Bacterial ApbC Can Bind and Effectively Transfer Iron−Sulfur Clusters[†,‡]

Jeffrey M. Boyd,[§] Antonio J. Pierik,[||] Daili J. A. Netz,[||] Roland Lill,[||] and Diana M. Downs*[,§]

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706, and Institut für Zytobiologie, Philipps-Universität Marburg, Robert-Koch-Strasse 6, D-35033 Marburg, Germany

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ABSTRACT: The metabolism of iron−sulfur ([Fe-S]) clusters requires a complex set of machinery that is still being defined. Mutants of *Salmonella enterica* lacking *apbC* have nutritional and biochemical properties indicative of defects in [Fe-S] cluster metabolism. ApbC is a 40.8 kDa homodimeric ATPase and as purified contains little iron and no acid-labile sulfide. An [Fe-S] cluster was reconstituted on ApbC, generating a protein that bound 2 mol of Fe and 2 mol of S^{2-} per ApbC monomer and had a UV−visible absorption spectrum similar to known [4Fe-4S] cluster proteins. Holo-ApbC could rapidly and effectively activate *Saccharomyces cerevisiae* apo-isopropylmalate isolomerase (Leu1) *in vitro*, a process known to require the transfer of a [4Fe-4S] cluster. Maximum activation was achieved with 2 mol of ApbC per 1 mol of apo-Leu1. This article describes the first biochemical activity of ApbC in the context of [Fe-S] cluster metabolism. The data herein support a model in which ApbC coordinates an [4Fe-4S] cluster across its dimer interface and can transfer this cluster to an apoprotein acting as an [Fe-S] cluster scaffold protein, a function recently deduced for its eukaryotic homologues.

Iron and sulfide are necessary for the survival of most, if not all, organisms. To avoid toxic effects of these molecules, the cellular metabolism of Fe and S requires multiple proteins to ensure their storage, trafficking, and delivery (reviewed in *1*). Cofactors comprised of both iron and sulfur ([Fe-S] clusters) exhibit a wide array of metabolic functions including substrate activation, electron transfer, DNA repair, and sensing environmental changes (reviewed in *2*). Research addressing the biosynthesis of the iron−molybdenum cofactor of nitrogenase in *Azotobacter vinelandii* led to the discovery of the first operon (*iscA*^{nif}*nifUScysE1*) involved in the biosynthesis of [Fe-S] clusters (reviewed in *3*). Subsequently, two more systems involved in the *de novo* biosynthesis of [Fe-S] clusters were discovered: the *isc* (*i*ron *s*ulfur *c*luster) and the *suf* (*s*ulfur *u*tilization *f*actor) systems (*4, 5*). Like *Escherichia coli*, the genome of *S. enterica* encodes the *isc* and *suf* [Fe-S] biosynthetic operons and cellular viability requires the presence of only one of the two operons. In *E. coli*, the *isc* operon is under the regulation of transcriptional repressor IscR and is the general house keeping [Fe-S] biosynthetic operon (*6–9*). The suf operon is a member of the OxyR and Fur regulons and is induced during times of limited Fe availability and oxidative stress (*5, 7, 8, 10–12*).

The three [Fe-S] biosynthetic systems have two functional components in common: a cysteine desulfurase and a molecular scaffold. The cysteine desulfurase enzymes (NifS, SufS, IscS, and CsdA) catalyze the removal of atomic sulfur from L-cysteine (*13–17*). Labile [Fe-S] clusters are built on the molecular scaffolding proteins (NifU, IscU, IscA(nif), IscA, and SufA) presumably from S provided by a cysteine desulfurase and Fe provided by a Fe trafficking molecule such as the frataxin homologue CyaY (*18*). These holo-scaffolds can then transfer their labile [Fe-S] clusters to apo-proteins (*19–23*). Additionally, the Isc and Suf systems contain energy producing proteins. Both the HscA and SufC proteins can hydrolyze ATP (*24–26*). Studies have shown that cluster transfer from IscU to apo-ferredoxin was stimulated more than 20-fold by the addition of HscA, HscB, and Mg•ATP (*25*).

Other loci beyond these operons encode proteins thought to have a role in [Fe-S] cluster metabolism. Work in our laboratory showed that strains lacking *apbC* display a number of mutant phenotypes reflecting a defect in [Fe-S] cluster metabolism including a requirement for thiamine, sensitivity to oxidants, and reduced specific activities of known [Fe-S] cluster enzymes (*27–29*). The presence of an additional mutation in *isc*, but not *suf*, exacerbates these effects (J. M. Boyd and D. M. Downs, unpublished work and (*28*)). Since this work, other groups have identified small P-loop ATPases with high similarity to ApbC and suggested these proteins are involved in [Fe-S] cluster metabolism in higher organisms (*30–33*). HCF101 was demonstrated to be involved in *Arabidopsis thaliana* chloroplast [Fe-S] cluster metabolism (*30*) and Cfd1 and Nbp35 were two of the first

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* To whom correspondence should be addressed. Department of Bacteriology, 420 Henry Mall, University of Wisconsin, Madison, WI 53706. Phone: (608) 265-4630. Fax: (608) 890-0785. E-mail: downs@bact.wisc.edu.

[§] University of Wisconsin.

[||] Philipps-Universität Marburg.

