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Lack of the ApbC or ApbE Protein Results in a Defect in Fe-S Cluster Metabolism in *Salmonella enterica* Serovar Typhimurium

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The *isc* genes function in the assembly of Fe-S clusters and are conserved in many prokaryotic and eukaryotic organisms. In most bacteria studied, the *isc* operon can be deleted without loss of cell viability, indicating that additional systems for Fe-S cluster assembly must exist. Several laboratories have described nutritional and biochemical defects resulting from mutations in the *isc* operon. Here we demonstrate that null mutations in two genes of unknown function, *apbC* and *apbE*, result in similar cellular deficiencies. Exogenous ferric chloride suppressed these deficiencies in the *apbC* and *apbE* mutants, distinguishing them from previously described *isc* mutants. The deficiencies caused by the *apbC* and *isc* mutations were additive, which is consistent with Isc and ApbC's having redundant functions or with Isc and ApbC's functioning in different areas of Fe-S cluster metabolism (e.g., Fe-S cluster assembly and Fe-S cluster repair). Both the ApbC and ApbE proteins are similar in sequence to proteins that function in metal cofactor assembly. Like the enzymes with sequence similarity to ApbC, purified ApbC protein was able to hydrolyze ATP. The data herein are consistent with the hypothesis that the ApbC and ApbE proteins function in Fe-S cluster metabolism in vivo.

Fe-S clusters are cofactors for many proteins and aid in a variety of functions, including electron transfer, protein structure maintenance, substrate binding, and regulation in response to small molecules (reviewed in references 3, 4, 13, 14, 26, and 46). In the last several years, significant strides have been made in our understanding of Fe-S cluster assembly. Expanded efforts in this area were prompted by the discovery of an operon in *Azotobacter vinelandii* that was involved in Fe-S metabolism (62). Homologues to the genes in this operon (*isc*) were subsequently demonstrated in *Escherichia coli* (49, 56, 57), *Salmonella enterica* (52), cyanobacteria (51), *Pseudomonas aeruginosa* (9), *Thermotoga maritima* (35), mitochondrial eukaryotes (reviewed in reference 34), and amitochondrial anaerobic eukaryotes (55). Genomic sequencing efforts continue to reinforce the conservation of these genes.

In *A. vinelandii*, the *isc* operon is essential for cell viability (62), but in others, *E. coli*, *S. enterica*, and *P. aeruginosa*, for instance, multiple *isc* genes or the entire *isc* gene cluster can be deleted. In each case, the resulting strains are viable but have various growth defects (9, 20, 49, 52, 57). In addition, elimination of the *isc* genes results in reduction but not complete loss of activity of several Fe-S proteins (49, 52, 57), suggesting that additional proteins or systems are involved in the assembly and repair of Fe-S clusters in vivo. One such system is likely to include the Suf proteins, which were hypothesized to have a role in Fe-S cluster metabolism based on their similarity to Isc proteins (41). The *suf* genes are located in an operon that is regulated by iron and oxidative stress (18, 39, 41, 63) and includes *sufA*, which has similarity to *iscA*; *sufB*, whose gene product interacts with the ABC ATPase, *sufC* (39, 43); *sufD,* which is required for stabilization of the 2Fe-2S cluster of the FhuF protein (41); *sufS*, which has similarity to *iscS* and *csdA*; and *sufE*, which is similar to the *E. coli ygdK* gene, which is located adjacent to *csdA*.

Genomic sequences continue to provide insight about the genetic (and metabolic) makeup of diverse organisms and reveal the large number of genes for which no function is known. Our research efforts have focused on describing and characterizing the integration of thiamine biosynthesis with other metabolic pathways in *Salmonella enterica* serovar Typhimurium. In the course of this work, two mutants defective in genes of unknown function (*apbC* and *apbE*) were identified and found to show a number of phenotypic similarities to *isc* mutants.

Strains defective in *apbC* (*mrp*) were isolated in *S. enterica* as the predominant class of mutants unable to synthesize thiamine in the absence of the PurF enzyme (42). Based on the distance between *apbC* and the adjacent *yehE* gene (278 nucleotides), *apbC* is likely monocistronic and has been designated *mrp* in *E. coli* to reflect its location near the divergently transcribed *metG* gene. At that time, this locus was hypothesized to encode a MetG-related protein, though no phenotype of the mutant or function of the gene was described (12). Subsequently, mutants that contained an insertion in the *apbC* homologue of *Haemophilus influenzae* were characterized and found to be defective in synthesis of the Galα(1-4)βGal component of *H. influenzae* strain RM7004 lipopolysaccharide (21). Loss of the *apbC* homologue in this strain also influenced the copy number of the 5′-CAAT-3′ repeat present in *lic2A*, a gene which is required for synthesis and phase-variable expression of the Galα(1-4)βGal lipopolysaccharide epitope (21). The actual function of ApbC in this process was not demonstrated. To the best of our knowledge, no further characterizations of *apbC* mutants in these or other organisms have been reported.

Mutant screens in *S. enterica* that identified *apbC* also identified lesions in an uncharacterized, monocistronic open reading frame designated *apbE* (1). ApbE was subsequently shown

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to be a periplasmic lipoprotein whose location but not membrane association was required for efficient thiamine synthesis (1, 2). Mutations in genes similar to *apbE* have been isolated in various organisms and result in a lack of nitrous oxide reductase activity (*nosX* mutants) in *Paracoccus denitrificans* (47) and *Rhizobium meliloti* (11) and a defect in nitrogen fixation (*rnfF* mutants) in *Rhodobacter capsulatus* (48).

