Archaeal ApbC/Nbp35 Homologs Function as Iron-Sulfur Cluster Carrier Proteins

Jeffrey M. Boyd,1 Randy M. Drevland,2 Diana M. Downs,1 and David E. Graham2,3*

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706; Department of Chemistry and Biochemistry, The University of Texas at Austin, Austin, Texas 78712; and Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712

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Iron-sulfur clusters may have been the earliest catalytic cofactors on earth, and most modern organisms use them extensively. Although members of the Archaea produce numerous iron-sulfur proteins, the major cluster assembly proteins found in the Bacteria and Eukarya are not universally conserved in archaea. Free-living archaea do have homologs of the bacterial apbC and eukaryotic NBP35 genes that encode iron-sulfur cluster carrier proteins. This study exploits the genetic system of Salmonella enterica to examine the in vivo functionality of apbC/NBP35 homologs from three archaea: Methanococcus maripaludis, Methanocaldococcus jannaschii, and Sulfolobus solfataricus. All three archaeal homologs could correct the tricarballylate growth defect of an S. enterica apbC mutant. Additional genetic studies showed that the conserved Walker box serine and the Cys-X-X-Cys motif of the M. maripaludis MMP0704 protein were both required for function in vivo but that the amino-terminal ferredoxin domain was not. MMP0704 protein and an MMP0704 variant protein missing the N-terminal ferredoxin domain were purified, and the Fe-S clusters were chemically reconstituted. Both proteins bound equimolar concentrations of Fe and S and had UV-visible spectra similar to those of known [4Fe-4S] cluster-containing proteins. This family of dimeric iron-sulfur carrier proteins evolved before the archaeal and eukaryal lineages diverged, representing an ancient mode of cluster assembly.

Members of the Archaea produce many proteins that require iron-sulfur cluster cofactors, including redox proteins, aconitase-like dehydratases, radical S-adenosylmethionine enzymes, and RNA polymerase (9, 13, 18, 32). Methanogenic archaea are obligate anaerobes, and many heterotrophic archaea grow anaerobically, indicating that oxidative stress has not limited the proliferation of iron-sulfur proteins in these lineages. Archaea must have a mechanism to assemble Fe-S clusters, but many members lack homologs of the known bacterial and eukaryotic Nif or Isc systems, suggesting that an alternative mechanism to assemble Fe-S clusters may have been the earliest catalytic cofactors on earth. The SufB and SufC proteins interact, and SufB stimulates the ATPase activity of SufC. We hypothesize that the Archaea share a common mechanism for Fe-S cluster biosynthesis, supplemented with genes acquired by horizontal gene transfer in some lineages.

A screen for Salmonella enterica bacteria defective in thiamine biosynthesis identified lesions in the apbC locus (28) that compromised Fe-S metabolism (33). An apbC mutant cannot grow with tricarballylate as a carbon and energy source, which may be due to a defect in assembling or repairing [4Fe-4S] clusters in the membrane-bound TcuB protein (21, 22). ApbC is a 40-kDa cytoplasmic protein with Walker A and B nucleotide-binding domains and two conserved carboxy-terminal cysteine residues separated by two amino acids (Cys-X-X-Cys). Mutational analyses have shown that ApbC proteins with directed changes in the Cys-X-X-Cys or Walker A motifs are not active in vivo (6). Suppressor analysis allowed the conclusion that a degree of functional redundancy between ApbC and the Fe-S scaffold protein IscU exists (4, 38). Although purified ApbC does not contain iron or sulfur, biochemical studies showed that ApbC can bind an Fe-S cluster and rapidly transfer it to an apoprotein (5).

It is thought that in eukaryotes, Fe-S clusters are assembled by the mitochondrial iron-sulfur cluster (ISC) system (23). The clusters are transported into the cytosol and delivered by the cytosolic iron-sulfur protein assembly system. Two components of this system, Nbp35 and Cfd1, are homologs of bacterial ApbC (Fig. 1) and act as intermediate Fe-S cluster-trafficking proteins in the cytosol (16, 27, 30). Electron paramagnetic resonance, Mössbauer, and absorbance spectra of the Saccharomyces cerevisiae, human, and Arabidopsis Nbp35 holopro-
proteins suggest that these holoproteins form dimers with stable amino-terminal [4Fe-4S] clusters and a shared carboxy-terminal [4Fe-4S] cluster (10, 34). 

Archaeal homologs of bacterial ApbC and eukaryotic Nbp35 are underannotated as nucleotide-binding proteins or misannotated as cobyrinic acid (10, 34). 