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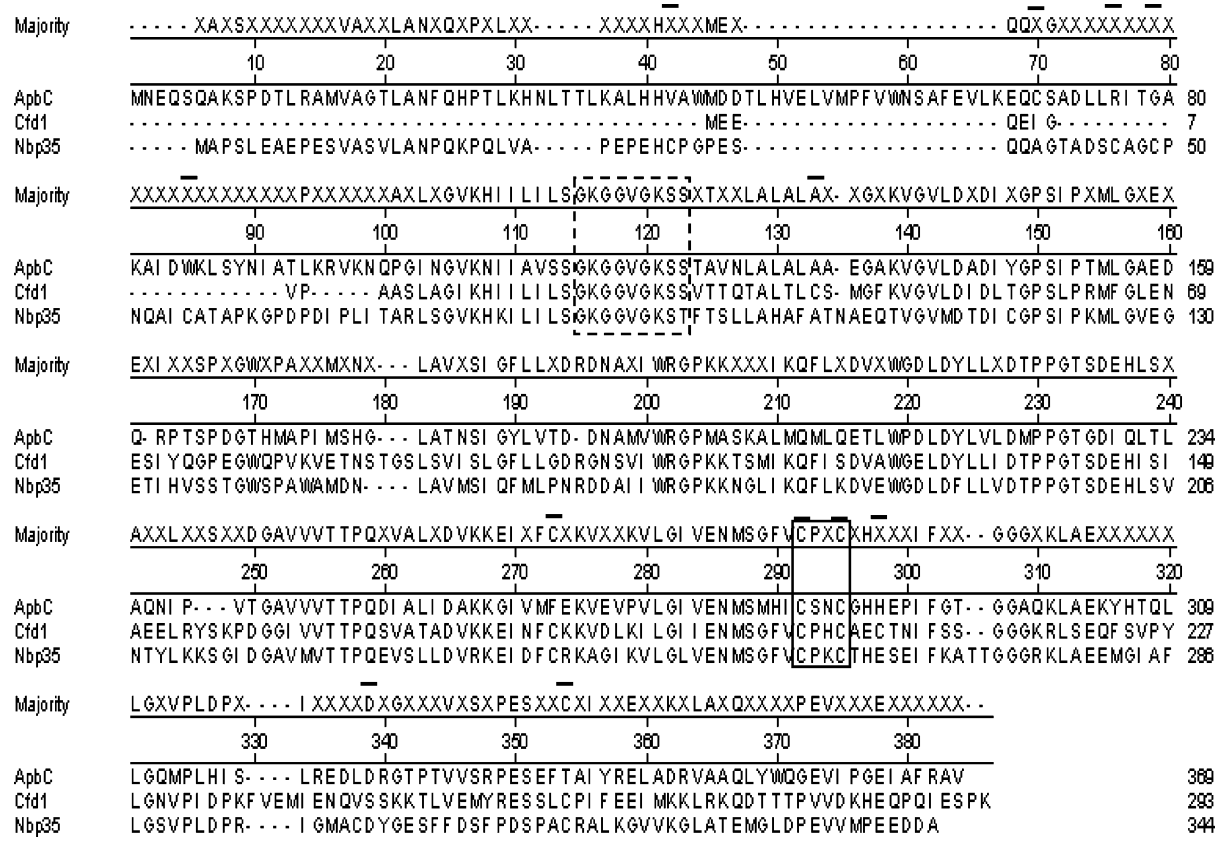

FIGURE 1: Protein sequence alignment of Nbp35, Cfd1, and ApbC. Protein alignments were assembled using the Clustal_W method in the Lasergene software. The sequence of interest is boxed: the Walker A box is dashed, and the Cys-X-X-Cys motif is solid. Cysteine residues are highlighted above the consensus sequence. Proteins are listed as follows: ApbC (*Salmonella enterica* Serovar Typhimurium LT2), Cfd1 (*Saccharomyces cerevisiae*), and Nbp35 (*Saccharomyces cerevisiae*).

components demonstrated to be involved in cytoplasmic [Fe-S] cluster metabolism in *Saccharomyces cerevisiae* (*31, 32*).

A sequence alignment of the ApbC, Nbp35, and Cfd1 proteins is presented in Figure 1. The three sequences contain two highly conserved domains: the Walker A box for ATP binding/hydrolysis (boxed-dashed) and a CXXC motif in the C-terminal third of the protein (boxed-solid). *In vivo* studies have shown that the Cys residues in the CXXC motif are required for in vivo function (*28, 31*). The three core protein sequences (without N-terminal tail) show 25% identity plus 31% similarity supporting the possibility of a common function. Cfd1 and Nbp35 share more similarity with each other than with ApbC, particularly apparent are two additional conserved Cys residues in the C-terminal tails of these proteins (*28, 31*).

Proteins Nbp35 and Cfd1 purify as a protein complex with a $\alpha_2\beta_2$ quaternary structure (*34*). These proteins individually bind [Fe-S] clusters *in vivo* and *in vitro*. Nbp35 copurified with a stable [Fe-S] cluster, thought to be ligated by an N-terminal cysteine rich motif (*32*). After anaerobic chemical reconstitution approximately 8 moles of Fe and 8 moles of S were associated with one mole of Nbp35, 4 moles of Fe and 4 moles S with one mole Cfd1, and 12 moles of Fe and 12 moles S with one mole of the heterodimeric complex. Further biochemical studies showed that both Nbp35 and Cfd1, individually, and the $\alpha_2\beta_2$ complex could transfer an [Fe-S] clusters to the an apo-form of the isopropylmalate dehydratase Leu1 (*34*).

In this study we investigated a function for ApbC in [Fe-S] cluster binding and transfer using a heterologous system. While the bacterial and yeast proteins were not functionally interchangeable *in vivo*, we show that ApbC can bind an [Fe-S] cluster and activate apo-Leu1 *in vitro*, in an ATP-independent way. This is the first report describing a function beyond ATP hydrolysis for a bacterial member of this conserved class of proteins involved in [Fe-S] cluster metabolism. Our findings emphasize the general function Mrp/Nbp35 proteins might have in [Fe-S] cluster assembly.

EXPERIMENTAL PROCEDURES

Materials. $FeCl_3$ (ACS grade), thiamine (>99%), Li_2S (>98%), $Fe(NH_4)_2(SO_4)_2$ (>99%), 3-(2-pyridyl)-5,6-di((2-furyl)-1,2,4-triazine-5′,5″ (>99%), ascorbic acid (>99%), $(C_2H_3O_2)_2Zn\cdot 2H_20$ (reagent grade), *N,N*-dimethyl-*p*-phenylenediamine sulfate (98%), and L-cysteine (>98%) were purchased from Sigma Aldrich, St. Louis, MO. 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, Rockford, IL. *Escherichia coli* BL21 (AI*) cells were purchased from Novagen.

Anaerobic Work. Anaerobic work was preformed using a Coy anaerobic glovebox (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 Cu column for removal of O_2. Solutions and plastic-ware was allowed to equilibrate for >6 h inside the glovebox before use. Outside

of the glovebox, all solutions were added to anaerobic cuvettes using gastight Hamilton syringes.

Recombinant Protein Over-Production. Escherichia coli strain BL21(AI*) containing the protein expression plasmids pCTH-ApbC (*28*), pET-15b-Leu1-His (*34*), or pIscS (P. Kiley, University of Wisconsin) were grown in standard LB medium at 37 °C in a 16 L fermenter or in a 1 L culture to an optical density (A_{650}) of 0.6. The media was then cooled and arabinose (1 mM) and IPTG (0.1 mM) was added. Protein overproduction from pIscS, pCTH-ApbC, or pET-15b-Leu1-His was at 32 °C (5 h), 32 °C (5 h), and 15 °C (14 h), respectively. Cells were harvested by centrifugation. Cell paste was flash frozen with liquid nitrogen and stored at −80 °C.

