The *cbiS* Gene of the Archaeon *Methanopyrus kandleri* AV19 Encodes a Bifunctional Enzyme with Adenosylcobinamide Amidohydrolase and α -Ribazole-Phosphate Phosphatase Activities

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Received 13 February 2006/Accepted 5 April 2006

Here we report the initial biochemical characterization of the bifunctional α -ribazole-P (α -RP) phosphatase, adenosylcobinamide (AdoCbi) amidohydrolase CbiS enzyme from the hyperthermophilic methanogenic archaeon *Methanopyrus kandleri* AV19. The *cbiS* gene encodes a 39-kDa protein with two distinct segments, one of which is homologous to the AdoCbi amidohydrolase (CbiZ, EC 3.5.1.90) enzyme and the other of which is homologous to the recently discovered archaeal α -RP phosphatase (CobZ, EC 3.1.3.73) enzyme. CbiS function restored AdoCbi salvaging and α -RP phosphatase activity in strains of the bacterium *Salmonella enterica* where either step was blocked. The two halves of the *cbiS* genes retained their function in vivo when they were cloned separately. The CbiS enzyme was overproduced in *Escherichia coli* and was isolated to >95% homogeneity. High-performance liquid chromatography, UV-visible spectroscopy, and mass spectroscopy established α -ribazole and cobyrinic acid as the products of the phosphatase and amidohydrolase reactions, respectively. Reasons why the CbiZ and CobZ enzymes are fused in some archaea are discussed.

In the last 10 years, we have seen an exponential increase in the number of gene and protein sequences deposited in databases of organisms from all three domains of life. Experimental studies, however, have had little chance to keep up with the numerous computational studies aimed at assigning functions to these new gene sequences. It is likely that the great majority of the genes in most species may not be studied experimentally and that our understanding of the biochemical and physiological roles of the products encoded by these genes will be derived from functionally characterized orthologs from model organisms (for a review, see reference 18). The wealth of information obtained from model organisms (e.g., *Escherichia coli*, *Saccharomyces cerevisiae*, and *Arabidopsis thaliana*) allows for the transfer of information gained from experimental studies. Archaea are a special case, since no equivalent model system has yet been established for this domain of life. The genome sequences of archaea have provided insight into their physiology and metabolism, but the majority of the gene functions have been predicted based solely on their similarity to genes from nonarchaeal model organisms. However, the function of proteins encoded by these archaeal genes may differ from their orthologous functions in bacteria and eukaryotes. Furthermore, these genes with assigned function comprise only about 50% of the total genes, and about half of those have no orthologs in the other two domains of life (1, 9). It is therefore necessary to determine the functions of archaeal gene products through experimental work so we can better understand the biochemistry and physiology of archaea.

One strategy used to assign function to new genes is comparative genomics. This approach identifies glaring gaps in

otherwise well-characterized metabolic pathways and uses classical approaches to determine what enzyme archaea use to fill the gap. The archaeal fructose-1,6-bisphosphate aldolase (6, 26), thymidylate synthase (7, 19), and phosphoglycerate mutase (14, 32) enzymes have been identified by using this strategy.

The coenzyme B₁₂ (also known as adenosylcobalamin [AdoCbl]) biosynthetic pathway has been extensively studied in bacteria and has been shown to require at least 25 genes for de novo biosynthesis of the complete molecule (AdoCbl) (34). Archaea are known to synthesize and require corrinoids to live. For example, methanogenic archaea use cobamides for methanogenesis from H₂ and CO₂, acetate, or methanol (3). Active cobamide-dependent ribonucleotide reductases have been purified from *Thermoplasma acidophilum* (29) and *Pyrococcus furiosus* (23), suggesting these organisms use cobamides for DNA synthesis. *Halobacterium* sp. strain NRC-1 has recently been shown to synthesize and require Cbl under certain growth conditions, but it is unknown why it is needed (37, 38).