The experiments described herein identified the first archaeal proteins that form functional Fe-S carrier proteins. The unique amino-terminal ferredoxin-like domains of the MMP0704 and MJ0283 proteins were not required. Purified MMP0704 proteins bind Fe-S clusters. Orthologs of ApbC/Nbp35 proteins were found in all of the available genomes of free-living archaea, identifying this protein family as an ancient part of the Fe-S cluster evolution that preceded the divergence of Archaea and Eukarya.

**MATERIALS AND METHODS**

**Strains, media, and DNA.** Chromosomal DNA was purified from *M. jannaschii* (MJP074; accession no. NP_578874.1), *Methanosarcina acetivorans* (MAA244; accession no. NP_619111.1), and *M. jannaschii* (MJ0283; accession no. NP_247256.1) and *M. maripaludis* (MMP0704; accession no. NP_987824.1). The two paralogs from *S. cerevisiae* are Nbp35 (accession no. NP_011424.1) and Cfd1 (accession no. NP_012263.1). 

**Cloning and mutagenesis.** *M. jannaschii* chromosomal DNA was purified from strain MJ0283 (RefSeq accession no. NP_987824.1) was amplified using primers 5MJ0283N and 3MJ0283X. The PCR product was ligated between the NdeI and BamHI sites of vector pET-11a to produce plasmid pDG499 (Table 1). The same PCR product was ligated into the HincII site of vector pSU18 to produce plasmid pJMB102. A PCR product was ligated into the HincII site of vector pSU18 to produce plasmid pJMB103.

**FIG. 1.** A protein sequence alignment of bacterial, archaeal, and eukaryotic ApbC/Nbp35 homologs was constructed using the ClustalW program (version 1.83) (37). The sequence of the *S. enterica* serovar Typhimurium protein (ApbC; RefSeq accession no. NP_461098.1) is shown without the amino-terminal domain that is not homologous to the amino-terminal domains of the archaeal and eukaryotic proteins. The archaeal homologs are from *S. solfataricus* (SSO0460; accession no. NP_341994.1), *P. furiosus* (PF1145; accession no. NP_578874.1), *Methanosarcina acetivorans* (MAA244; accession no. NP_619111.1), *M. jannaschii* (MJ0283; accession no. NP_247256.1), and *M. maripaludis* (MMP0704; accession no. NP_987824.1). The two paralogs from *S. cerevisiae* are Nbp35 (accession no. NP_011424.1) and Cfd1 (accession no. NP_012263.1). 

Conserved amino acid residues are shown in white on a black background. Similar residues are shown in black on a gray background. The four conserved amino-terminal cysteine residues shared by the MMP0704 and Nbp35 proteins are boxed. Asterisks above the sequences indicate MMP0704 amino acid residues that are replaced by mutagenesis in this study. A vertical bar indicates the N termini of the truncated proteins MJ0283(19-290) and MMP0704(20-289).
TABLE 1. List of microorganisms and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid (parent plasmid)</th>
<th>Description and/or partial genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli BL21(DE3)</td>
<td>Expression strain with T7 RNA polymerase gene</td>
<td>Novagen</td>
</tr>
<tr>
<td>DHEs</td>
<td>General cloning host</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>M. jannaschii 3AL-1</td>
<td>Wild type</td>
<td>DSM 2261</td>
</tr>
<tr>
<td>M. maripaludis 900</td>
<td>Wild type</td>
<td>DSM 1617</td>
</tr>
<tr>
<td>S. enterica DM10300</td>
<td>ara-9 apbC55;Tn10(eetr)</td>
<td></td>
</tr>
<tr>
<td>S. solfatarius P2</td>
<td>Wild type</td>
<td></td>
</tr>
</tbody>
</table>

