenesis as follows: the Walker A motif, a conserved Ser, and the Cys residues present in a C*XX*C motif. Walker A motifs have been examined in detail and are used to bind and to hydrolyze ATP and transfer the message of ATP binding/hydrolysis to a second biochemical activity elsewhere in the protein (reviewed in Ref. 28). Motifs consisting of two Cys residues separated by two residues (C*XX*C) are common in proteins that bind [Fe-S] clusters.

The noncomplementing variant proteins examined herein were defined by three distinct classes. As seen previously, wild-type ApbC complemented an *apbC* null mutant, hydrolyzed ATP, bound [Fe-S] cluster, and rapidly transferred the cluster to apo-Leu-1; each class of variant ApbC proteins was defective in one or more of these four areas. The classification of these proteins is summarized in Table 6.

The first class of ApbC proteins consisting of ApbC$_{K116A}$ and ApbC$_{K121A}$ hydrolyzed ATP with wild-type kinetics and were capable of binding an [Fe-S] cluster. However, cluster transfer from ApbC to Leu-1 was compromised in these variants.

Both reconstituted ApbC$_{K116A}$ and reconstituted ApbC$_{K121A}$ are dimers (data not shown) and display some degree of negative kinetic cooperativity (Table 5). One interpretation of these data suggest that variant proteins can still communicate ATP binding to adjacent monomers, but may be lacking the ability to communicate ATP binding and/or hydrolysis with the second function of the protein, which is necessary for *in vivo* function.

Other members of this ATPase superfamily of proteins that have mutations directed to the Walker motif have also been shown to hydrolyze ATP (general (28, 53), Lys-116 (54–57), Ser-122 (58), and Lys-121 (59–61)). In some proteins within this family such as NifH, MinD, ArsA, UvrB, and the F1-ATPase, making directed amino acid substitutions in the Walker A motif resulted in the absence of a signal between the ATPase domain and elsewhere within the protein (24, 25, 53, 59–63). In these cases this signal is necessary for the second function of the protein such as electron transfer or protein interaction. These parallels suggest the exciting possibility that further analysis of these ApbC variants will provide clues as to the signals causing cluster transfer from this protein *in vivo*.

Variants of two other bacterial proteins have been shown to be defective in [Fe-S] cluster transfer. The variants IscU$_{D39A}$ and Nfu-1$_{D39A}$ were shown to be compromised in [Fe-S] cluster transfer to apo-aconitase and apo-dinitrogen reductase, respectively (64, 65). These protein variants have been useful in dissecting the mechanism of [Fe-S] cluster biosynthesis and transfer (36, 66–69). There is no evidence to suggest that either of these proteins bind or hydrolyze ATP.

The second class of variant ApbC proteins consisting of ApbC$_{S122A}$ and ApbC$_{S182A}$ had no detectable ATPase activity, but could bind and rapidly transfer [Fe-S] cluster to Leu-1. The Ser-182 residue is conserved in all known ApbC homologs, and the Ser-122 residue resides in the Walker A motif. One explanation for the behavior of these variants is that the ATPase activity is required for loading the cluster *in vivo*, and thus, the *in vitro* system of [Fe-S] cluster reconstitution bypassed the need for this activity.

Interestingly, the ApbC$_{S182A}$ variant was able to complement under growth conditions in which the demand for [Fe-S] cluster-containing proteins was low (thiamine biosynthesis), but not when the demand for [Fe-S] proteins was high (tricarbally-late utilization). It is plausible that ApbC$_{S182A}$ has a low rate of ATP hydrolysis that was out of the sensitivity range of the coupled assay, yet sufficient for the low demand of thiamine synthesis. Alternatively, the protein is not capable of hydrolyzing ATP, but the ApbC$_{S182A}$ variant is locked into a conformation that allows for a single turnover, which may fulfill the ApbC requirement in the low flux thiamine requirement condition.

The third class of variant ApbC proteins, consisting of ApbC$_{C283A}$ and ApbC$_{C286A}$, could hydrolyze ATP and transfer an [Fe-S] cluster that was reconstituted on the proteins *in vitro*. Our hypothesis is that ApbC binds an [Fe-S] cluster that bridges the dimer interface ligated by four Cys residues, two cysteine ligands from each ApbC monomer. Therefore, we anticipated that these cysteines would be required for *in vivo* ApbC function, as they were. Similarly, studies by others have shown that the Cys residues that are proposed [Fe-S] cluster ligands in cluster scaffolding proteins, the proposed intermediate [Fe-S] cluster carrier NfuA, and eukaryotic ApbC homologs are essential for *in vivo* function (11, 31, 68, 70–73). Therefore, it was surprising that these ApbC variant proteins could bind an [Fe-S] cluster when artificially reconstituted *in vitro*. To the best of our knowledge there is no report of whether similar variants of the other proteins can bind an [Fe-S] cluster *in vitro*.