Comparative genomics, however, has revealed the absence of several genes required by bacteria to synthesize Cbl that are conserved in bacteria. One missing protein, CobU, is a bifunctional ATP:AdoCbi kinase/GTP:AdoCbi-P guanylyltransferase (Fig. 1A) (20). The two functional “gaps” were solved by the identification of the CobY and CbiZ enzymes (Fig. 1B). The CobY protein was shown to be the nonorthologous replacement of the CobU GTP:AdoCbi-P guanylyltransferase activity (30, 36), and the adenosylcobinamide (AdoCbi) amidohydrolase (CbiZ) enzyme was shown to salvage AdoCbi using a new mechanism by converting it to the de novo intermediate adenosylcobyrinic acid (AdoCby) (35).

We recently identified CobZ, the archaeal nonorthologous replacement of the bacterial α -ribazole-P (α -RP) phosphatase enzyme (CobC in *Salmonella enterica*) (40). This step involves the removal of a phosphate from the activated lower α -ligand

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of AdoCbl and is required for the assembly of the nucleotide loop of AdoCbl (21) (Fig. 1C).

This paper reports the identification of the *cbiS* gene encoding a protein that is the result of a fusion of the *cobZ* and *cbiZ* genes. The CbiS enzyme from the methanogenic hyperthermophilic archaeon *Methanopyrus kandleri* was shown to have both AdoCbi amidohydrolase (CbiZ) activity and α -RP phosphatase (CobZ) activity when expressed in *S. enterica* and in vitro.

MATERIALS AND METHODS

Strains and plasmids. The genotypes of the *S. enterica* strains and the plasmids used in this work are described in Table 1.

Chemicals, culture media, and growth conditions. Unless otherwise stated, all chemicals used in this work were commercially available, high-purity compounds. The given pH of buffers corresponded to the temperature at which they were used. When added to the medium, corrinoids were present at 10 nM. All corrinoids were added in their cyano form. AdoCbi was synthesized as previously described (30). Cobinamide dicyanide (Cbi) and cyanocobalamin (Cbl) were purchased from Sigma. Cbi-phosphate (Cbi-P) and Cbi-GDP were synthesized as previously described (30). Cobyrilic acid dicyanide (Cby) was a gift from Paul Renz (Universität-Hohenheim, Stuttgart, Germany); α -RP was synthesized as described previously (17); 5,6-dimethylbenzimidazole (DMB) was purchased from Sigma.

Bacterial strains used for protein overproduction. Overproduction of CbiS-chitin-binding protein fusion was performed in *E. coli* strain ER2566 (New England Biolabs).

Plasmid constructions. Plasmids were propagated in *E. coli* strain DH5 α except where noted. *M. kandleri* AV19 genomic DNA (a gift from M. Thomm) was used as the DNA template in every PCR. We used T4 ligase (MBI Fermentas) for all DNA ligation reactions. All primers were purchased from Integrated DNA Technologies, Inc.

Plasmid pMkCBIS2. To clone the *M. kandleri* *cbiS* gene into an overexpression vector, *M. kandleri* *cbiS* was first cloned into the multicloning vector pGEM-T (Promega). The 5' primer Mk1672-NdeI-5' (TCTAGACATATGGA GTTCTTTTCACGTGATC) and the 3' reverse primer MkCBIS-SalI-3' #2 (GT CGACGGAATGCGGCGGTCATG) were used to PCR amplify a 1,080-bp fragment from genomic DNA (italic portions of all primer sequences indicate introduced restriction sites). The fragment was cloned into pGEM-T according to manufacturer's instructions to yield the plasmid pMkCBIS2.

Plasmid pMkCBIS3. Plasmid pMkCBIS3 contains *M. kandleri* *cbiS* under the control of an inducible promoter. The overproduced protein has a C-terminal chitin-binding protein fusion that can be cleaved resulting in native CbiS protein. The plasmid was constructed as follows. A DNA fragment containing the *cbiS* gene was obtained by digesting plasmid pMkCBIS2 with SalI restriction enzyme, blunt ending with Klenow fragment (Promega), digesting with NdeI restriction enzyme, and then gel purifying with a QIA Quick Gel extraction kit (QIAGEN). The fragment was then ligated into the NdeI/SmaI restriction site of plasmid pTYB2 (New England Biolabs) to generate plasmid pMkCBIS3.