Plasmids

| pDG499 (pET-11a)                  | MJ0283 (wild type)                  | This work            |
| pDG530 (pET-11a)                  | Δ1-18 MJ0283; encodes MMP0704(19-290) protein | This work            |
| pDG547 (pET-20b)                  | Δ1-19 MMP0704; encodes MMP0704(20-289)-His6 protein | This work            |
| pDG549 (pET-20b)                  | MMP0704(20-289) protein             | This work            |
| pDG592 (pET-20b)                  | Expression vector                   | Novagen              |
| pET-20b                            | Expression vector                   | Novagen              |
| pMB100 (pSU18)                    | S. enterica apbC (wild type)        | This work            |
| pMB102 (pSU18)                    | MJ0283 (wild type)                  | This work            |
| pMB103 (pSU18)                    | Δ1-18 MJ0283                       | This work            |
| pMB104 (pSU18)                    | MMP0704 (wild type)                | This work            |
| pMB105 (pSU18)                    | Δ1-19 MMP0704                      | This work            |
| pMB107 (pSU18)                    | MMP0704(Ser55Ala)                  | This work            |
| pMB108 (pSU18)                    | MMP0704(Cys218Ala)                 | This work            |
| pMB109 (pSU18)                    | MMP0704(Cys5,12,18Ala)             | This work            |
| pMB110 (pSU18)                    | Δ1-19 MMP0704                      | This work            |
| pMB111 (pSU18)                    | Δ1-19 MMP0704(Cys218Ala)           | This work            |
| pMB112 (pSU18)                    | MMP0704(Cys220Ala)                 | This work            |
| pMB113 (pSU18)                    | MMP0704(Cys218,220Ala)             | This work            |
| pMB114 (pSU18)                    | Δ1-19 MMP0704(Cys218,220Ala)       | This work            |
| pMB115 (pSU18)                    | Δ1-19 MMP0704(Cys218,220Ala)       | This work            |
| pMB116 (pSU18)                    | SS00480                            | This work            |
| pSU18                              | Complementation vector             | 2                    |

PCR product was ligated between the NdeI and XhoI sites of vector pET-20b to produce plasmid pDG549. The ligation of the same product into the HindIII site of vector pSU18 afforded plasmid pMB104. Primers 5'MMP0704(19-290) were used to amplify a truncated sequence that lacked codons 1 to 19 (Δ1-19 MMP0704). The ligation of this PCR product into pET-20b formed plasmid pDG547, and ligation into pSU18 formed pMB105.

Primers C218A and C218A-rev were used to perform site-directed mutagenesis on plasmid pMB108, producing plasmid pMB114. Plasmids pMB110, pMB111, pMB114, and pMB115 were created by the amplification of Δ1-19 MMP0704 mutant sequences from the full-length constructs with the primers 5'MMP0704ΔN2 and 3'MMP0704ΔS2 and subsequent subcloning into pSU18. To replace the four amino-terminal cysteine residues in the MMP0704 protein with alanine residues, primers 5'MMP0704ΔN2 and 3'MMP0704ΔS2 were annealed to each other and extended by Deep Ventα DNA polymerase (New England Biolabs) to produce a 77-bp product. Together with primer 5'MMP0704ΔS2, this product was used to amplify the MMP0704 gene from pDG549. The resulting PCR product was ligated into the NdeI and XhoI sites of pET-20b to produce plasmid pDG592. The same PCR product was ligation into pSU18 to form plasmid pMB109. The DNA sequences of all plasmid inserts and mutations were confirmed by sequencing (at the University of Wisconsin Biotechnology Center or the Institute for Cellular and Molecular Biology DNA facility at the University of Texas at Austin). Primers – 40 FORWARD and – 48 REVERSE were used to sequence pSU18 inserts.

Phenotypic analysis. Nutritional requirements were assessed by the quantification of growth on solid and liquid media (4). Liquid growth experiments were performed with 96-well plates, and growth was monitored with an ELX808 high-throughput spectrophotometer (Bio-Tek Instruments). The optical density at 600 nm (OD600) was recorded every 30 min for 48 h, and the incubation chamber was maintained at 37°C. The starting OD600 was routinely between 0.03 and 0.08, with a final OD600 between 0.5 and 1.1. Each culture had at least three replicates. Growth on solid medium was scored after replica printing onto relevant medium and incubation at 37°C for 24 to 48 h.