Previous work by Golbeck and co-workers (74) showed that externally supplied organic molecules such as 2-mercaptoethanol could act as an artificial ligand to a [4Fe-4S] cluster. Similar bio-inorganic studies have examined the ability of a 16-amino acid ferredoxin maquette peptide containing four Cys residues to bind a [4Fe-4S] cluster. The authors found that when one or more of these Cys residues were changed to Ala, the ferredoxin maquettes could still bind an [4Fe-4S] cluster, although the yield of [4Fe-4S] cluster binding was lower (75, 76). DTT was present in all *in vitro* ApbC reconstitution assays, making it feasible that the thiolate moieties of DTT were providing ligands for an [Fe-S] cluster that was bound by ApbC variants within an advantageous proteinaceous cavity. In this case,

[Fe-S] cluster binding in the Cys → Ala variant ApbC proteins would be an artifact of the *in vitro* reconstitution.

A strength of this study lies in the combination of *in vivo* and *in vitro* approaches. This approach has allowed us to dissect the biochemical properties of ApbC within the context of biological activity. The resulting data showed that ApbC has two biochemical activities, and both activities are required for function *in vivo*. Identification of protein variants with no defect in the biochemical assays, but nonfunctional *in vivo*, indicates that some aspect of the *in vitro* assays is not physiologically relevant. We suggest that the chemical reconstitution of a cluster on ApbC does not reflect the process that occurs to generate holo-ApbC *in vivo*. The results described here with variant proteins provide a framework for continuing experiments intended to determine the function of ApbC in the network of [Fe-S] cluster metabolism.

REFERENCES

1. Beinert, H. (2000) *J. Biol. Inorg. Chem.* **5,** 2–15
2. Frazzon, J., and Dean, D. R. (2003) *Curr. Opin. Chem. Biol.* **7,** 166–173
3. Zheng, L., Cash, V. L., Flint, D. H., and Dean, D. R. (1998) *J. Biol. Chem.* **273,** 13264–13272
4. Takahashi, Y., and Tokumoto, U. (2002) *J. Biol. Chem.* **277,** 28380–28393
5. Layer, G., Ollagnier-de Choudens, S., Sanakis, Y., and Fontecave, M. (2006) *J. Biol. Chem.* **281,** 16256–16263
6. Vivas, E., Skovran, E., and Downs, D. M. (2006) *J. Bacteriol.* **188,** 1175–1179
7. Bou-Abdallah, F., Adinolfi, S., Pastore, A., Laue, T. M., and Dennis Chasteen, N. (2004) *J. Mol. Biol.* **341,** 605–615
8. Cui, Q., Thorgersen, M. P., Westler, W. M., Markley, J. L., and Downs, D. M. (2006) *Proteins Struct. Funct. Bioinformat.* **62,** 578–586
9. Gralnick, J. A., and Downs, D. M. (2003) *J. Biol. Chem.* **278,** 20708–20715
10. Loiseau, L., Gerez, C., Bekker, M., Ollagnier-de Choudens, S., Py, B., Sanakis, Y., Teixeira de Mattos, J., Fontecave, M., and Barras, F. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104,** 13626–13631
11. Bandyopadhyay, S., Naik, S. G., O'Carroll, I. P., Huynh, B. H., Dean, D. R., Johnson, M. K., and Dos Santos, P. C. (2008) *J. Biol. Chem.* **283,** 14092–14099
12. Angelini, S., Gerez, C., Ollagnier-de Choudens, S., Sanakis, Y., Fontecave, M., Barras, F., and Py, B. (2008) *J. Biol. Chem.* **283,** 14084–14091
13. Jin, Z., Heinnickel, M., Krebs, C., Shen, G., Golbeck, J. H., and Bryant, D. A. (2008) *J. Biol. Chem.* **283,** 28426–28435
14. Justino, M. C., Almeida, C. C., Goncalves, V. L., Teixeira, M., and Saraiva, L. M. (2006) *FEMS Microbiol. Lett.* **257,** 278–284
15. Justino, M. C., Almeida, C. C., Teixeira, M., and Saraiva, L. M. (2007) *J. Biol. Chem.* **282,** 10352–10359
16. Loiseau, L., Ollagnier-de Choudens, S., Lascoux, D., Forest, E., Fontecave, M., and Barras, F. (2005) *J. Biol. Chem.* **280,** 26760–26769
17. Skovran, E., and Downs, D. M. (2003) *J. Bacteriol.* **185,** 98–106
18. Petersen, L., and Downs, D. M. (1996) *J. Bacteriol.* **178,** 5676–5682
19. Koo, M. S., Lee, J. H., Rah, S. Y., Yeo, W. S., Lee, J. W., Lee, K. L., Koh, Y. S., Kang, S. O., and Roe, J. H. (2003) *EMBO J.* **22,** 2614–2622
20. Beck, B. J., Connolly, L. E., De Las Penas, A., and Downs, D. M. (1997) *J. Bacteriol.* **179,** 6504–6508
21. Beck, B. J., and Downs, D. M. (1998) *J. Bacteriol.* **180,** 885–891
22. Gralnick, J., Webb, E., Beck, B., and Downs, D. (2000) *J. Bacteriol.* **182,** 5180–5187
23. Skovran, E., Lauhon, C. T., and Downs, D. M. (2004) *J. Bacteriol.* **186,**