Plasmid pMkCBIS4. Plasmid pMkCBIS4 contains a wild-type allele of *M. kandleri* *cbiS* under the control of a T7 promoter and was constructed as follows. The 5' primer Mk1672-NdeI-5' and the 3' reverse primer Mk1672-SalI-3' (TC TAGAGTCGACTTTTCAGGAATCCACC) were used to PCR amplify a 1,130-bp DNA fragment from genomic DNA. The fragment was digested with NdeI and SalI restriction enzymes and ligated into the NdeI/SalI restriction site of plasmid pT7-7 to generate plasmid pMkCBIS4.

Plasmid pMkCBIS6. Plasmid pMkCBIS6 contains only the first 621 bp of the *M. kandleri* *cbiS* open reading frame (ORF) followed by a TGA stop codon. This is predicted to encode only the amidohydrolase "domain" of the CbiS protein under the control of a T7 promoter. The plasmid was constructed as follows. The

TABLE 1. Strains and plasmids used in this study^a

Strain ^b or plasmid	Relevant genotype and description	Reference or source
Plasmids		
pT7-7	<i>bla</i> ⁺ , cloning vector	28
pSU39	<i>kan</i> ⁺ , cloning vector	Laboratory collection
pJO52	<i>bla</i> ⁺ <i>cobU</i> ⁺	20
pMmCBIZ1	<i>bla</i> ⁺ <i>cbiZ</i> ⁺	35
pJO46	<i>bla</i> ⁺ <i>cobC</i> ⁺	21
pMkCBIS3	<i>bla</i> ⁺ <i>cbiS</i> ⁺	
pMkCBIS4	<i>bla</i> ⁺ <i>cbiS</i> ⁺	
pMkCBIS6	<i>bla</i> ⁺ 5' half of <i>cbiS</i>	
pMkCBIS7	<i>bla</i> ⁺ 3' half of <i>cbiS</i>	
pCOBY38	<i>kan</i> ⁺ <i>cobY</i> ⁺	
Strains		
TR6583	<i>metE250 ara-9</i>	K. Sanderson via J. Roth
JE824	<i>cbiP236::Tn10d</i> (Tet)	Laboratory collection
	<i>cobU330</i>	
JE2119	<i>cobC1175::Tn10d</i> (Tet)	Laboratory collection
JE2192	<i>cobC1175::Tn10d</i> (Tet)	Laboratory collection
	<i>cobT109::MudJ</i>	
JE4724	<i>cobC1175::Tn10d</i> (Tet)	Laboratory collection
	<i>cobT109::MudJ</i>	
JE6984	<i>cbiP236::Tn10d</i> (Tet)	Laboratory collection
	<i>cobD1230::Tn10d</i> (Chl)	
	<i>cobU330</i>	

^a Unless otherwise stated, strains and plasmids were constructed during the course of this study.

^b All strains are derivatives of *S. enterica* serovar Typhimurium strain LT2.

5' primer Mk1672-NdeI-5' and the 3' primer MkCBIS-SalI-3' #3 (TCTACTGT CGAGTACCCCTGCGGCTCTCC) were used to PCR amplify a 640-bp DNA fragment from genomic DNA. The fragment was digested with NdeI and SalI restriction enzymes and ligated into the NdeI/SalI restriction site of plasmid pT7-7 to generate plasmid pMkCBIS6.

Plasmid pMkCBIS7. Plasmid pMkCBIS7 contains only the last 453 bp of the *M. kandleri* *cbiS* ORF preceding an ATG start site; *cbiS* expression was placed under the control of a T7 promoter. The 3' half of *cbiS* (3' *cbiS*) was predicted to encode the phosphatase region of the CbiS protein with phosphatase activity. The plasmid was constructed as follows. The 5' primer MkCBIS-NdeI-5' #2 (TCTACTCATATGTCTTCCCAACACGCTCG) and the 3' primer Mk1672-SalI-3' were used to PCR amplify a 500-bp DNA fragment from genomic DNA. The fragment was cut with NdeI and SalI restriction enzymes and ligated into the NdeI/SalI restriction site of plasmid pT7-7 to generate plasmid pMkCBIS7.