Protein expression and purification. Escherichia coli BL21(DE3) cells were transformed with expression vectors by electroporation. Growth aerobically at 37°C inuria-Bertani Mmiller medium supplemented with ampicillin (100 μg ml⁻¹) were cultured in mid-logarithmic-growth phase with 0.1% L-lactose. Cells were harvested and lysed as described previously, and the MMP0704 proteins were purified by nickel affinity chromatography using standard methods (13). Fractions containing the MMP0704-His6 protein were combined in dialysis tubing (SpectraPor4, 14,000-molecular-weight cut-off) and dialyzed for 15 h in 2 liters of buffer containing 50 mM KCl and 20 mM Tris-HCl (pH 8.0) at 4°C. Dialysis was continued for 5 h in fresh buffer before the protein inside the dialysis tubing was concentrated by dehydration with 20,000-Da polyethylene glycol. The MJ0283 proteins were purified by heat treatment of cell extracts prepared in a mixture of 50 mM KCl and 20 mM Tris-HCl (pH 8.0) at 4°C. The purified apoproteins were diluted to a final concentration of 2.5 mg ml⁻¹ for the His-tagged MMP0704 protein comprising residues 20 to 289 [MMP0704(20-289)-His6] or 1.0 mg ml⁻¹ for (full-length MMP0704-His6) in 625-μl reaction mixtures sealed under argon gas (13). The reconstitution buffer contained 200 mM KCl, 3 mM dithiotreitol, 0.64 mM ATP, 1.3 mM MgCl2, and 50 mM Tris-HCl (pH 8.0). FeCl3 was added to the stirred reaction mixtures at 0°C to obtain a concentration of 500 μM. After 10 min, Na2S was added dropwise to reconstitute clusters in holoproteins. The MMP0704 holoproteins were desalted using a PD-10 column (GE Healthcare) equilibrated with an anoxic solution of 50 mM Tris-HCl (pH 8.0) (12). Iron and sulfide analyses were

Table 2. Oligodeoxyribonucleotide primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>5MJ0283BN....</td>
<td>...CGAGGATCCCATATAGCTGATTGATTTGAAGAAATTG</td>
</tr>
<tr>
<td>3MJ0283B....</td>
<td>...CGAGGATCCCTTTTCTTACCTTCGACC</td>
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<td>5MJ0283BN2....</td>
<td>...CGAGGATCCATATAGCTGATTGATTTGAAGAAATTG</td>
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<tr>
<td>5MMPE0704N....</td>
<td>...CGAGGATCCATATAGCTGATTGATTTGAAGAAATTG</td>
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<td>5MMPE0704X....</td>
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<tr>
<td>5MMPE0704C....</td>
<td>...CGAGGATCCATATAGCTGATTGATTTGAAGAAATTG</td>
</tr>
<tr>
<td>5MMPE0704C....</td>
<td>...CGAGGATCCATATAGCTGATTGATTTGAAGAAATTG</td>
</tr>
</tbody>
</table>

* Restriction sites are underlined, and the initiator codon is shown in boldface.
performed using standard methods (3, 41). The standard errors for each mean value were calculated by propagating errors from each analysis.

**Phylogenetic analysis.** An alignment of seven amino acid sequences from eukaryotic Nbp35 proteins was combined with an alignment of the eight euryarchaeal proteins that have ferredoxin-like domains. The remaining sequences were added to this alignment. All alignments were constructed using the ClustalW program (version 1.83) (37). The full alignment of 47 sequences was manually edited using the Jalview program (version 2.3) (11) to remove positions that were aligned with low confidence, resulting in an average of 303 amino acids per sequence. This alignment was analyzed using the proml program from the Phylib package (version 3.67; J. Felsenstein, University of Washington) to infer a maximum-likelihood phylogeny from this alignment, with the Jones-Taylor-Thornton model of amino acid changes and a -distribution of rates (σ = 2.4) approximated by three states. Bootstrap analysis was performed with 100 replicates. The full organism names and accession numbers corresponding to the aligned sequences are listed in the supplemental material.

### RESULTS

**Identification of ApbC/Nbp35 homologs in the Archaea.** *M. maripaludis* has numerous paralogs of genes from the P-loop-containing nucleoside triphosphate hydrolase family that includes the *apbC* and NBP35 genes, but only the MMP0704 sequence encodes a protein conserving the key cysteine residues of this subfamily. To date, all of the known genome sequences of free-living archaea encode homologs of the MMP0704 protein (Fig. 2). Although the phylogeny of these ApbC/Nbp35 homologs does not recapitulate organismal phylogenies inferred from small-subunit rRNA or ribosomal protein sequences, most major archaeal groups are conserved (8). Notable exceptions include the grouping of the low-temperature crenarchaeal homologs with the haloarchaeal homologs and the gammaproteobacterial ApbC homologs. These exceptions may be explained by horizontal gene transfer events, but the direction and frequency of this transfer are obscure.