Protein Purification. Frozen cell paste was suspended in an equal volume of buffer A (50 mM Tris-HCl, pH 8.0) containing DNase (0.03 mg/mL). Cell suspensions were passed three times through a chilled French pressure cell at 4 °C. Cell lysates were clarified by centrifugation (39,000 × g for 40 min at 4 °C). All overproduced proteins had poly-*hexa* or *deca*histidine tags. The clarified cell extract was loaded onto a 1.6 × 10 cm pre-equilibrated Ni^{2+}-loaded Chelating Sepharose Fast Flow (GE healthcare) column and washed with 20 column volumes of 50 mM Tris, pH 8.0, 1 M NaCl. The column was again equilibrated with buffer A and recombinant protein was eluted during a 30 column volume linear gradient from 0 to 100% elution buffer (50 mM Tris, pH 8.0, 250 mM imidazole). Fractions that contained the protein of interest at >95% purity by SDS−PAGE analysis were pooled and concentrated over a 30,000 Da molecular mass cutoff membrane (Amicon YM30). After concentration, ApbC or IscS were dialyzed overnight in 50 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, and 150 mM NaCl. After concentration Leu1 was dialyzed in 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl. Finally, the proteins were pelleted into liquid nitrogen and stored at −80 °C until needed. All steps were performed at 4 °C and buffers used for dialysis had the pH adjusted at 4 °C.

For anaerobic purification of ApbC, cells were lysed using a sonicator placed inside of the anaerobic glovebox. Cell extract was placed in anaerobic centrifuge tubes, removed from the glovebox, and centrifuged before returning lysates to the anerobic atmosphere for decanting the supernatant. All other purification procedures were carried out as described above.

Protein Concentration Determination. Protein concentration was determined using a colorimetric assay or an empirically determined extinction coefficient (Apo-ApbC-poly $His_{280} = 43.1\ mM^{-1}\ cm^{-1}$). 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.

Quaternary Structure Determination. The quaternary structure of ApbC was determined using a Superose 6 PC 3.2/30 size exclusion column (GE-Healthcare). Aerobically purified ApbC was placed inside of the anaerobic glovebox and the sample was exposed to the anaerobic atmosphere for 1 h prior to injection onto the column. The mobile phase for the analysis was 50 mM Tris-HCl, pH 8.0, 200 mM NaCl. One-hundred microliters of a 5 mg/mL solution was injected onto the column. To determine the quaternary structure for the reduced sample, 1 mM DTT was added 15 min prior to injection onto the column. The migration of low- and high-molecular mass protein standards (GE-Healthcare) were used to create a graph of retention time vs log molecular mass. The linear fit had an $R^2 > 0.93$.

Metal Analysis. Inductively coupled plasma mass spectrometry (ICP-MS) was conducted by the University of Wisconsin-Madison soil and plant analysis laboratory. The concentration of nonheme iron and acid labile sulfide was also determined as described elsewhere (*35*).

Fe-S Cluster Reconstitution. ApbC (2.1 mg/mL; 52 μM) was reduced anaerobically with 5 mM DTT for at least 1 h prior to Fe^{3+} and S^{2-} addition. After prereduction, $FeCl_3$ was added to 5-fold excess and incubated for approximately 2 min before a 5-fold excess of Li_2S was slowly added over 15 min. The solution was incubated for various times (typically 1 h) before excess salts were removed by desalting with a PD-10 column (GE Healthcare). Post desalting 0.5 mM DTT was added to the sample. For reconstitutions using IscS, 5 mM L-cysteine and IscS (20 μM) were added in place of Li_2S.

The UV−visible absorption spectra were recorded with a Perkin-Elmer Lambda Bio 40 spectrophotometer using 1.5 mL Sterna Cell cuvettes that can be anaerobically sealed (Atascadero, CA).

Leu1 Activation Assays. Apo-isopropylmalate isomerase (apo-Leu1) (25 μM) was prereduced with 5 mM DTT in the anaerobic glovebox for at least 1 h prior to assay initiation. The Leu1 activation assays contained 3−5 μM Leu1, 5 mM DTT, 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl in a total volume of 250 or 500 μL. Assays were initiated by the addition of either ApbC or Fe^{3+} and S^{2-}. Ten microliter aliquots of the assay mixture were removed at time points and Leu1 was assayed for the ability to convert 3-isopropylmalate to dimethylcitraconate acid spectrophotometrically (dimethylcitraconate $\varepsilon_{235} = 4.35\ mM^{-1}\ cm^{-1}$) (*36*).

The linear part of the apo-Leu1 activation as a function of the time was used to estimate the rate constant of reconstitution (expressed as % Leu1 activity per min under standard conditions). The exponential decay of reconstituted ApbC to a form with a lower rate constant of apo-Leu1 reconstitution was simulated using eq 1:

$$v\text{activation} = v\text{less active form} + (v\text{active form} - v\text{less active form}) \times \exp(-t \times \ln(2)/t1/2) \quad (1)$$

where $v$activation is the rate of apo-Leu1 activation at a given time, $v$active form is the rate of apo-Leu1 activation with reconstituted ApbC, $v$less active form is the velocity of apo-Leu1 activation after breakdown of reconstituted ApbC, t is time in minutes, and $t1/2$ is the time required to convert half of the reconstituted ApbC to form with a lower proficiency to activate apo-Leu1.

Assessment of ApbC Cluster Stability. ApbC was reconstituted, desalted and the ability of ApbC (without added DTT) to activate Leu1 was assessed.

To determine the effect of oxygen on the [Fe-S] cluster the spectrum of the anaerobic sample was taken immediately post desalting prior to opening the cuvette to the atmosphere. Air was introduced into the sample by removing the cap and pipetting the sample up and down once. The UV−visible absorbance was taken at fixed time points and the data used to construct a plot of A_{400} vs time. The decay constant for

Table 1: One Mole of ApbC Binds Two Moles of Iron and Two Moles of Sulfur Atoms[a]

additions to reconstitution	μmol iron/μmol ApbC	μmol sulfide/μmol ApbC
none	0.1 ± 0.1	BDL[b]
Fe^{3+}	0.5 ± 0.3	BDL
S^{2-}	0.1 ± 0.1	BDL
Fe^{3+} and S^{2-}	1.9 ± 0.1	2.0 ± 0.0
Fe^{3+} and S^{2-}	2.1 ± 0.1	2.1 ± 0.1
Fe^{3+} and S^{2-}	2.2 ± 0.1	2.3 ± 0.1
Fe^{3+} and S^{2-}	2.4 ± 0.1	2.4 ± 0.0
Fe^{3+} and S^{2-}	1.9 ± 0.0	2.0 ± 0.1
average Fe^{3+} S^{2-}	2.2 ± 0.1	2.1 ± 0.1

[a] ApbC was reconstituted with a 5-fold excess of S^{2-} and Fe^{3+} as described in Experimental Procedures. Iron and sulfide were quantified as described elsewhere (*35*). Values shown for individual reconstitutions are the average of two independent measurements. Errors are reported as standard deviations. [b] Below detectable limit.