In *S. enterica*, the phenotypes (with respect to thiamine synthesis) caused by mutations in *apbC* and *apbE* were similar to each other and to those found for mutants defective in two known loci, *gshA* and *isc* (17, 52). Independent of other individual phenotypes, each of the four mutants displayed (i) a requirement for the 4-methyl-5-(β-hydroxyethyl)-thiazole monophosphate precursor to thiamine pyrophosphate, (ii) correction of the 4-methyl-5-(β-hydroxyethyl)-thiazole monophosphate requirement by L-tyrosine, (iii) suppression of the 4-methyl-5-(β-hydroxyethyl)-thiazole monophosphate requirement by anaerobic growth, and (iv) suppression of the 4-methyl-5-(β-hydroxyethyl)-thiazole monophosphate requirement by overexpression of the YggX protein (16, 17).

Characterization of these phenotypes suggested a model in which the thiamine requirement in these strains was caused indirectly by a defect in the synthesis and/or repair of the putative Fe-S cluster of ThiH (17, 52). ThiH is a thiazole biosynthetic enzyme and a member of the *S*-adenosylmethionine family of proteins, the characterized members of which have been shown to contain oxygen-labile Fe-S clusters (54). Based on a known or predicted role for the *isc*, *gshA*, and *yggX* gene products in Fe-S cluster assembly, repair, and/or protection (15, 16, 22–24, 27–30, 32, 35, 37, 38, 40, 49, 50, 52, 53, 56–58, 61, 62), we hypothesized that the ApbC and ApbE proteins were involved in some step of this process (17).

In this paper, we describe the biochemical and nutritional properties associated with *apbC* and *apbE* mutant strains that reflect a defect in Fe-S cluster metabolism. These results, the sequence homology of ApbC and ApbE to proteins involved in metal cofactor assembly, and the demonstration of ATP hydrolysis activity for ApbC are consistent with a role for the ApbC and ApbE proteins in the assembly, protection, and/or repair of Fe-S clusters.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. All strains used in this study are derived from *S. enterica* LT2 and are listed with their respective genotypes in Table 1. The NCE medium of Berkowitz et al. (6) supplemented with 1 mM $MgSO_4$ was used as the minimal medium, with 11 mM glucose or 10 mM gluconate added as a sole carbon source. When present in the culture media, these compounds were used at the following final concentrations: thiamine, 100 nM; cysteine, 10 mg/ml; cystine, 10 mg/ml; glutathione, 1 mM; and $FeCl_3$ (Sigma Chemical Co., St. Louis, Mo.), 5 to 50 μM as indicated in the text. Luria broth (LB) and Difco nutrient broth (NB) (8 g/liter) with NaCl (5 g/liter) were used as rich media, with Difco BiTek agar added to a final concentration of 1.5% for solid media. The final concentrations of antibiotics were as follows: tetracycline, 20 μg/ml; kanamycin, 50 μg/ml; ampicillin, 30 μg/ml; and chloramphenicol, 20 μg/ml.

Phenotypic analysis. Nutritional requirements were assessed by quantification of growth in liquid medium. All analysis was done in triplicate from three independent cultures of each strain. Strains to be analyzed were grown overnight at 37°C in minimal glucose medium with limiting thiamine (10 nM). Cells were harvested and resuspended in an equal volume of NCE medium and subsequently inoculated (150 μl of resuspended culture) into 5 ml of the appropriate medium. Culture tubes (18 by 150 mm) were placed in an air shaker at 37°C, and growth was monitored by following the optical density at 650 nm on a Bausch & Lomb Spectronic 20. Doubling times were determined as described previously (42). In growth curves, the starting A_{650} was routinely between 0.02 and 0.06.

TABLE 1. Strains[a]

Strain	Relevant genotype[b]	Plasmid(s)
LT2	Wild type	
DM5647	*yggX*::Gm	
DM5986	*yggX*::Gm *apbC55*::Tn*10* (Tc)	
DM5988	*yggX*::Gm *apbE64*::MudJ	
DM5990	*yggX*::Gm *gshA102*::MudJ	
DM5994	*yggX*::Gm *iscA2*::MudJ	
DM6086	*yggX*::Gm *iscA2*::MudJ *apbC55*::Tn*10*	
DM6374	*yggX*::Gm *apbC55*::Tn*10* (Tc)	pET20b (Ap)
DM6375	*yggX*::Gm *apbC55*::Tn*10* (Tc)	pCTH-ApbC
DM6376	*yggX*::Gm *apbC55*::Tn*10* (Tc)	pCTH-ApbC(K121A)
BL21(DE3)		pLysS
DM5477	BL21(DE3)/pLysS	pCTH-ApbC, pLysS
DM6403	BL21(DE3)/pLysS	pCTH-ApbC(K121A), pLysS

[a] Unless indicated otherwise, strains were constructed for this study. The *yggX*::Gm allele has been described (16) Tn*10*d refers to the transposition-defective mini-Tn*10* (Tn*10*D-16 D-17) (60). MudJ refers to the MudI1734 transposon (10). *E. coli* BL21(DE3)/pLysS was obtained from Novagen, Madison, Wis.

[b] Resistances are indicated in parentheses: Tc, tetracycline; Ap, ampicillin.

Generation of C-terminal His_6 tag fusion construct. The following primers (Sigma Genosys) were used to amplify *apbC* from *S. enterica*, creating an N-terminal *Nde*I site and a C-terminal *Nco*I site: ApbC*Nde*IForward, 5′-CTACA AAAGGAGTCCATATGAACGAACAATCCCAG-3′, and CTermApbC, 5′-G GATTACACCTCGAGGAAGGCGATTC-3′. The amplified PCR product was ligated into the *Sma*I site of pSU19(Cm) (36). pCTH-ApbC was created by digesting this clone with *Nco*I, followed by partial digestion with *Nde*I, purifying the fragment, and ligating it into appropriately digested pET20b(Ap) vector (Novagen, Madison, Wis.). Plasmid inserts were confirmed by sequence analysis and restriction digestion. pCTH-ApbC was transformed into expression strain *E. coli* BL21(DE3)/pLysS(Cm) (Novagen, Madison, Wis.).