Most crenarchaeal homologs, including the *S. solfataricus* SSO0460 protein represented in Fig. 1, are missing the cysteine residue that corresponds to MMP0704 Cys220. In these sequences, an acidic amino acid replaces cysteine. Three crenarchaeal sequences, from the deeply branching organisms *Thermofilum pendens*, “Cenarchaeum symbiosum,” and “Candidatus Nitrosopumilus marinus,” do contain Cys220. From these data, we conclude that the archaeal ancestor contained a homolog of the MMP0704 gene (encoding both conserved Cys218 and Cys220 residues) and that this gene has been vertically inherited in most archaeal lineages.

The *M. maripaludis* *apbC/NBP35* homolog complements an *S. enterica* *apbC* mutation. An *S. enterica* *apbC* mutant will not grow with tricarboxylate as the sole carbon and energy source (4). This growth defect was used to examine the in vivo functionality of methanogen *apbC/NBP35* homologs. The *M. maripaludis* MMP0704 locus and the *M. jannaschii* MJ0283 locus were cloned into a multicopy vector compatible with *S. enterica* (pSU18). *S. enterica* strain DM10300 was transformed with the resulting plasmids, and the abilities of these genes to complement an *apbC* null mutant was examined. As shown in Fig. 3, strain DM10300 that had pJM100 (apbC) grew on tricarboxylate whereas strain DM10300 that had pSU18 (empty vector) did not. Strain DM10300(pJM102), expressing MJ0283, did not grow on tricarboxylate medium but did grow on glucose medium (Table 3). However, strain DM10300(pJM104), expressing MMP0704, did grow on tricarboxylate despite a significant lag before the start of exponential growth (Fig. 3). The doubling time for this strain was nearly twice as long as that for strain DM10300(pJM100). Although the MMP0704 and MJ0283 proteins show 73% amino acid identity and 91% similarity, these data indicate that only the MMP0704 protein can replace ApbC for tricarboxylate metabolism.

The amino-terminal domain of MMP0704 protein is not required for activity. Eukaryotic Nbp35 protein copurifies with an oxygen-stable Fe-S cluster thought to be coordinated by the four cysteine residues in the N-terminal ferredoxin domain. These cysteines are absent in the ApbC protein but appear to be conserved in some archaeal sequences (Fig. 1). Most archaea have truncated forms of these proteins (data not shown). To examine the necessity of the cysteine-rich ferredoxin-like domain of MMP0704 protein, an MMP0704 mutant gene with a deletion removing the first 19 codons (Δ1-19 MMP0704) was constructed. An MJ0283 gene with a similar deletion removing the first 18 codons (Δ1-18 MJ0283) was also produced (Fig. 1 and Table 1). Strain DM10300 with either pJM103 (Δ1-18 MJ0283) or pJM105 (Δ1-19 MMP0704) grew on tricarboxylate with doubling times slightly longer than that of the *apbC* control (Fig. 3 and Table 3). Additionally, the long lag before...
exponential growth seen with strain DM10300(pJMB104) was not seen with cells carrying either pJMB103 or pJMB105. We conclude that the truncated forms of MMP0704 and MJ0283 complemented an apbC mutation more efficiently than their full-length counterparts.

To determine whether the ferredoxin-like domain of wild-type MMP0704 protein reduced the effectiveness of the protein in complementation, a mutant protein in which four Cys residues were replaced by Ala was constructed. Figure 3 shows that strain DM10300(pJMB109), expressing the MMP0704 (Cys5,9,12,18Ala) variant, was able to grow with tricarboxylate. The lag before logarithmic growth and the doubling time of DM10300(pJMB109) were intermediate relative to those of strains with the full-length and truncated constructs.

Mutational analysis of the Walker A box and Cys-X-X-Cys domain of MMP0704. To determine whether nucleotide binding and hydrolysis are required for in vivo function, the Ser55 residue in the Walker A motif of MMP0704 was replaced with Ala. The corresponding ApbC variant is not able to function in vivo or hydrolyze ATP in vitro (6), and the crystal structure model of the homologous MinD cell division protein shows that the corresponding Thr17 hydroxyl group coordinates an Mg2+ ion required for the hydrolysis of ATP (17). *S. enterica* DM10300 strains with pJMB107 [MMP0704(Ser55Ala)] or pJMB110 [Δ1-19 MMP0704(Ser55Ala)] could not grow with tricarboxylate, but both strains grew with glucose (Table 3).