Plasmid pCOBY38. Plasmid pCOBY38 contains the wild-type allele of the *Methanosarcina mazei* Gö1 *cobY* gene under the control of a T7 promoter and was constructed as follows. A DNA fragment containing *cobY*⁺, a T7 promoter, and a ribosome-binding site was excised from plasmid pCOBY10 (35) using BglII and SalI restriction enzymes. The DNA fragment was gel purified and ligated into the BamHI/SalI restriction site of plasmid pSU39 to yield plasmid pCOBY38.

Growth behavior studies. Cultures of *S. enterica* strains were grown and monitored as previously described (35). Where indicated, DMB (300 μ M) was added to the *S. enterica* medium. All plasmids introduced into *S. enterica* were first passed through a restriction enzyme-deficient strain (31).

Overproduction and purification of recombinant CbiS protein. *M. kandleri* CbiS protein fused to a C-terminal chitin-binding protein tag was overproduced by using plasmid pMkCBIS3 in the overproducing *E. coli* strain ER2566 (Strata-

FIG. 1. Relevant biochemical pathways of corrinoid biosynthesis and salvaging in prokaryotes. Shown are the late steps of de novo Cbl biosynthesis and Cbi salvaging (panel A), de novo cobamide biosynthesis and Cbi salvaging in archaea (panel B), and α -ligand synthesis in *S. enterica* (panel C). Intermediates are boxed and indicated below structures. Enzymes catalyzing the given reaction are in bold below arrows. CobA, ATP:co(I)rrinoid adenosyltransferase; CbiB, adenosylcobinamide phosphate synthase; CobS, cobalamin (5'-P) synthase; NMP, nucleotide monophosphate; NTP, nucleotide triphosphate; NaMN, nicotinate mononucleotide. Superscript letters: a, the archaeal CobA ortholog (N. R. Buan and J. C. Escalante-Semerena, unpublished results); b, the archaeal CbiB ortholog (38); c, the archaeal CobS ortholog (16).

gene). Three milliliters of an overnight culture of the overproducing strain carrying the plasmid pMkCBIS3 in LB broth containing ampicillin (100 µg/ml) was used to inoculate six 500-ml batches of fresh medium. Cultures were grown at 18°C with shaking (200 rpm) to a cell density (optical density at 600 nm) of 0.35, at which point IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the final concentration of 300 µM. IPTG-containing cultures were incubated under the same conditions for an additional 18 h. CbiS protein was purified and stored as previously described for the CbiZ protein (35), except that the final storage conditions contained 30% glycerol (wt/vol). The final concentration of protein was 0.23 mg per ml.

In vitro CbiS amidohydrolase activity assay. Corrinoid amidohydrolase activity assays were performed using a modification of a previously published protocol (35). Assays were performed in 40-µl reaction mixtures containing 0.9 mg of CbiS protein, 1.5 M phosphate buffer, pH 9.0, 360 µM corrinoid substrate. Reaction mixtures were incubated anaerobically at 90°C in dim light for one hour. The amidohydrolase activity of CbiS was equally effective with adenosylated and nonadenosylated substrates. To facilitate the performance of the experiments, nonadenosylated substrates were used in the reaction mixture.

In vitro CbiS phosphatase activity assay. α-RP phosphatase activity assays were performed in 50-µl volumes containing CbiS protein (1.1 µg), 50 mM Na-HEPES, pH 8.0, MgCl (10 mM), and α-RP (190 µM). Reaction mixtures were incubated anaerobically at 90°C for one hour.