Site-directed mutagenesis. With primer P-loopKA (5′-CGTGGACGATGCC CCCACCCCG-3′) and previously described methods (31), the lysine residue in the nucleotide-binding motif of ApbC (K121) in pCTH-ApbC was mutated to alanine, creating pCTH-ApbC(K121A). The mutation was verified by sequence analysis, and pCTH-ApbC(K121A) was transformed into expression strain *E. coli* BL21(DE3)/pLysS(Cm) (Novagen, Madison, Wis.).

Expression and purification of C-terminally His_6-tagged ApbC and ApbC (K121A). *E. coli* BL21(DE3)/pLysS(Cm) strains carrying the ApbC fusion constructs were grown in LB medium with ampicillin and chloramphenicol to an optical density at 650 nm of 0.6, and expression was induced for 3 h by adding isopropylthiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Cells were harvested, and protein was purified with Ni affinity chromatography (Novagen, Madison, Wis.) according to the manufacturer's specifications except that ApbC and ApbC(K121A) were eluted with a 0 to 250 mM imidazole gradient. Fractions (3 ml) of eluant were collected, and protein was visualized by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. Fractions found to contain ApbC were pooled and dialyzed into 50 mM Tris buffer (pH 8) containing 20% glycerol, frozen with liquid nitrogen, and stored at −80°C. ApbC and ApbC(K121A) were judged to be >95% pure with Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, Md.).

Enzyme assays. (i) ATP hydrolysis assays. ApbC and ApbC(K121A) (0 to 20 μg of protein) were analyzed for ATP hydrolysis in a 20-μl reaction containing CFB buffer (15 mM Tris-HCl [pH 7.5], 100 mM KCl, 10 mM $MgCl_2$), 2 mM ATP (including 1 μCi of [γ-^{32}P]ATP), and 0.1 mg of bovine serum albumin per ml. Assay mixtures were incubated at 37°C for 5 min, and reactions were initiated by addition of ATP. The reaction proceeded for 30 min and was stopped by addition of 1 M formic acid (200 μl). An aliquot of the reaction (5 μl) was spotted on a polyethyleneimine-cellulose thin-layer chromatography plate, and inorganic phosphate was separated from unhydrolyzed ATP with a 1:1 ratio of 0.4 M K_2HPO_4 and 0.7 M boric acid. ATP hydrolysis was visualized and quantified by phosphor-image analysis (Storm 860 phosphorimaging system and Image Quant software; Molecular Dynamics, Sunnyvale, Calif.). Percent hydrolysis was calculated by dividing the area corresponding to liberated phosphate by the total area of radioactivity detected for each reaction [$P_i/(P_i$ + unhydrolyzed ATP)].

(ii) Aconitase assays. Aconitase activity was determined as previously described (52) with the following exceptions. For each experiment, cell extracts (six to nine per strain) from six independent cultures were prepared from cells grown in minimal gluconate-thiamine medium with either 1 μM or 50 μM $FeCl_3$ to an optical density at 650 nm of 0.6, and cells were sonicated for a total of 5 s, with 0.5-s pauses between bursts. Each full experiment was repeated at least once.

(iii) Succinate dehydrogenase assays. For the succinate dehydrogenase assays, cells were grown, resuspended, and sonicated as described as above for the aconitase assays. Assays were preformed as described previously (52).

(v) Threonine deaminase. Cells were grown as described for the aconitase assays, washed, and resuspended in 300 μl of 100 mM Tris (pH 8)–0.4 mM dithiothreitol. Threonine deaminase activity was measured by adapting a protocol from Burns and LaRossa (8, 33). Cells were sonicated in two rounds with 0.5-s pauses between bursts, for a total burst time of 10 s. Reaction components consisted of 0.1 mM pyridoxal-5′-phosphate, 20 mM NH_4Cl, 0.5 mM dithiothreitol, and 10 to 30 μg of protein in 100 mM Tris-Cl (pH 8). The reaction mix was incubated at 37°C for 5 min, and the reaction was initiated with 10 mM L-threonine in a total volume of 50 μl. The reaction proceeded for 20 min at 37°C, derivatized with dinitrophenyl hydrazine (150 μl of a 0.025% solution in 0.6 N HCl) at room temperature for 15 min, and stopped by adding 50 μl of 40% KOH. Relative specific activity was calculated by measuring the A_{600} of α-ketobutyrate in a microtiter plate with a Spectramax Plus (Molecular Devices, Sunnyvale, Calif.) plate reader, subtracting the background from a no-threonine control reaction, and dividing by the milligrams of protein in the reaction [(A_{600} of the reaction − A_{600} of the control)/mg of protein]. Protein concentration was determined by the method of Bradford (7).

Antibody preparation and Western analysis. Aconitase A and B proteins were donated by Jorge Escalante-Semerena and used to generate rabbit polyclonal antibodies at the animal care unit of the University of Wisconsin Medical School. Standard SDS-PAGE was used, and Western analysis was performed as described previously with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Promega, Madison, Wis.). Band intensity was determined with Gel-Pro Analyzer software from Media Cybernetics, Silver Spring, Md.

RESULTS

Phenotypic characterization of *apbC* and *apbE* mutants. Because *apbC* and *apbE* mutants were initially grouped with *isc* and *gshA* mutants based on qualitative phenotypic observations, a quantitative phenotypic analysis was done to confirm and extend this grouping. In earlier studies, a frequently arising point mutation (*yggX** [16, 17]) that suppressed the phenotypes of mutants in this class had obscured phenotypic analyses. In the work presented here, all strains contained a *yggX* null mutation to simplify phenotypic interpretations.