Roy et al. reported that the replacement of yeast Cfd1 cysteine residues in the Cys-X-X-Cys motif with serine prevented protein function in vivo (30). These residues are predicted to be ligands for a carboxy-terminal Fe-S cluster. To test whether the corresponding cysteine residues in MMP0704 protein are essential, Cys218Ala and Cys220Ala variants were constructed. DM10300 strains with both pJMB111 [Δ1-19 MMP0704 (Cys218Ala)] and pJMB112 [Δ1-19 MMP0704(Cys220Ala)] grew in tricarboxylate medium (Fig. 3 and Table 3), but strain DM10300 with pJMB113 [Δ1-19 MMP0704(Cys218,220Ala)] could not. Variant full-length MMP0704 proteins in which both Cys218 and Cys220 were changed to Ala could not compensate for the loss of ApbC in vivo (Fig. 3 and Table 3).

A crenarchaeal ApbC/Npb35 homolog complements an *S. enterica* apbC mutation. Like other crenarchaeal homologs, the *S. solfataricus* SSO0460 protein is missing the four amino-terminal Cys residues present in MMP0704 and MJ0283 proteins, and the second Cys residue of the Cys-X-X-Cys motif is replaced with Asp (Fig. 1). Additionally, the *S. solfataricus* homolog has a glutamine- and proline-rich amino-terminal domain that is not found in euryarchaeal homologs.

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**TABLE 3.** Archaeal ApbC/Npb35 homologs can function in place of *S. enterica* ApbC in vivo

<table>
<thead>
<tr>
<th>Vector</th>
<th>Vector insert</th>
<th>Doubling time* (h) with:</th>
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<tbody>
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<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>pJMB100</td>
<td><em>apbC</em></td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>pJMB102</td>
<td>MJ0283</td>
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</tr>
<tr>
<td>pJMB103</td>
<td>Δ1-18 MJ0283</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>pJMB104</td>
<td>MMP0704</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>pJMB107</td>
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<td>MMP0704(Cys218Ala)</td>
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</tr>
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<td>MMP0704(Cys220Ala)</td>
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<td>pJMB114</td>
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<td>pJMB109</td>
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</tr>
<tr>
<td>pJMB106</td>
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</tr>
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</table>

* Complementation was conducted as outlined in Materials and Methods.
*Doubling times were calculated using the following formulas: \( t = \frac{\ln X}{X_0} T \), where \( \mu \) is the growth rate, \( X \) is the OD650 value at a given time point, \( X_0 \) is the OD650 value at time zero, and \( T \) is the time between readings \( X \) and \( X_0 \). The numbers shown represent the averages of results for three independent cultures. Minimal media with the indicated carbon sources were used. The media were supplemented with thiamine. NG, no growth.

**FIG. 3.** *M. maripaludis* MMP0704 constructs can complement an *S. enterica* apbC mutant. Strains were grown at 37°C in no-carbon essential salts medium supplemented with thiamine, with tricarboxylate as the sole carbon and energy source. (A) The growth of strain DM10300 with pJMB100 (apbC) (open squares), pJMB105 (Δ1-19 MMP0704) (open circles), pJMB109 [MMP0704(Cys5,9,12,18Ala)] (closed triangles), pJMB104 (MMP0704) (filled circles), and empty vector (pSU18) (filled squares) was determined by measuring the OD650. (B) Mutational analysis of conserved cysteine residues in the Δ1-19 MMP0704 gene product. The growth of strain DM10300 with pJMB105 (Δ1-19 MMP0704) (open circles), pJMB111 [Δ1-19 MMP0704(Cys218Ala)] (open squares), and pJMB115 [Δ1-19 MMP0704(Cys218,220Ala)] (closed circles) was monitored as described in the legend to panel A.
differences raised doubts about the functional similarities between ApbC and the crenarchaeal homologs. Strain DM10300 with pJMB116 (SSO0460) grew on tricarballylate, and cells doubled every 2.7 \( \pm \) 0.1 h and displayed a lag before logarithmic growth that was similar to that of strain DM10300 with pJMB105 (MJ0283). These complementation data indicate that the \( S. solfataricus \) SSO0460 protein has Fe-S carrier activity, supporting our observation that one of the cysteine residues of the Cys-X-X-Cys motif in the MMP0704 protein is dispensable.

**Purification of methanogen ApbC/Nbp35 proteins and reconstitution of the protein iron-sulfur centers.** High levels of MMP0704-His\(_6\), truncated MMP0704(20-289)-His\(_6\), untagged MJ0283, and truncated MJ0283(19-290) proteins were produced in \( E. coli \) BL21(DE3) cells as soluble proteins. From a 500-ml culture of BL21(DE3)(pDG547) cells, 7 mg of MMP0704(20-289)-His\(_6\) protein was purified by nickel affinity chromatography (Fig. 4). Heating cell extracts containing either the full-length MJ0283 or truncated MJ0283(19-290) protein at 70°C for 10 min produced a substantially pure protein solution after centrifugation.