7626–7634

24. Igarashi, R. Y., and Seefeldt, L. C. (2003) *Crit. Rev. Biochem. Mol. Biol.* **38,** 351–384

25. Lutkenhaus, J., and Sundaramoorthy, M. (2003) *Mol. Microbiol.* **48,** 295–303

26. Leonard, T. A., Butler, P. J., and Lowe, J. (2005) *EMBO J.* **24,** 270–282

27. Friedman, S. A., and Austin, S. J. (1988) *Plasmid* **19,** 103–112

28. Koonin, E. V. (1993) *J. Mol. Biol.* **229,** 1165–1174

29. Lezhneva, L., Amann, K., and Meurer, J. (2004) *Plant J.* **37,** 174–185

30. Stockel, J., and Oelmuller, R. (2004) *J. Biol. Chem.* **279,** 10243–10251

31. Roy, A., Solodovnikova, N., Nicholson, T., Antholine, W., and Walden, W. E. (2003) *EMBO J.* **22,** 4826–4835

32. Hausmann, A., Aguilar Netz, D. J., Balk, J., Pierik, A. J., Muhlenhoff, U., and Lill, R. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 3266–3271

33. Bych, K., Kerscher, S., Netz, D. J., Pierik, A. J., Zwicker, K., Huynen, M. A., Lill, R., Brandt, U., and Balk, J. (2008) *EMBO J.* **27,** 1736–1746

34. Boyd, J. M., Pierik, A. J., Netz, D. J., Lill, R., and Downs, D. M. (2008) *Biochemistry* **47,** 8195–8202

35. Boyd, J. M., Lewis, J. A., Escalante-Semerena, J. C., and Downs, D. M. (2008) *J. Bacteriol.* **190,** 4596–4602

36. Raulfs, E. C., O'Carroll, I. P., Dos Santos, P. C., Unciuleac, M. C., and Dean, D. R. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105,** 8591–8596

37. Lewis, J. A., Horswill, A. R., Schwem, B. E., and Escalante-Semerena, J. C. (2004) *J. Bacteriol.* **186,** 1629–1637

38. Gross, S. R., Burns, R. O., and Umbarger, H. E. (1963) *Biochemistry* **2,** 1046–1052

39. Balk, J., Pierik, A. J., Netz, D. J., Muhlenhoff, U., and Lill, R. (2004) *EMBO J.* **23,** 2105–2115

40. Chan, K. M., Delfert, D., and Junger, K. D. (1986) *Anal. Biochem.* **157,** 375–380

41. Kreuzer, K. N., and Jongeneel, C. V. (1983) *Methods Enzymol.* **100,** 144–160

42. Bates, P. A., Kelley, L. A., MacCallum, R. M., and Sternberg, M. J. (2001) *Proteins* **5,** S39–S46

43. Bates, P. A., and Sternberg, M. J. (1999) *Proteins* **3,** S47–S54

44. Contreras-Moreira, B., and Bates, P. A. (2002) *Bioinformatics* (*Oxf.*) **18,** 1141–1142

45. Holmgren, A. (1985) *Annu. Rev. Biochem.* **54,** 237–271

46. Enos-Berlage, J. L., and Downs, D. M. (1999) *J. Bacteriol.* **181,** 841–848

47. Leonardi, R., Fairhurst, S. A., Kriek, M., Lowe, D. J., and Roach, P. L. (2003) *FEBS Lett.* **539,** 95–99

48. Martinez-Gomez, N. C., Robers, M., and Downs, D. M. (2004) *J. Biol. Chem.* **279,** 40505–40510

49. Lewis, J. A., and Escalante-Semerena, J. C. (2006) *J. Bacteriol.* **188,** 5479–5486

50. Lewis, J. A., and Escalante-Semerena, J. C. (2007) *Biochemistry* **46,** 9107–9115

51. Netz, D. J., Pierik, A. J., Stumpfig, M., Muhlenhoff, U., and Lill, R. (2007) *Nat. Chem. Biol.* **3,** 278–286

52. Segel, I. H. (1975) *Enzyme Kinetics*, John Wiley & Sons, Inc., New York

53. Karkaria, C. E., Chen, C. M., and Rosen, B. P. (1990) *J. Biol. Chem.* **265,** 7832–7836

54. Seefeldt, L. C., Morgan, T. V., Dean, D. R., and Mortenson, L. E. (1992) *J. Biol. Chem.* **267,** 6680–6688

55. Tian, G. C., Yan, H. G., Jiang, R. T., Kishi, F., Nakazawa, A., and Tsai, M. D. (1990) *Biochemistry* **29,** 4296–4304