Rich medium. Growth in rich medium (LB) was determined for the *apbC*, *apbE,* and *isc* mutant strains and their double mutant derivatives. The *isc* mutant used in these and subsequent studies contained an insertion in the *iscA* gene that is likely to be partially polar on downstream genes *hscB, hscA, fdx,* and *orf3* (52). As has been reported (49, 52, 57), a mutation in the *isc* operon caused a reduction in doubling time (*isc*, 3.5 ± 0.1 h; wild type, 1.5 ± <0.1 h). The doubling times of the *apbC*, *apbE,* and *apbC apbE* double mutant strains were equal to that of the parental wild-type strain. The presence of an *apbC* mutation exacerbated the defect of the *isc* mutant strain, causing an hour increase in doubling time (6.3 ± 0.1 h). A mutation in *apbE* imparted no additional growth defect to an *isc* mutant (5.4 ± 0.1 h). These results suggested that defects caused by mutations in *apbC* and *isc* may be additive.

Carbon source. Because growth of the *isc apb* double mutants in defined medium was severely impaired, only the single mutants were analyzed for growth in minimal medium. The severity of the thiamine requirement in *apbC* and *apbE* mutants differed based on the carbon source used (Table 2), both mutants having a more severe requirement for thiamine when glucose was the sole carbon source. When gluconate was the sole carbon source, the *apbC* and *apbE* mutants had doubling times similar to that of a wild-type strain, though there was a 3-h increase in lag phase (data not shown). In contrast to these results, the *isc* mutant strain could grow without thiamine in both cases. The previously reported thiamine requirement of this *isc* mutant was seen on solid medium (52) and by increasing the aeration in liquid medium with beveled Klett flasks (Michael Dougherty, personal communication). The discrepancy between solid and liquid medium is unclear but may result from differences in oxygen availability. Growth curve analysis (5-ml shaking cultures in 18-by-150 mm tubes) is likely semianaerobic, whereas the increased surface area-to-volume ratio and beveled bottom of a Klett flask are likely to increase oxygen content.

TABLE 2. Lesions in *isc, apbC,* and *apbE* cause detectable growth defects[a]

Genotype	Aerobic growth (doubling time, h)			Anaerobic growth (glucose)
	Gluconate	Glucose	Glucose + thiamine	
Wild type	1.7 ± 0.2	2.4 ± 0.1	1.9 ± 0.1	3.5 ± 0.3
apbC	2.2 ± 0.2	No growth[b]	1.9 ± 0.2	3.5 ± 0.2
apbE	1.7 ± 0.1	No growth	1.8 ± 0.1	3.6 ± 0.2
iscA	2.5 ± 0.3	2.8 ± 0.1	1.9 ± 0.2	3.6 ± 0.1

[a] Strains were grown overnight in minimal glucose medium supplemented with 10 nM thiamine, subcultured into the appropriate medium, and analyzed for growth as described in Materials and Methods. Cultures were grown aerobically unless specified. Anaerobic growth was monitored as previously described (42). Results are the mean of three independent experiments with standard deviations noted. All strains contained an insertion in the *yggX* gene, as explained in the Discussion.

[b] No growth, strains had a doubling time of <10 h and a final OD_{650} of <0.05 after 15 h.

Anaerobic growth. Based on the previous findings that the thiamine requirement in *isc* and *gshA* mutants was suppressed by anaerobic growth (17, 52) and that oxygen conditions altered the thiamine requirement of the *isc* mutant, doubling times for the *apbC, apbE,* and *isc* mutants in anaerobic minimal glucose medium were determined (Table 2). In the absence of oxygen and thiamine, each of the mutants grew as well as the parental strain, suggesting that the presence of oxygen may adversely affect thiamine synthesis. Because the defect in thiamine synthesis in the *isc, apbC,* and *apbE* mutant strains is conditional, the role of their gene products in thiamine synthesis is likely indirect. Previously reported results, in addition to the effect of oxygen availability on the thiamine requirement in these mutant strains, are consistent with the formally proposed model that the ThiH biosynthetic enzyme, a protein thought to contain an oxygen-labile Fe-S cluster (25, 60), is not fully functional in these strain backgrounds.

Activities of Fe-S proteins are decreased in *apbC* and *apbE* mutants. *isc* and *gshA* mutants have defects in multiple Fe-S cluster proteins (15, 49, 52, 56, 57); therefore, key enzymes were assayed in the *apbC* and *apbE* mutants to determine if these strains were also impaired in Fe-S cluster metabolism. The specific activity of aconitase was determined in cell extracts of strains with lesions in *apbE* and *apbC* in addition to the wild-type and *isc* mutant control strains. Results from these experiments are shown in Fig. 1A and demonstrated that strains defective in either *apbC* or *apbE* had reduced aconitase activity compared to a wild-type strain. The expected drop in

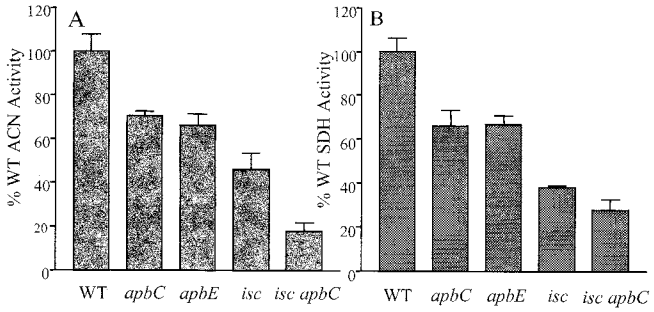

FIG. 1. Mutations in *apbC* and *apbE* result in lower aconitase and succinate dehydrogenase activities that are exacerbated by a mutation in *isc*. Activities of aconitase (ACN, panel A) and succinate dehydrogenase (SDH, panel B) are expressed as a percentage of wild-type (WT) activity present in crude cell extracts as described in Materials and Methods.

activity was obtained with the *isc* mutant. Two points were taken from these experiments. First, lesions in *apbC* or *apbE* reproducibly lowered aconitase activity (70% and 66% of wild-type activity, respectively). Second, the aconitase defect in the *apbC* and *apbE* mutants was less severe than that found in the *isc* mutant, which had 46% of wild-type activity.