the difference in absorbance was determined by fitting the data to eq 2:

$$A = y_o + ae^{(-bt)} \quad (2)$$

where *A*is the change in absorbance at time *t*, y_o is the residual absorbance after decay, *a* is the maximal decay, *b* is the decay constant, and *t* is time post exposure to air. The half-life of the absorbance change was determined using eq 3:

$$\text{half-life} = \ln(2)/b \quad (3)$$

RESULTS

ApbC Can Bind an [Fe-S] Cluster in Vitro. The ApbC protein is 40,800 Da (with poly histidine tag) and can be overproduced to high levels (approximately 25% cellular protein) in *E. coli*, as stable, soluble protein. Inductively coupled plasma mass spectroscopy (ICP-MS) metal analysis showed an excess of Zn^{2+} (~0.5 Zn per monomer) associated with the protein but little iron (<0.1 Fe per monomer, Table 1) when the protein was isolated aerobically or anaerobically. To determine whether ApbC could bind either iron or sulfur, the protein was reduced for one hour with 5 mM DTT and then either a 5-fold excess of Fe^{3+} or a 5-fold excess of S^{2-} was added. The samples were incubated for one additional hour, desalted, and the nonheme iron and acid-labile sulfide content was determined. As shown in Table 1, no sulfide was associated with the protein, however, ~0.5 moles of Fe remained bound per mole of ApbC.

In an effort to chemically reconstitute a Fe-S cluster on ApbC, the protein was incubated with up to a 10-fold excess of Fe^{3+} and S^{2-} after pretreatment with DTT. Iron and sulfur analysis revealed that ApbC bound equimolar amounts of Fe and S (2.2 ± 0.1 and 2.1 ± 0.1 mols/mol ApbC, respectively) (Table 1).

The UV−visible absorption spectrum of the aerobically or anaerobically isolated protein showed a weak shoulder at ~400 nm with a calculated extinction coefficient of ε_{400} = 0.3 ± 0.1 mM^{-1} cm^{-1} (data not shown). The spectrum of chemically reconstituted ApbC protein had no discernible features except a broad peak at 400 nm and slight shoulders at 325 and 650 nm (Figure 2). The peak at 400 nm is a typical feature for proteins with an $[4Fe\text{-}4S]^{2+}$ cluster(s) and reconstituted ApbC had a calculated extinction coefficient of ε_{400} = 7.0 mM^{-1} cm^{-1} and a A_{400}/A_{280} ratio of 0.15. The

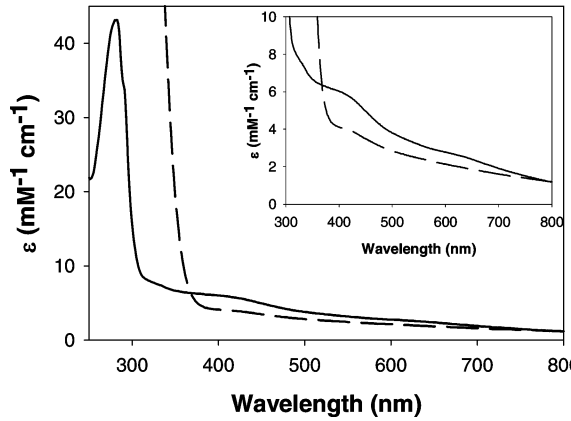

FIGURE 2: UV−visible absorption spectra of holo-ApbC shows spectral features similar to known [Fe-S] cluster proteins. Spectra of ApbC after chemical reconstitution without (solid), and with 2 mM sodium dithionite (long dash) in 50 mM Tris, pH 8.0, and 150 mM NaCl. ApbC was reconstituted as outlined in Experimental Procedures. The concentration of ApbC is 1.1 mg/mL for the reconstituted, and reconstituted-reduced protein samples. The Figure includes an enlargement of the spectra from 300−800 nm (inset) to view spectroscopic details.

absorbance at 400 nm was diminished in comparison to the oxidized spectra upon reduction with 2 mM dithionite (Figure 2). In addition to chemical reconstitution, an [Fe-S] cluster could be reconstituted on ApbC by adding a 5-fold excess of Fe^{3+}, 5 mM cysteine, and IscS after prereduction with DTT. ApbC reconstituted using IscS and cysteine had properties indistinguishable from the chemically reconstituted protein (data not shown). Reconstituted ApbC protein was EPR silent with and without the presence of 2 mM dithionite (data not shown).

The Quaternary Structure of ApbC Protein Is in a Dynamic Equilibrium. On the basis of the spectral characteristics, the two essential cysteine residues, and the Fe and S stoichiometry after chemical reconstitution, we hypothesized that the [Fe-S] cluster of ApbC bridged the dimer interface using two Cys ligands from each protein. As isolated, ApbC was a mixture of monomers and dimers (Figure 3A). The ratio of A_{280} for the monomer and dimer peaks was 1:2, indicating that the molar ratio of monomeric species to dimeric species is approximately 1:1 as isolated. After anaerobic reduction with 1 mM DTT, ApbC eluted as a dimer (Figure 3A). Fractions containing the reduced dimer were pooled, desalted, concentrated, and incubated aerobically at 4 °C for 5 h. The resulting protein sample eluted once again as a mixture of monomers and dimers (data not shown). Chemically reconstituted ApbC eluted as a mixture of dimers and tetramers (Figure 3B) but, a majority of holo-ApbC was dimeric. These data indicate that ApbC protein is in a dynamic equilibrium between monomeric, dimeric, and tetrameric species.

The Holoform of ApbC Can Activate Leu1 Enzyme Activity. The isopropylmalate isomerase Leu1 is an 87.8 kDa dehydratase from *Saccharomyces cerevisiae* that requires a [4Fe-4S] cluster to convert (2*S*)- into (2*R*,3*S*)-isopropylmalate (*34, 37*). Aerobically isolated apo-Leu1 contained <0.1 moles of Fe and no detectable sulfide per mole of protein, and had no detectable activity (Figure 4). Incubation of holo-ApbC and apo-Leu1 restored enzyme activity of Leu1 in a time dependent manner (Figure 4). The efficiency of the ApbC mediated activation of Leu1 is 100% as compared to