The iron-sulfur centers of the MMP0704 apoproteins were reconstituted under anaerobic conditions. Without ATP in the reconstitution mixture, the proteins aggregated to form high-mass complexes. However, when a fivefold excess of Mg-ATP was added, the proteins remained soluble. The aerobically purified MMP0704 apoproteins were colorless, with no significant UV-visible absorption peaks in the 400-nm region that are characteristic of major \( S \rightarrow Fe \) charge transfer bands. The UV-visible absorption spectra of chemically reconstituted MMP0704(20-289)-His\(_6\) and full-length MMP0706-His\(_6\) holo-proteins had a broad peak at 400 nm, a typical feature for proteins with \([4Fe-4S]^{2+}\) clusters, and a slight shoulder at 325 nm (Fig. 5). The reconstituted MMP0706-His\(_6\) protein had an absorption coefficient at 400 nm of 75 \( \pm \) 2 equivalents of iron and 7.7 \( \pm \) 1.6 equivalents of sulfide per monomer. The truncated MMP0704(20-289)-His\(_6\) protein had an absorption coefficient at 400 nm of 13 mM\(^{-1}\) cm\(^{-1}\) and an absorption condition of 0.47. This full-length MMP0704-His\(_6\) protein bound 7.5 \( \pm \) 1.2 equivalents of iron and 7.7 \( \pm \) 1.6 equivalents of sulfide per monomer. These values are consistent with either a monomeric [2Fe-2S] center or a dimeric [4Fe-4S] center.

**DISCUSSION**

The eukaryotic ISC biosynthesis system has a clear prokaryotic origin (35, 39), and it is too sporadically distributed in the *Archaea* to be an ancestral pathway for Fe-S cluster biosynthesis (see Table S1 in the supplemental material). Those archaea with Isc or Nif systems, such as the *Methanosaetales* and *Methanomicrobiales* lineages, probably acquired them by horizontal gene transfer. Alternatively, most archaea...
have homologs of the bacterial SuBc proteins, but it is not
clear how this system could function without other Fe-S
cluster biosynthetic proteins. The archaean ApbC/Nbp35
protein may be the intermediate carrier protein that re-
ceives an Fe-S cluster from the SuBc complex and delivers
it to an apoprotein.

The archaean Fe-S carrier proteins described herein unify an
ancient family of cluster-binding proteins that evolved before
the divergence of the Archaea and Eukarya. Despite the sig-
nificant sequence similarity among the gammaproteobacterial
ApbC proteins, the eukaryotic Nbp35 proteins, and the ar-
chaean homologs, it was not clear that archaea shared an an-
cestral Fe-S carrier prior to this work. All members of the large
nucleoside triphosphate hydrolase superfamily that includes
these proteins adopt highly conserved P-loop folds, making it
difficult to identify signature motifs or distinguish orthologs at
the primary sequence level.

A remarkable number of archaea have acquired paralo-
gous genes through recent duplications of the Mmp0704
ortholog (Fig. 2). Most members of the Methanomicrobiales
have duplicate copies that evolved independently in many
genera. Similar duplications occurred in the haloarchaeal
strain Natronomonas pharaonis and in the sulfate reducer
Archeoglobus fulgidus. In Methanoculleus marisnigri and
Methanospirillum hungatei, one copy lost the sequence encod-
ing the amino-terminal ferredoxin-like domain, reminiscent of
the Cfd1 gene in yeast. It remains to be determined whether
describing these paralogous genes encode proteins that interact, as in
yeast (27), or are differentially expressed to activate different
proteins.

The eukaryotic Nbp35 homologs share an amino-terminal
ferredoxin-like domain, an ATP-binding motif, and two con-
served carboxy-terminal cysteine residues that are believed to
bind an Fe-S cluster. The S. cerevisiae Nbp35 protein contains
an oxygen-stable [4Fe-4S] cluster when overexpressed in and
purified from E. coli (34). A truncated Nbp35 variant missing
the N-terminal ferredoxin-like domain is not purified with Fe
and S (16). Comparative sequence analyses show that all bac-
terial homologs and most archaean homologs lack the ferre-
doxin-like domain present in Mmp0704 protein. S. enterica
mutants expressing the truncated form of the Mmp0704 pro-
tein grew faster than cells producing wild-type Mmp0704 pro-
tein during tricarballylate-dependent growth assays, demon-
strating that the amino-terminal domain is dispensable. To test
whether this difference in growth rate was due to decreased
gene expression or thiol-mediated complexes of the full-length
protein, all four amino-terminal cysteines were replaced by
alanine. Cells producing this variant protein grew faster than
cells with wild-type protein, suggesting that interactions due to
the cysteine thiols caused reduced activity. Therefore, the amino-
terminal domain is not required for Mmp0704 activity in S.
enterica, although genetic analysis of M. maripaludis will be
required to determine whether this domain is essential in the
native host.