HPLC analysis of CbiS reactions. Corrinoids present in the amidohydrolase reaction mixtures were analyzed via high-performance liquid chromatography (HPLC) as described previously (35). For CbiS phosphatase reactions, α-RP and α-ribazole (α-R) were separated via HPLC as described previously (17).

Mass spectrometry. Corrinoid samples were prepared for mass spectrometry analysis as previously described (35). CbiS phosphatase reaction products were prepared for mass spectrometry by drying HPLC-purified peaks under vacuum. All samples were submitted for analysis to the mass spectrometry facility at the University of Wisconsin—Madison Biotechnology Center. The mass spectra of corrinoids were obtained using a Bruker Daltonics (Billerica, MA) BIFLEX III matrix-assisted laser desorption/ionization–time of flight mass spectrometer. Mass spectra of products from the CbiS phosphatase reaction were obtained using a Perkin-Elmer Sciex API365 triple quadrupole instrument with an ion spray source (positive-ion mode).

RESULTS

Identification of the gene encoding both AdoCbi amidohydrolase (CbiZ-like) and α-RP phosphatase (CobZ-like) activities. The gene encoding the AdoCbi amidohydrolase enzyme (CbiZ) is conserved in all available archaeal genome sequences (38). In some hyperthermophilic archaea, however, *cbiZ* is predicted to be part of a much larger gene (referred to as *cbiS* in this paper), for which only the N terminus of the encoded peptide has similarity to CbiZ. The C terminus of the CbiS protein is currently annotated as a member of clusters of orthologous groups (COG) number 1267, which includes phosphatidylglycerophosphatase A (PgpA)-related enzymes. The C-terminal half of CbiS is also found encoded by a separate gene (*cobZ*) recently identified as encoding the nonorthologous replacement in several archaea of the α-RP phosphatase (CobC) enzyme of *S. enterica* (40). We did not find that the CbiS or CobZ protein shares any significant similarity with the bacterial PgpA enzyme or to any other characterized enzyme (data not shown). *cbiS* orthologs in the NCBI database were identified in the hyperthermophilic archaea *Aeropyrum pernix* (APE2032; gene identification number [gi] 14601794), *Pyrobaculum aerophilum* (PAE0371; gi 18311898), and *M. kandleri* (Mk1672; gi 20095108) (Fig. 2). In all three organisms, the *cbiS* gene was included in a putative operon predicted to encode orthologs to other corrinoid biosynthetic enzymes. In *M. kandleri*, *cbiS* (annotated as *pgpA*) is the first gene in a putative operon encoding orthologs to the cobalamin synthase (CobS, EC 2.7.8.26; Mk1671), the GTP:adenosylcobinamide-P guanylyl-

FIG. 2. Organization of the *cbiS* gene in genomes of hyperthermophilic archaea. NaMN, nicotinate mononucleotide.

transferase (CobY, EC 2.7.1.156; Mk1670), and a hypothetical protein (Mk1669). A nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase (CobT, EC 2.4.2.21) ortholog is predicted to be encoded distally by ORF Mk0224. *M. kandleri* CbiS amino acid residues 1 to 207 have 45% similarity and 26% identity to *M. mazei* CbiZ (EC 3.5.1.90), and amino acid residues 208 to 357 have 52% similarity and 33% identity to *M. mazei* Gö1 CobZ (40).