Succinate dehydrogenase was a second Fe-S-containing enzyme that had reduced activity in *isc* mutants (49, 52, 57). The activities of succinate dehydrogenase were determined in cell extracts of the *apbC*, *apbE*, and *isc* mutant strains. The results of these experiments are shown in Fig. 1B and demonstrated that mutations in *apbC* and *apbE* also affected the specific activity of succinate dehydrogenase. Similar to the pattern of aconitase activity, *isc* mutations caused a more significant drop in succinate dehydrogenase activity than did lesions in *apbC*. As indicated in Fig. 1B, an *isc* mutant strain retained only 38% of wild-type activity, while the *apbC* and *apbE* mutants retained 66 and 67%, respectively. Mutants defective in *apbE* retained 67% of wild-type succinate dehydrogenase activity (data not shown). While mutations in either *apbC* or *apbE* resulted in lower aconitase and succinate dehydrogenase activities, the activity of threonine deaminase, a non-Fe-S cluster protein, was not affected by these mutations (data not shown).

To determine whether the aconitase and succinate dehydrogenase defects in the *apb* and *isc* mutant strains were additive, combinations of the *isc, apbC,* and *apbE* mutations were analyzed for these activities. When the activity of either aconitase or succinate dehydrogenase was measured, the *apbC apbE* double mutant strain did not differ significantly from the parental strains. However, the *isc apbC* double mutant had less aconitase and succinate dehydrogenase activity than either of the single parental mutant strains. As the data in Fig. 1A and B show, the double mutant had ≈18% of wild-type activity aconitase and ≈28% of wild-type succinate dehydrogenase activity. This result was interpreted to mean that the *isc* and *apbC* mutations were cumulative with respect to the defects in aconitase and succinate dehydrogenase, although more so with aconitase. This result was consistent with the growth defect of these mutant strains in LB.

These results suggest that ApbC may have a function that is partially redundant with that of one of the proteins coded by the *isc* operon or that ApbC may facilitate a different part of Fe-S cluster metabolism than Isc, such as Fe-S repair or iron scavenging. Similar to what was shown with growth in LB, the *isc* and *apbE* mutations were not additive with respect to aconitase activity. The *isc apbE* double mutant contained approximately the same activity as the *isc* single mutant. The succinate dehydrogenase activity of the *isc apbE* double mutant was slightly lower than that of the *isc* single mutant (31 versus 38% of wild-type activity, respectively).

Although a simple working model attributed the decreased enzyme activity to a reduced specific activity, it was formally possible that the levels of the aconitase and succinate dehydrogenase proteins were reduced in the mutant strains. This possibility was addressed specifically for aconitase. The amounts of both the AcnA and AcnB proteins were compared by Western blot analysis in the wild-type, *apbC*, *isc*, and *apbC isc* double mutant strains. In these experiments, the quantities of both AcnA and AcnB varied by less than 5% between these strains (data not shown), supporting the conclusion that decreased enzymatic activity in the mutant strains was due to lowered specific activity.

Exogenous $FeCl_3$ corrects defects associated with *apbC* and *apbE* mutations. To gain insight into the roles of ApbC and ApbE, multiple nutrients were screened for their ability to eliminate or exacerbate the thiamine requirement of both *apbC* and *apbE* mutants. The additive that best stimulated thiamine-independent growth was $FeCl_3$ (Fig. 2), although this addition had no effect on the growth rate of the *isc* mutant. The data in Fig. 2 show growth of the *apb* mutants under conditions of iron limitation (no addition of iron) and iron excess (50 μM). The doubling times of the *apbC* and *apbE* mutant strains in the presence of excess $FeCl_3$ were similar to those of a wild-type strain, though the mutants had an increased lag phase (3 h). The growth of the wild-type strain was significantly increased in the presence of iron, reflecting iron limitation in the minimal medium used. Titration experiments indicated that detectable thiamine-independent growth in the *apbC* and *apbE* mutant strains could be restored when at least 0.5 μM $FeCl_3$ (up to 250 μM was tested) was added to the growth medium (data not shown).

Because it was hypothesized, based on phenotypic similari-

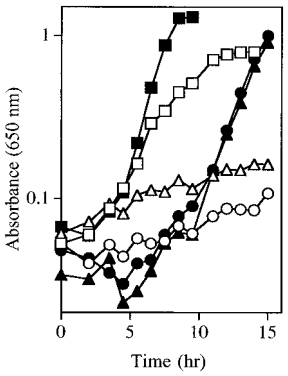

FIG. 2. Exogenous $FeCl_3$ corrects the thiamine auxotrophy of *apbC* and *apbE* mutants. Growth was monitored over time as the log A_{650}. Strains were grown at 37°C in minimal NCE salts medium with glucose as the sole carbon source. Open symbols, growth in minimal medium; solid symbols, growth in minimal medium supplemented with 50 μM $FeCl_3$. Strains: wild type, squares; *apbC*, circles; *apbE*, triangles.

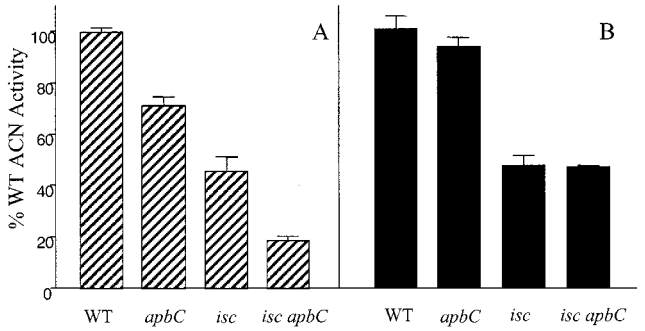

FIG. 3. Iron restores aconitase activity to an *apbC* mutant. Activity of aconitase (ACN) is expressed as a percentage of wild-type (WT) activity present in crude cell extracts as described in Materials and Methods. Cells were grown at 37°C in minimal NCE salts medium with gluconate, 100 nM thiamine, and either 5 μM (A) or 50 μM (B) $FeCl_3$.

ties, that the Apb proteins were involved in some aspect of Fe-S cluster metabolism, components of Fe-S cluster metabolism in addition to iron were tested to see if they could restore thiamine-independent growth to the *apbC* and *apbE* mutants. Neither exogenous cysteine nor cystine was able to stimulate growth of these mutants in NCE-glucose medium (data not shown). It was possible that a mutation in *apbC* or *apbE* inhibited glutathione synthesis or recycling, thus explaining the phenotypic similarity to *gshA* mutants. Exogenous glutathione also failed to restore thiamine-independent growth to *apbC* and *apbE* mutants, eliminating this possibility (data not shown).