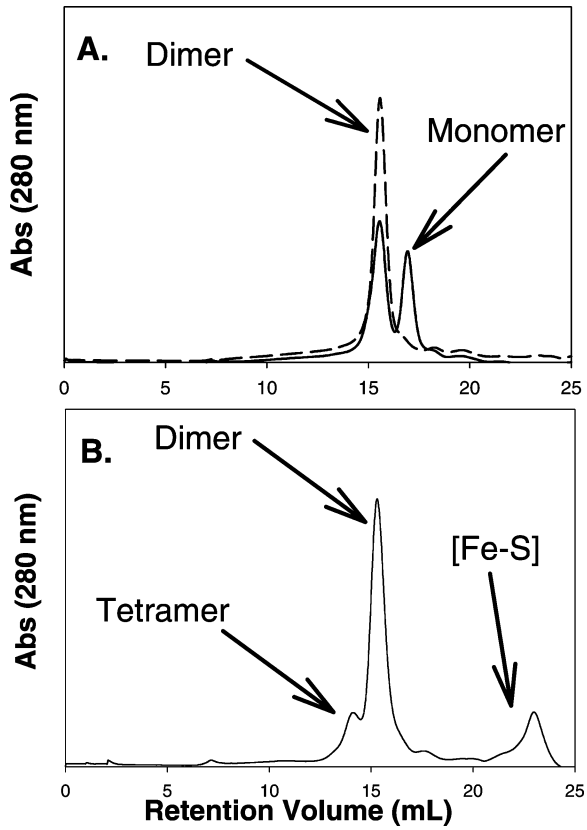

FIGURE 3: Quaternary structure of ApbC protein is in a dynamic equilibrium. (Panel A) As isolated, ApbC is a mixture of monomers and dimers but is a dimer when reduced with 1 mM DTT. ApbC (0.5 mg) air oxidized (solid) and reduced with 1 mM DTT (dashed) is shown. (Panel B) Holo-ApbC (0.5 mg) migrated as a mixture of dimers and tetramers. The gel filtration conditions are as follows: solid phase, Superose 6; mobile phase, 50 mM Tris-HCl at pH 8.0 and 200 mM NaCl; flow-rate, 0.1 mL/min.

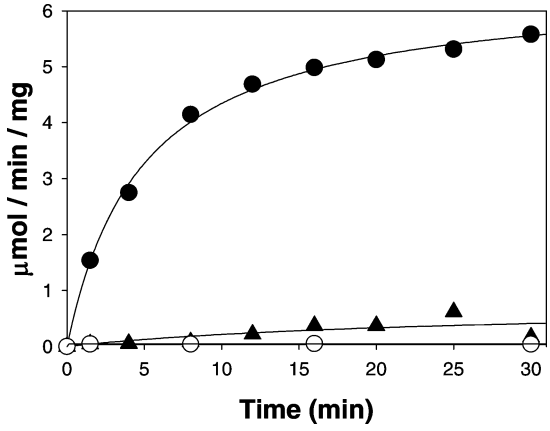

FIGURE 4: Holo-ApbC can activate the isopropylmalate isomerase Leu1. Time-course of Leu1 activation upon the addition of ApbC. All Leu1 activation reactions contained 2.9 μM apo-Leu1 in 50 mM Tris, pH 8.0, and 150 mM NaCl, but Leu1 activity was monitored at fixed time points by the formation of isopropylmaleate, which was monitored at A_{235} in 20 mM Tris, pH 7.4, and 50 mM NaCl. The reaction was started by adding either Apo-(○) or holo-(●) ApbC (5.8 μM final concentration) to the reaction vessel. As a control, Leu1 was assayed at fixed time points after the addition of Fe^{3+} and S^{2-} (▲) at the same concentration (11 μM final) that bound to holo-ApbC in the Leu1 activation assays.

chemical reconstitution (data not shown). Addition of apo-ApbC had no effect. Nonheme iron and acid-labile sulfide content was determined for the holo-ApbC used in this

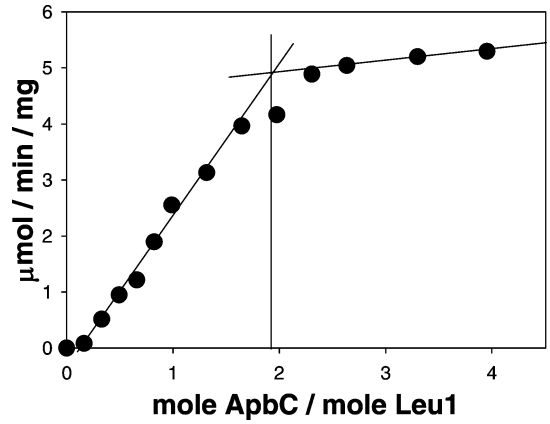

FIGURE 5: Stoichiometry of Leu1 activation by holo-ApbC. Specific activity of Leu1 as a function of ApbC dimer present in each assay. Holo-ApbC and apo-Leu1 were incubated for 15 min, and Leu1 activity was assessed. Leu1 activation assays contained 2.9 μM Leu1, 0−13 μM ApbC, 50 mM Tris, pH 8.0, and 150 mM NaCl. Leu1 activity was assessed by the absorption change at 235 nm resulting from the production of dimethylcitraconate. Data points shown are the average of two assays.

experiment (1.9 Fe and 1.8 S per ApbC). When this amount of Fe^{3+} and S^{2-} was added to apo-Leu1 in the absence of ApbC weak activation was observed. Activation by holo-ApbC was unaffected by the addition of ATP (and/or Mg; 5 mM) to the reaction (data not shown).

Two Moles of Holo-ApbC Are Required to Activate One Mole of Leu1. ApbC was reconstituted and increasing amounts of ApbC were incubated with apo-Leu1 for 15 min before Leu1 activity was assayed. The results of this experiment are shown in Figure 5, and showed that about 2 moles of ApbC were required to fully activate 1 mol of Leu1 (Figure 5, inset). The holo-ApbC protein used in this experiment was analyzed for metal and found to have 1.9 iron atoms and 2 sulfide atoms per ApbC. The presence of about 4 moles of iron atoms was necessary to fully activate 1 mol of Leu1, consistent with the requirement for a [4Fe-4S] cluster for activity (*34, 37*).

Leu1 was chemically reconstituted and incubated with increasing amounts of prereduced apo-ApbC (up to 50-fold excess of apo-ApbC) for 1.5 h. The activity of Leu1 was then assessed and found to be unaffected by the presence of apo-ApbC (data not shown). On the basis of observations reported in this study, eq 4 can be used to describe the holo-ApbC dependent activation of apo-Leu1.

$$\text{Apo}-\text{Leu1} + [4\text{Fe}-4\text{S}] - (\text{ApbC})_2 \rightarrow [4\text{Fe}-\text{S}] - \text{Leu1} + 2\text{Apo}-\text{ApbC} \quad (4)$$

The [Fe-S] Cluster Bound by ApbC Is Labile. After ApbC was chemically reconstituted and desalted, 0.5 mM DTT was added to stabilize the [Fe-S] cluster. Storage of the protein anoxically in the presence of DTT for up to 6 h did not change the UV−visible absorption spectrum of the ApbC or the ability to activate Leu1. Following the 6 h incubation, the protein was desalted and found to retain 2 Fe and 2 S per monomer (data not shown). However, if DTT was not added after desalting, the A_{650} increased and the A_{325} decreased over time, while the A_{400} remained unchanged (Figure 6B). The absorbance at 325 and 650 nm was normalized to the absorbance at 400 nm and a plot was constructed for change in absorbance as a function of time