An archaeal *cbiS* gene restores Cbi salvaging in an *S. enterica* mutant. To determine whether CbiS was involved in Cbi salvaging in archaea, the ability of an archaeal *cbiS* gene to complement an *S. enterica* strain defective in Cbi salvaging was tested. *S. enterica* strain JE824 (*metE205 cobU330*) was used to test for *M. kandleri* *cbiS*⁺ function. Growth of this strain depends on B₁₂-dependent methylation of homocysteine by the B₁₂-dependent methionine synthase enzyme (4). Because the de novo pathway in *S. enterica* is blocked under aerobic conditions, aerobic growth in defined medium lacking methionine requires corrinoid salvaging (11). Mutation *cobU330* eliminates both the ATP:AdoCbi kinase and the GTP:AdoCbi-P guanylyltransferase activities and therefore blocks both de novo corrin ring biosynthesis and Cbi salvaging in this bacterium (5). To restore CbiS- and Cbi-dependent growth of strain JE824, an NTP:AdoCbi-P nucleotidyltransferase was provided. For this purpose, plasmid pCOBY38 (*M. mazei* *cobY*⁺) (36) was transformed (24) into strain JE824. Plasmid pJO52 (*cobU*⁺) was used as a positive control, whereas plasmid pT7-7 (cloning vector used to construct plasmid pJO52) was used as a negative control. pMkCBIS4 (*M. kandleri* *cbiS*⁺) and/or pCOBY38 (*cobY*⁺) was introduced into strain JE824 by transformation. The resulting strains were tested for aerobic growth in medium supplemented with Cbi. Under aerobic conditions, growth depended on the ability of the strain to salvage Cbi. Cbi-dependent growth was only observed when *S. enterica* harbored *S. enterica* *cobU* (Fig. 3A) or both *M. kandleri* *cbiS* and *M. mazei* *cobY* (Fig. 3A). These data supported the conclusion that *cbiS* function restored Cbi salvaging.

CbiS restores Cbi salvaging via the archaeal amidohydrolase pathway. Although CbiS restored Cbi salvaging in strain JE824, these results did not differentiate between CbiS acting as a kinase or as an amidohydrolase. To identify an entry point for Cbi, we used an *S. enterica* strain carrying a mutation in the

FIG. 3. Nutritional analysis using full-length CbiS and separate N-terminal and C-terminal segments of CbiS. Cbi-salvaging-dependent growth of *S. enterica* strains in chemically defined liquid medium at 37°C. OD₆₅₀, optical density at 650 nm. Panel A. All strains were derivatives of strain JE824 (*metE205 cobU330*). Panel B. All strains were derivatives of strain JE6984 (*metE205 cobU330 cobD1272*). Strains are indicated by genotype. Panel C. All strains were derivatives of strain JE2192 (*metE cobC cobT*). DMB was added to 300 μ M except where noted. Panel D. All strains were derivatives of strain JE2119 (*metE cobC*). Strains are indicated by genotype. In all cases, corrinoids were added to 10 nM. Plasmids used were pT7-7 (control vector), pJO52 (*cobU*⁺), pCOBY38 (*cobY*⁺), pMkCBIS4 (*cbiS*⁺), pMkCBIS6 (5' *cbiS*⁺), pMkCBIS7 (3' *cbiS*⁺), and pJO46 (*cobC*⁺).

L-threonine-P decarboxylase (CobD) enzyme (2), which decarboxylates L-threonine-P, yielding 1-amino-2-propanol-phosphate (AP-P), a substrate for the CbiB enzyme (Fig. 1A). A block in this step would not affect Cbi salvaging if the entry point for the product of the CbiS reaction was AdoCbi-P. If, however, CbiS converted Cbi to the earlier intermediate AdoCby, CobD function would be required for Cbi salvaging.

As expected, the control plasmid pJO52 (*cobU*⁺) restored Cbi-dependent growth of strain JE6984 (*metE205 cobU330 cobD1272*) (Fig. 3B). Together, plasmids pMkCBIS4 (*cbiS*⁺) and pCOBY38 (*cobY*⁺) failed to restore growth of the same strain unless AP was added to the medium (Fig. 3B). This result was expected since AP compensates for the lack of CobD function (2, 8). These results were consistent with the

prediction that *cbiS* encoded an AdoCbi amidohydrolase enzyme with CbiZ-like activity.