Correction by $FeCl_3$ ties together the in vivo and in vitro defects of an *apbC* mutant. Since exogenous $FeCl_3$ restored wild-type growth properties to the *apbC* and *apbE* mutant strains, the ability of $FeCl_3$ to correct the biochemical defects in these strains was tested. Cells of the relevant strains were grown in the presence of either 5 μM or 50 μM $FeCl_3$. In each case, cell extracts were prepared and assayed for aconitase activity. Results from these experiments are shown in Fig. 3. These data showed that when an *apbC* mutant strain was grown in the presence of 50 μM $FeCl_3$, no significant defect in aconitase activity was detected (Fig. 3B). Under these growth conditions, the *apbC* mutant extract had ≈97% of the aconitase activity in the wild-type strain grown under the same conditions. This was in contrast to the result seen when cells were grown with low $FeCl_3$ (5 μM) (Fig. 3A). Consistent with the growth results, exogenous $FeCl_3$ restored aconitase activity in an *apbE* mutant to ≈100% (data not shown) but did not affect the aconitase activity of the *isc* mutant strain (Fig. 3B). When grown in the presence of excess $FeCl_3$, the *isc apbC* double mutant had aconitase activity similar to that of the *isc* single mutant. These results indicated that $FeCl_3$ specifically corrected the defect caused by the *apb* lesions.

Inhibition by anthranilic acid. While screening through different compounds for stimulation or inhibition of thiamine-independent growth, it was noticed that anthranilic acid (640 μM), an intermediate in tryptophan biosynthesis, inhibited the ability of *apbC*, *apbE*, and *isc* mutants to grow in the absence of thiamine when gluconate was the sole carbon source (Fig. 4). In each case, addition of thiamine restored full growth, suggesting that anthranilic acid was specifically affecting thiamine synthesis in these strains. At this concentration of anthranilic acid, growth of the wild-type strain was not affected. Exogenous tryptophan did not inhibit these strains, suggesting that growth inhibition was not due to increased production of this amino acid (data not shown). Several analogues of anthranilic acid, methyl benzoate and 2-chlorobenzoic acid, also inhibited growth of these strains in a thiamine-dependent manner (which implied a structural basis for the mechanism of inhibition). These results allowed visualization of the thiamine requirement of the *isc* mutant in growth curve analysis and suggested that sensitivity to anthranilic acid and its analogues may be an additional feature of strains that are defective in Fe-S metabolism (which could be used to dissect this process in vivo).

ApbC is similar to members of NifH and MinD protein families. The deduced amino acid sequence of ApbC contains a Walker P-loop motif indicative of a nucleotide-binding protein (59). Homology searches with ApbC suggested an ATPase function for this protein (12). Figure 5 shows proteins of known (or predicted) function that are most similar to ApbC, primarily members of the MinD and NifH/FdxC families of proteins. With the alignment method of ClustalW 1.8 at the Baylor College of Medicine search launcher (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html), ApbC aligned with the NifH/FdxC and MinD families of proteins starting from the P-loop motif. This motif was found 115 bp from the N terminus of ApbC and at the beginning of the MinD, NifH, and CooC proteins (7 to 38 bp from the N terminus).

Individual alignments showed that, over this stretch, ApbC was 40% identical and 58% similar to MinD from *Clostridium acetobutylicum*, 20% identical and 34% similar to NifH from *Methanococcus voltae*, and 17% identical and 29% similar to CooC from *Rhodospirillum rubrum*. Proteins in each of these families have demonstrated ATPase activity (19, 25, 44). The first 115 amino acids of ApbC showed no significant homology with other proteins and contained no defined protein motifs. Given the phenotypes of *apbC* mutants described above, of particular note were the similarities to NifH, a protein required for maturation of the iron-molybdenum cofactor of and electron donation to dinitrogenase (45), and to CooC, a pro-

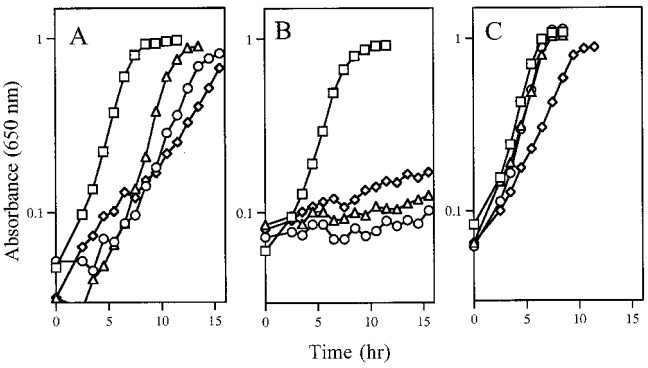

FIG. 4. Anthranilic acid inhibits thiamine-independent growth of *apbC*, *apbE*, and *isc* mutant strains. Growth was monitored over time as the log A_{650}. Strains were grown at 37°C in minimal NCE salts medium with gluconate (A), gluconate plus 640 μM anthranilic acid (B), or gluconate plus 640 μM anthranilic acid and 100 nM thiamine (C). Strains: wild type, □; *apbC*, ○; *apbE*, △; *isc*, ◇.