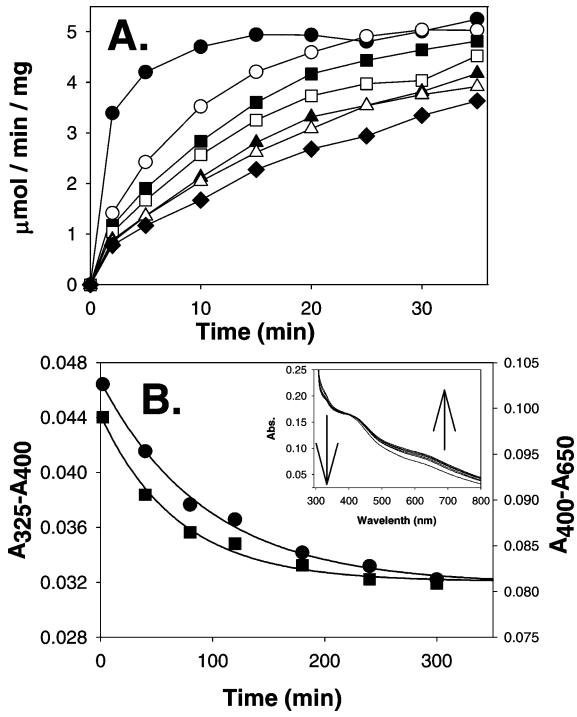

FIGURE 6: The [Fe-S] cluster associated with ApbC rapidly changes form under anoxic conditions in the absence of DTT. (Panel A) ApbC-dependent Leu1 activation 2 (●), 40 (○), 80 (■), 120 (□), 180 (▲), 240 (△), and 300 (◆) minutes after desalting reconstituted ApbC. ApbC was reconstituted as described in Experimental Procedures, but DTT was not added back to the sample after desalting. (Panel B) The normalized absorbance changes at 325 nm (●, left axis) and 650 nm (■, right axis) as a function of time postdesalting. The data in panel B were fit by eq 2. (Panel B inset) The UV−visible absorption spectra of reconstituted ApbC after 2, 40, 80, 120, 180, 240, and 300 min postdesalting.

post desalting. The data were fit with eq 2, and half-lives of 65 and 48 min were determined for the changes in absorbance at 325 and 650 nm, respectively. The ability of reconstituted ApbC to activate Leu1 at fixed times after desalting in the absence of DTT was tested. Figure 6A shows that the rate of Leu1 activation by reconstituted ApbC decreased over time. A plot of the estimated rate of activation at of apo-Leu1 as a function of time post desalting was created and fit to eq 1. An accurate rate of Leu1 activation could not be determined because ~65% of the Leu1 was active by the first time point at 2 min. A half-life of inactivation of ApbC (with respect to the ability to activate apo-Leu1) of 21 ± 4 min was determined. It is possible that after the removal of excess DTT a cluster rearrangement occurs and this form of ApbC is not as efficient at activating Leu1. This interpretation is based on the following two observations. First, after removing DTT, reconstituted ApbC activated Leu1 at a rate greater than that seen by iron and sulfide in the absence of ApbC (Figures 4 and 6A). Second, although changes were seen in the UV−visible absorption spectra of reconstituted ApbC after removing DTT, the A_{400} of reconstituted ApbC did not change over the 300 min it was examined (inset Figure 6B).

In a different experiment, ApbC was reconstituted and DTT was not added after desalting. The sample was removed from the glovebox in an anaerobic cuvette, a spectrum was taken, and the cuvette was opened to the oxic atmosphere. The spectra recorded every few minutes over 1 h showed a gradual decrease in absorption between the wavelengths of 800 to 300 nm (data not shown). A plot of A_{400} vs time post aeration was generated, the data were fit by eq 2 and a half-life of 17 min was determined for the absorbance at 400 nm. This value is similar to the anaerobic half-life of ApbC dependent Leu1 activation (Figure 6). These data are consistent with ApbC binding an [Fe-S] cluster that has similar lability as that of known [Fe-S] cluster scaffold/delivery proteins (*20, 25, 34, 38–41*).

DISCUSSION

Previous genetic studies implicated ApbC in the metabolism of [Fe-S] clusters (*27, 28*). This study was initiated to investigate the biochemical function of ApbC in the context of [Fe-S] cluster maturation, based on similar studies with the eukaryotic homologues Nbp35 and Cfd1 (*34*). ApbC is a 369 amino acid protein with two relevant motifs; i) a Walker A box for ATP binding/hydrolysis and ii) a conserved CXXC motif. Both motifs are conserved in Cfd1 and Nbp35. ApbC was not active in assays to detect detoxification of Fenton chemistry byproducts, peroxidase activity, or reduction of disulphide bonds (data not shown). However, ApbC could bind an [Fe-S] cluster and efficiently activate the isopropylmalate dehydratase Leu1, presumably by transfer of a [4Fe-4S] cluster.

Data presented herein contributed to the hypothesis that an ApbC homodimer binds one [4Fe-4S] cluster that can be transferred to Leu1 *in vitro*. Iron and sulfide analysis found that holo-ApbC bound equimolar quantities of Fe and S (2 mols/mol ApbC) consistent with these elements being present as an [Fe-S] cluster. The visible spectrum exhibits features similar to that of known [4Fe-4S] proteins and the extinction coefficient (A_{400}) is approximately one-half of what would be expected for one [4Fe-4S] cluster per monomer (*34, 42*). Leu1 requires a [4Fe-4S] cluster for catalysis and the addition of holo-ApbC led to rapid Leu1 activation, with maximum activation requiring a 2:1 ratio of holo-ApbC to Leu1. We conclude that ApbC shares important properties of scaffold proteins such as IsuU and Cfd1/Nbp35.

The [Fe-S] cluster bound by ApbC shows lability in the presence and absence of oxygen. Characterized scaffolding proteins with solvent exposed transient [Fe-S] clusters also show a sensitivity to oxygen (*20, 25, 34, 38–41*). Although ApbC does not share significant sequence homology with the molecular scaffold components of the Suf or Isc systems, it has similarity to the NfU-type scaffolding proteins that are defined by a conserved CXXC motif (*41, 43*). ApbC differs from NfU-type, U-type, and A-type scaffolding proteins by its ability to hydrolyze ATP (*28*). For Fe-S cluster transfer from both Cfd1-Nbp35 and ApbC to Leu1 ATP hydrolysis was not required even though in both cases residues in the Walker nucleotide binding motif are necessary for *in vivo* function (Boyd and Downs, W. Walden, unpublished data). We hypothesize that ATP hydrolysis is necessary to build or (re)load an [Fe-S] cluster onto ApbC *in vivo*.