56. Reinstein, J., Schlichting, I., and Wittinghofer, A. (1990) *Biochemistry* **29,** 7451–7459

57. Sigal, I. S., Gibbs, J. B., D'Alonzo, J. S., Temeles, G. L., Wolanski, B. S., Socher, S. H., and Scolnick, E. M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 952–956

58. Seefeldt, L. C., and Mortenson, L. E. (1993) *Protein Sci.* **2,** 93–102

59. Zhou, H., Schulze, R., Cox, S., Saez, C., Hu, Z., and Lutkenhaus, J. (2005) *J. Bacteriol.* **187,** 629–638

60. Seeley, T. W., and Grossman, L. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6577–6581

61. Parsonage, D., Al-Shawi, M. K., and Senior, A. E. (1988) *J. Biol. Chem.* **263,** 4740–4744

62. Ryle, M. J., and Seefeldt, L. C. (1996) *Biochemistry* **35,** 4766–4775

63. Ryle, M. J., Lanzilotta, W. N., Mortenson, L. E., Watt, G. D., and Seefeldt, L. C. (1995) *J. Biol. Chem.* **270,** 13112–13117

64. Agar, J. N., Krebs, C., Frazzon, J., Huynh, B. H., Dean, D. R., and Johnson, M. K. (2000) *Biochemistry* **39,** 7856–7862

65. Smith, A. D., Jameson, G. N., Dos Santos, P. C., Agar, J. N., Naik, S., Krebs, C., Frazzon, J., Dean, D. R., Huynh, B. H., and Johnson, M. K. (2005) *Biochemistry* **44,** 12955–12969

66. Shimomura, Y., Wada, K., Fukuyama, K., and Takahashi, Y. (2008) *J. Mol. Biol.* **383,** 133–143

67. Shimomura, Y., Kamikubo, H., Nishi, Y., Masako, T., Kataoka, M., Kobayashi, Y., Fukuyama, K., and Takahashi, Y. (2007) *J. Biochem.* (*Tokyo*) **142,** 577–586

68. Johnson, D. C., Unciuleac, M. C., and Dean, D. R. (2006) *J. Bacteriol.* **188,** 7551–7561

69. Foster, M., Mansy, S., Hwang, J., Penner-Hahn, J., Surerus, K. K., and Cowan, J. A. (2000) *J. Am. Chem. Soc.* **122,** 6805–6806

70. Vitale, G., Fabre, E., and Hurt, E. C. (1996) *Gene* (*Amst.*) **178,** 97–106

71. Dos Santos, P. C., Smith, A. D., Frazzon, J., Cash, V. L., Johnson, M. K., and Dean, D. R. (2004) *J. Biol. Chem.* **279,** 19705–19711

72. Kaut, A., Lange, H., Diekert, K., Kispal, G., and Lill, R. (2000) *J. Biol. Chem.* **275,** 15955–15961

73. Lu, J., Yang, J., Tan, G., and Ding, H. (2008) *Biochem. J.* **409,** 535–543

74. Antonkine, M. L., Maes, E. M., Czernuszewicz, R. S., Breitenstein, C., Bill, E., Falzone, C. J., Balasubramanian, R., Lubner, C., Bryant, D. A., and Golbeck, J. H. (2007) *Biochim. Biophys. Acta* **1767,** 712–724

75. Gibney, B. R., Mulholland, S. E., Rabanal, F., and Dutton, P. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 15041–15046

76. Mulholland, S. E., Gibney, B. R., Rabanal, F., and Dutton, P. L. (1998) *J. Am. Chem. Soc.* **120,** 10296–10302

77. Gralnick, J., and Downs, D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 8030–8035

78. Way, J. C., Davis, M. A., Morisato, D., Roberts, D. E., and Kleckner, N. (1984) *Gene* (*Amst.*) **32,** 369–379

79. Neidhardt, F. C., Ingraham, J. L., and Schaechter, M. (1990) *Physiology of the Bacterial Cell*, pp. 198–199, Sinauer Associates, Inc., Sunderland, MA

80. Martinez-Gomez, N. C., and Downs, D. M. (2008) ThiC is an [Fe-S] Cluster protein that requires AdoMet to generate the 4-amino-5-hydroxymethyl-2-methylpyrimidine moiety in thiamin synthesis. *Biochemistry* **47,** 9054–9056