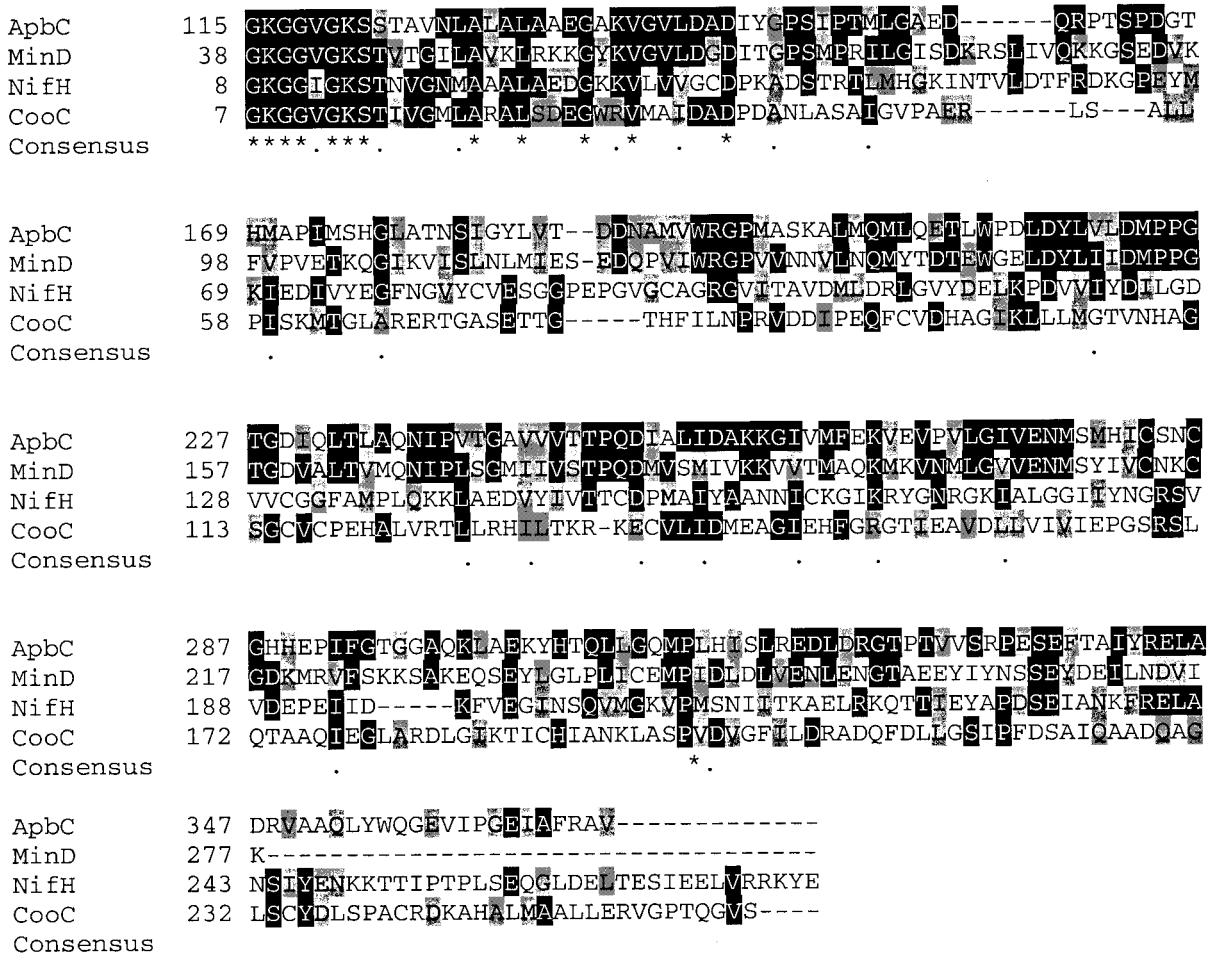

FIG. 5. Sequence alignment of the ApbC protein in *S. enterica* serovar Typhimurium. The amino acid sequence of ApbC, beginning with the Walker P-loop motif (GX_4GKT/S), was aligned with MinD from *Clostridium acetobutylicum*, NifH from *Methanococcus voltae*, and CooC from *Rhodospirillum rubrum* with the BMC search launcher at http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html. Black boxes indicate identical amino acid residues, and gray boxes indicate similar amino acid residues. Conserved amino acids are indicated with an asterisk for identical and a dot for similar residues.

tein involved in nickel cofactor maturation for carbon monoxide dehydrogenase (25).

ApbC hydrolyzes ATP in vitro. Biochemical characterization of the ApbC protein was facilitated by construction of an ApbC-His_6 fusion protein. A clone expressing the C-terminally His_6-tagged fusion construct was able to complement the phenotypes associated with an *apbC* mutant, indicating that the fusion protein was active in vivo. The fusion protein was purified via nickel affinity chromatography, dialyzed into 50 mM Tris buffer (pH 8) containing 20% glycerol, and judged to be >95% homogenous by band densitometry analysis of an SDS-PAGE gel. ATP hydrolysis was analyzed with [γ-^{32}P]ATP. The results shown in Fig. 6A demonstrated that ApbC had the ability to hydrolyze ATP. The quantity of phosphate liberated increased linearly with the amount of ApbC tested (2.5 μg to 20 μg). In control experiments, either boiling the sample or treating the protein preparation with proteinase K prior to incubation eliminated ATP hydrolysis (data not shown).

Targeted mutagenesis of the P-loop motif was performed to address whether the ATP hydrolysis activity of ApbC was physiologically relevant. A construct encoding the ApbC-His_6 fusion protein containing a K121A substitution was generated. The K121A mutant clone failed to complement the phenotypes of an *apbC* mutant (data not shown), supporting the conclusion that the ATP hydrolysis activity is significant to the in vivo function of ApbC. The K121A form of the protein was purified and assayed for ATP hydrolysis activity. ATP hydrolysis was detected with the mutant protein, but under the conditions tested, the quantity of phosphate released was fourfold lower than with the wild-type protein (Fig. 6B). It is formally possible that this low level of activity is due to a contaminating ATPase. An alternative explanation, which we favor, is that the lowered ATPase activity detected for the mutant protein in vitro is insufficient to fulfill the role of ApbC in vivo.