The ATPase activities of full-length and truncated Mmp0704
proteins are necessary for function in vivo. Both the truncated
and full-length Mmp0704 proteins require Mg-ATP for effi-
cient in vitro chemical reconstitution of their Fe-S clusters. Key
residues in the ATP-binding motif of ApbC are also essential
for function in vivo but not for in vitro Fe-S cluster binding (5,
6). In the presence of Mg-ATP, the full-length Mmp0704
holoprotein formed a mixture of high-mass species (analyzed
by size exclusion chromatography), while the Mmp0704(20-
289)-His4 holoenzyme formed primarily dimers (R. Drevland,
unpublished data). Our interpretation of these data is that
ATP binding or hydrolysis is required for cluster transfer to
Mmp0704 protein or cluster construction on Mmp0740 pro-
tein.

Chemically reconstituted, truncated Mmp0704 protein
bound approximately 2 mol of Fe and S per mol of protein,
formed dimers, and had an absorption spectrum similar to that
of proteins known to have [4Fe-4S] clusters. These data are
consistent with the hypothesis that truncated Mmp0704
protein binds a [4Fe-4S] cluster that forms the interface of two
subunits. The ligands for the essential Fe-S cluster in ApbC/
Nbp35 proteins were assumed to be two conserved cysteine
thiols, Cys218 and Cys220, near the carboxy terminus. A prelim-
nary model of the A. fulgidus AF2382 homolog (Protein Data
Bank accession no. 2PH1) shows that the conserved cysteine
residues are found in a β turn. The two cysteine sulfur atoms
are 4.3 Å apart, coordinating a Zn2+ ion at the dimer interface
(F. Forouhar and L. Tong, unpublished data). In most cran-
archaeal homologs, an Asp or Glu residue replaces Cys218, yet
the S. solfataricus homolog complemented the Salmonella
apbC mutation. Aspartate is also a ligand for the [4Fe-4S]
cluster of the P. furiosus ferredoxin, where it slightly increases
the cluster’s reduction potential compared to a cysteine ligand
substituent (7). Yet both the Δ1-19 Mmp0704/Cys220Ala and
Δ1-19 Mmp0704/Cys218Ala alleles complemented an apbC
mutant, suggesting that individual cysteine residues in the Cys-
X-X-Cys motif are dispensable. Amino acid insertions in this
region of the plant Nbp35 homologs also indicate variable
modes of coordination (10). A similar result was reported for
variants of the Azotobacter vinelandii NitF Fe-S scaffold pro-
tein, where mutations in conserved cysteine codons reduced,
but did not abolish, diazotrophic growth (1). Additional elec-
tron paramagnetic resonance and Mössbauer studies will be
required to determine which residues are ligands for an Fe-S
cluster.

Almost all modern organisms have Fe-S proteins, and
these cofactors represent the interface of biological chem-
istry with the complex marine geochemistry of iron sulfides
(29). FeS minerals, particularly mackinawite, greigite, and
pyrite, feature prominently in several hypotheses for the
chemical origin of life, as Lewis acid catalysts or primitive
inorganic membranes (19, 24, 31, 40). As iron became less
bioavailable, cells’ iron acquisition systems became sophis-
ticated and a variety of Fe-S cluster assembly systems
evolved. Among archaea with completely sequenced ge-
omes, only the obligate parasite “Nanoarchaeum equitans”
lacks an apbC/Nbp35 gene. The loss of most iron-sulfur-
dependent metabolic proteins from N. equitans may be as-
associated with this gene loss, since the remaining iron-sulfur
proteins must be assembled using host-derived clusters. This
report represents the first genetic and biochemical study
examining Fe-S cluster biosynthesis in archaea. The data
herein support the hypothesis that archaean ApbC/Nbp35
homologs function as Fe-S cluster carrier proteins.
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