The eukaryotic ApbC homologues Cfd1 and Nbp35 are homotetrameric and have cysteine-rich C-termini that can independently bind 4 Fe and 4 S per monomer after chemical reconstitution (*34*). These holo-proteins can rapidly and efficiently activate apo-Leu1 *in vitro* (*34*). Despite their similar activities *in vitro*, there are significant functional

differences *in vivo*. In *S. cerevisiae* both *NBP35* and *CFD1* are essential for yeast cell viability and for cystosolic and nuclear Fe-S protein biogenesis. Further, expression of *S. cerevisiae NBP35* or *CFD1* in *S. enterica* was not able to suppress the growth defects of an *apbC* null mutant (data not shown). The three proteins share sequence homology from the Walker box to the C-terminus but differ in there N-termini (Figure 1), suggesting the N-terminal tails of these proteins could contribute to the inherent specificity *in vivo*.

While ApbC and the molecular [Fe-S] scaffolding protein IscU do not share sequence homology, genetic experiments have described conditions of functional overlap *in vivo* (*29*). Like ApbC, wild-type IscU does not purify with [Fe-S] clusters but, it can bind Fe and S *in vitro*. IscU can bind two [2Fe-2S] clusters per homodimer and transfer these clusters to apo-ferredoxin (*20, 25, 44, 45*). Recently it was shown that the two [2Fe-2S] clusters can be reductively coupled yielding one [4Fe-4S] cluster per homodimer (*42*). The [4Fe-4S] loaded form of IscU, but not the [2Fe-2S] form, rapidly transferred its cluster to apo-aconitase which requires a [4Fe-4S] cluster for catalysis (*19*). Biophysical approaches are required to determine the extent of the mechanistic similarity between cluster transfer from ApbC and IscU.

ApbC is a representative member of a unique class of evolutionarily conserved proteins that can bind a transient [Fe-S] cluster and activate apo-proteins, presumably by cluster transfer. This study provides the first functional assay for a bacterial member of this class. These findings are complementary to similar studies with eukaryotic homologues and will provide a means to compare and contrast both structure and function of these proteins *in vivo* and *in vitro*.

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REFERENCES

1. Lill, R., and Muhlenhoff, U. (2006) Iron-sulfur protein biogenesis in eukaryotes: components and mechanisms. *Annu. Rev. Cell Dev. Biol. 22*, 457–486.
2. Beinert, H. (2000) Iron-sulfur proteins: ancient structures, still full of surprises. *J. Biol. Inorg. Chem. 5*, 2–15.
3. Frazzon, J., and Dean, D. R. (2003) Formation of iron-sulfur clusters in bacteria: an emerging field in bioinorganic chemistry. *Curr. Opin. Chem. Biol. 7*, 166–173.
4. Takahashi, Y., and Tokumoto, U. (2002) A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. *J. Biol. Chem. 277*, 28380–28393.
5. Zheng, M., Wang, X., Templeton, L. J., Smulski, D. R., LaRossa, R. A., and Storz, G. (2001) DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J. Bacteriol. 183*, 4562–4570.
6. Schwartz, C. J., Giel, J. L., Patschkowski, T., Luther, C., Ruzicka, F. J., Beinert, H., and Kiley, P. J. (2001) IscR, an Fe-S cluster-containing transcription factor, represses expression of *Escherichia coli* genes encoding Fe-S cluster assembly proteins. *Proc. Natl. Acad. Sci. U.S.A. 98*, 14895–14900.
7. Hantke, K. (2002) Members of the Fur protein family regulate iron and zinc transport in *E. coli* and characteristics of the Fur-regulated fhuF protein. *J. Mol. Microbiol. Biotechnol. 4*, 217–222.
8. Masse, E., Vanderpool, C. K., and Gottesman, S. (2005) Effect of RyhB small RNA on global iron use in *Escherichia coli*. *J. Bacteriol. 187*, 6962–6971.
9. Tokumoto, U., and Takahashi, Y. (2001) Genetic analysis of the isc operon in *Escherichia coli* involved in the biogenesis of cellular iron-sulfur proteins. *J. Biochem. 130*, 63–71.
10. Nachin, L., El Hassouni, M., Loiseau, L., Expert, D., and Barras, F. (2001) SoxR-dependent response to oxidative stress and virulence of *Erwinia chrysanthemi*: the key role of SufC, an orphan ABC ATPase. *Mol. Microbiol. 39*, 960–972.
11. Patzer, S. I., and Hantke, K. (1999) SufS is a NifS-like protein, and SufD is necessary for stability of the [2Fe-2S] FhuF protein in *Escherichia coli*. *J. Bacteriol. 181*, 3307–3309.
12. Lee, J. H., Yeo, W. S., and Roe, J. H. (2004) Induction of the sufA operon encoding Fe-S assembly proteins by superoxide generators and hydrogen peroxide: involvement of OxyR, IHF and an unidentified oxidant-responsive factor. *Mol. Microbiol. 51*, 1745–1755.
13. Loiseau, L., Ollagnier-de Choudens, S., Lascoux, D., Forest, E., Fontecave, M., and Barras, F. (2005) Analysis of the heteromeric CsdA-CsdE cysteine desulfurase, assisting Fe-S cluster biogenesis in *Escherichia coli*. *J. Biol. Chem. 280*, 26760–26769.
14. Zheng, L., Cash, V. L., Flint, D. H., and Dean, D. R. (1998) Assembly of iron-sulfur clusters. Identification of an*iscSUA-hscBA-fdx* gene cluster from *Azotobacter vinelandii*. *J. Biol. Chem. 273*, 13264–13272.
15. Kambampati, R., and Lauhon, C. T. (1999) IscS is a sulfurtransferase for the in vitro biosynthesis of 4- thiouridine in *Escherichia coli* tRNA. *Biochemistry 38*, 16561–16568.
16. Outten, F. W., Wood, M. J., Munoz, F. M., and Storz, G. (2003) The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for Fe-S cluster assembly in *Escherichia coli*. *J. Biol. Chem. 278*, 45713–45719.
17. Loiseau, L., Ollagnier-de-Choudens, S., Nachin, L., Fontecave, M., and Barras, F. (2003) Biogenesis of Fe-S cluster by the bacterial Suf system: SufS and SufE form a new type of cysteine desulfurase. *J. Biol. Chem. 278*, 38352–38359.
18. Layer, G., Ollagnier-de Choudens, S., Sanakis, Y., and Fontecave, M. (2006) Iron-sulfur cluster biosynthesis: characterization of *Escherichia coli* CyaY as an iron donor for the assembly of [2Fe-2S] clusters in the scaffold IscU. *J. Biol. Chem. 281*, 16256–16263.
19. Unciuleac, M. C., Chandramouli, K., Naik, S., Mayer, S., Huynh, B. H., Johnson, M. K., and Dean, D. R. (2007) In vitro activation of apo-aconitase using a [4Fe-4S] cluster-loaded form of the IscU [Fe-S] cluster scaffolding protein. *Biochemistry 46*, 6812–6821.
20. Bonomi, F., Iametti, S., Ta, D., and Vickery, L. E. (2005) Multiple turnover transfer of [2Fe2S] clusters by the iron-sulfur cluster assembly scaffold proteins IscU and IscA. *J. Biol. Chem. 280*, 29513–29518.
21. Krebs, C., Agar, J. N., Smith, A. D., Frazzon, J., Dean, D. R., Huynh, B. H., and Johnson, M. K. (2001) IscA, an alternate scaffold for Fe-S cluster biosynthesis. *Biochemistry 40*, 14069–14080.
22. Ollagnier-de Choudens, S., Nachin, L., Sanakis, Y., Loiseau, L., Barras, F., and Fontecave, M. (2003) SufA from *Erwinia chrysanthemi*. Characterization of a scaffold protein required for iron-sulfur cluster assembly. *J. Biol. Chem. 278*, 17993–8001.
23. Dos Santos, P. C., Smith, A. D., Frazzon, J., Cash, V. L., Johnson, M. K., and Dean, D. R. (2004) Iron-sulfur cluster assembly: NifU-directed activation of the nitrogenase Fe protein. *J. Biol. Chem. 279*, 19705–19711.
24. Nachin, L., Loiseau, L., Expert, D., and Barras, F. (2003) SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. *EMBO J. 22*, 427–437.
25. Chandramouli, K., and Johnson, M. K. (2006) HscA and HscB stimulate [2Fe-2S] cluster transfer from IscU to apoferredoxin in an ATP-dependent reaction. *Biochemistry 45*, 11087–11095.
26. Silberg, J. J., and Vickery, L. E. (2000) Kinetic characterization of the ATPase cycle of the molecular chaperone Hsc66 from *Escherichia coli*. *J. Biol. Chem. 275*, 7779–7786.
27. Skovran, E., Lauhon, C. T., and Downs, D. M. (2004) Lack of YggX results in chronic oxidative stress and uncovers subtle defects in Fe-S cluster metabolism in *Salmonella enterica*. *J. Bacteriol. 186*, 7626–7634.
28. Skovran, E., and Downs, D. M. (2003) Lack of the ApbC or ApbE protein results in a defect in Fe-S cluster metabolism in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol. 185*, 98–106.
29. Boyd, J. M., Lewis, J. A., Escalante-Semerena, J. C., and Downs, D. M. (2008) *Salmonella enterica* requires ApbC function for growth on tricarballylate: Evidence of functional redundancy between ApbC and IscU. *J. Bacteriol. 190*, 4596–4602.