DISCUSSION

The *isc* locus (*iscRSUA hscBA fdx orf3*) has been shown to have a role in the assembly of Fe-S clusters (22–24, 27–30, 32, 37, 38, 40, 49, 50, 52, 53, 56–58, 61, 62). In many organisms, the *isc* genes can be deleted without loss of viability (9, 20, 49, 52, 57), making it reasonable to think that another component(s)

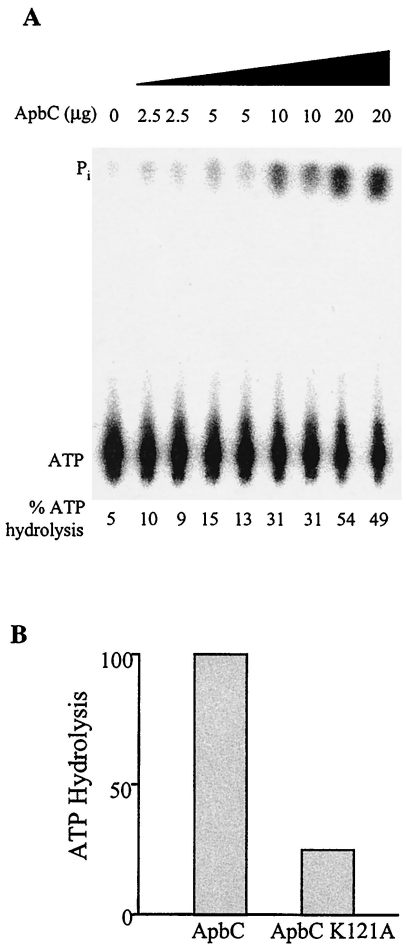

FIG. 6. ApbC has ATP hydrolysis activity. ATP hydrolysis was initiated with [γ-^{32}P]ATP and allowed to proceed for 30 min as described in Materials and Methods (A). Liberated phosphate was separated by thin-layer chromatography, and percent hydrolysis was calculated as described in Materials and Methods. Reaction mixes contained 0 to 20 μg of ApbC, as indicated. ATP hydrolysis by the wild-type and K126A mutant forms of ApbC was analyzed (B). In this experiment, the reaction mix contained 20 μM ATP. ATP hydrolysis by wild-type ApbC under these conditions was set at 1.

and/or system(s) exists to assemble Fe-S clusters in vivo. Data presented here support our hypothesis that the products of two loci, *apbC* and *apbE,* have a role in the assembly and/or repair of Fe-S clusters.

It is important to note that in all strains analyzed, the *yggX* gene was eliminated. In the *S. enterica* LT2 strain background used routinely in our laboratory, YggX protein is not detectable by Western blot analysis (Jeff Gralnick, personal communication). However, a frequently arising mutation occurs which causes *yggX* to be expressed at levels easily detected by Western analysis. We have recently found that *yggX* is expressed at high levels in *E. coli* K-12 and additional *S. enterica* LT2 strains (J. Gralnick and D. M. Downs, unpublished data). Because high levels of YggX suppress many phenotypes of the *apbC* mutant class, the defects described here may not be detected without elimination of *yggX*.

The activities of at least two Fe-S-containing enzymes were reduced in cell extracts of mutants defective in either *apbC* or *apbE*. Lesions in the *isc* operon, known for its requirement in Fe-S cluster assembly, caused a reduction in the same activities. The finding that lesions in *apbC* and *apbE* had less of an effect on the activity of the Fe-S cluster enzymes than that caused by mutations in *isc* is consistent with the ApbC and ApbE proteins providing a supplementary role in Fe-S cluster metabolism. Alternatively, these proteins could be involved in the metabolism of a subset of Fe-S clusters and as such provide functions partially redundant with those encoded by the *isc* operon. The severe reduction in aconitase and succinate dehydrogenase activities demonstrated for the *isc apbC* double mutant would be consistent with either scenario.

The *isc* and *apb* mutants were again distinguished from one another by the ability of iron to restore aconitase activity. Importantly, Benov and Fridovich have demonstrated the ability of iron to correct nutritional and biochemical defects associated with *sod* mutants (5). Specifically, in *sod* mutants, iron was able to increase the activity of 4Fe-4S oxygen-labile dehydratases without affecting transcript levels. They proposed that in the *sod* mutants, dehydratases damaged by superoxides followed by reactivation with iron serve as a sink for superoxides, thereby preventing damage to other difficult-to-repair cellular components (5). It is possible that the *apbC* and *apbE* mutants, like a *sod* mutant, are also defective in preventing or repairing damage due to oxidative stress. The addition of iron may compensate for their absence by increasing repair of oxygen-labile Fe-S clusters, similar to what was shown for *sod* mutants. This could explain why iron eliminated the thiamine requirement and restored aconitase activity to the *apb* mutants; both aconitase and ThiH have or are predicted to have oxygen-labile Fe-S clusters.

Consistent with the presence of a Walker-P motif, the ApbC protein was shown to have ATP hydrolysis activity. The targeted mutagenesis of the P-loop resulted in a protein that was both unable to complement the defects of an *apbC* mutant and defective in the hydrolysis of ATP. Together, these results implicated ATP hydrolysis in the in vivo function of the ApbC protein. Considering the results presented here, one possibility is that ApbC functions to facilitate Fe insertion into at least some Fe-S clusters during assembly or repair. The documentation that ApbC is similar at the amino acid level to proteins with a known role in metal cofactor assembly (e.g., NifH and CooC) is consistent with this hypothesis. Results presented here support a role for *apbC* and *apbE*, two loci originally identified in the context of thiamine biosynthesis, in the metabolism of Fe-S clusters.

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