30. Lezhneva, L., Amann, K., and Meurer, J. (2004) The universally conserved HCF101 protein is involved in assembly of [4Fe-4S]-cluster-containing complexes in Arabidopsis thaliana chloroplasts. *Plant J.: Cell Mol. Biol. 37*, 174–185.
31. Roy, A., Solodovnikova, N., Nicholson, T., Antholine, W., and Walden, W. E. (2003) A novel eukaryotic factor for cytosolic Fe-S cluster assembly. *EMBO J. 22*, 4826–4835.
32. Hausmann, A., Aguilar Netz, D. J., Balk, J., Pierik, A. J., Muhlenhoff, U., and Lill, R. (2005) The eukaryotic P loop NTPase Nbp35: an essential component of the cytosolic and nuclear iron-sulfur protein assembly machinery. *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) The iron−sulphur protein Ind1 is required for effective complex I assembly. *EMBO J. 27* (12), 1736–1746.
34. Netz, D. J., Pierik, A. J., Stumpfig, M., Muhlenhoff, U., and Lill, R. (2007) The Cfd1-Nbp35 complex acts as a scaffold for iron-sulfur protein assembly in the yeast cytosol. *Nat. Chem. Biol. 3*, 278–286.
35. Balk, J., Pierik, A. J., Netz, D. J., Muhlenhoff, U., and Lill, R. (2004) The hydrogenase-like Nar1p is essential for maturation of cytosolic and nuclear iron-sulphur proteins. *EMBO J. 23*, 2105–15.
36. Gross, S. R., Burns, R. O., and Umbarger, H. E. (1963) The Biosynthesis of leucine. II. The enzymic isomerization of beta-carboxy-beta-hydroxyisocaproate and alpha-hydroxy-beta-carboxy-isocaproate. *Biochemistry 2*, 1046–1052.
37. Jang, S., and Imlay, J. A. (2007) Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. *J. Biol. Chem. 282*, 929–937.
38. Agar, J. N., Krebs, C., Frazzon, J., Huynh, B. H., Dean, D. R., and Johnson, M. K. (2000) IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU. *Biochemistry 39*, 7856–7862.
39. Yuvaniyama, P., Agar, J. N., Cash, V. L., Johnson, M. K., and Dean, D. R. (2000) NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein. *Proc. Natl. Acad. Sci. U.S.A. 97*, 599–604.
40. Muhlenhoff, U., Gerber, J., Richhardt, N., and Lill, R. (2003) Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p. *EMBO J. 22*, 4815–4825.
41. 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) NifS-mediated assembly of [4Fe-4S] clusters in the N- and C-terminal domains of the NifU scaffold protein. *Biochemistry 44*, 12955–12969.
42. Chandramouli, K., Unciuleac, M. C., Naik, S., Dean, D. R., Huynh, B. H., and Johnson, M. K. (2007) Formation and properties of [4Fe-4S] clusters on the IscU scaffold protein. *Biochemistry 46*, 6804–6811.
43. Nishio, K., and Nakai, M. (2000) Transfer of iron-sulfur cluster from NifU to apoferredoxin. *J. Biol. Chem. 275*, 22615–22618.
44. Mansy, S. S., Wu, G., Surerus, K. K., and Cowan, J. A. (2002) Iron-sulfur cluster biosynthesis: *Thermatoga maritima* IscU is a structured iron-sulfur cluster assembly protein. *J. Biol. Chem. 4*, 21397–21404.
45. Wu, G., Mansy, S. S., Wu Sp, S. P., Surerus, K. K., Foster, M. W., and Cowan, J. A. (2002) Characterization of an iron-sulfur cluster assembly protein (ISU1) from *Schizosaccharomyces pombe*. *Biochemistry 41*, 5024–5032.

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