The 5' fragment of the *cbiS* gene is necessary and sufficient for Cbi salvaging. Because only the first 207 amino acid residues of the *M. kandleri* CbiS protein were similar to CbiZ, we investigated whether a 5' fragment of the *cbiS* gene encoding only residues 1 to 207 would retain its CbiZ-like activity. To this end, plasmid pMkCBIS6 (5' *cbiS*⁺) or pMkCBIS7 (3' *cbiS*⁺) was introduced into strain JE824 (*cobU330*) carrying plasmid pCOBY38 (*cobY*⁺). Cbi-dependent growth was observed when the 5' fragment of *cbiS* was provided but not when a 3' fragment (encoding residues 208 to 250) was provided (Fig. 3A). The 5' fragment of *cbiS* also allowed Cbi-dependent growth of strain JE6984 when *cobY* was provided in *trans* and the medium was supplemented with AP (Fig. 3B). These results suggested that the first 207 codons of *cbiS* were necessary and sufficient for Cbi amidohydrolase function.

***cbiS* restores B₁₂ synthesis in an *S. enterica* α -RP phosphatase (*cobC*) strain.** Archaea lack an ortholog to the bacterial CobC enzyme, but some species have a nonorthologous replacement encoded by the *cobZ* gene (40). The C-terminal segment of the CbiS protein is homologous to the CobZ protein. Based on this homology, we tested whether *cbiS* would correct the phenotype of an *S. enterica* α -RP phosphatase (*cobC*) mutant. Plasmid pMkCBIS4 (*cbiS*⁺) was introduced into strain JE2192 (*metE cobT cobC*), and the cobamide-dependent growth of the resulting strain was tested. As positive and negative controls, plasmids pJO46 (*cobC*⁺) and pT7-7 (empty cloning vector) were provided, respectively. Cbi and DMB were present in the medium, which should allow only growth of strains with α -RP phosphatase activity. As expected, *cobC* restored growth of strain JE6984 (Fig. 3C). *M. kandleri cbiS* also restored growth (Fig. 3C) and was dependent on DMB (Fig. 3C). Plasmids pMkCBIS6 and pMkCBIS7 were also introduced into strain JE6984 to test if only the 3' fragment of *cbiS* was required for *cobC* complementation. As expected, 3' *cbiS* completely restored growth of strain JE6984 (Fig. 3C), whereas 5' *cbiS* did not increase growth significantly above background levels (Fig. 3C). These results suggested that *M. kandleri cbiS* did encode α -RP phosphatase activity and that only the last 150 codons were required for this function.

***cbiS* restores Cby-dependent growth of an *S. enterica cobC* mutant.** Another notable phenotype of an *S. enterica cobC* mutant is its inability to salvage Cby (C. L. Zayas and J. C. Escalante-Semerena, unpublished results). To test whether *cbiS* function is capable of complementing this phenotype, plasmid pMkCBIS4 (*cbiS*⁺) was introduced into strain JE2119 (*metE205 cobC*) and cobamide-dependent growth was tested. Plasmids pJO46 (*cobC*⁺) and pT7-7 (empty cloning vector) were used as controls. Cby was provided in the medium, which should allow only growth of strains with the ability to salvage this known intermediate of the de novo corrin ring biosynthetic branch. As expected, *cobC* and *cbiS* restored growth of strain JE2119 (Fig. 3D). Plasmids pMkCBIS6 and pMkCBIS7 were also introduced into strain JE2119 to test whether the 3' fragment of *cbiS* would suffice. As shown in Fig. 3D, 3' *cbiS* completely restored growth of strain JE2119, whereas 5' *cbiS* did not. These results indicated that *M. kandleri cbiS* encoded a CobC-like activity in the 3' end.

FIG. 4. HPLC analysis of the CbiS amidohydrolase and phosphatase activity products. Chromatograms of components of amidohydrolase reaction mixtures monitored at 367 nm without enzyme (panel A) and the complete reaction mixture with enzyme (panel B). The inset in panel B shows the UV-visible spectrum of cobyric acid, the product of the amidohydrolase activity of CbiS. λ_{max} , maximum wavelength (nm). Chromatograms of components of the phosphatase reaction mixtures monitored at 280 nm without enzyme (panel C) and the complete reaction mixture with enzyme (panel D). The inset in panel D shows the UV-visible spectrum of α -ribose-phosphate, the product of the phosphatase activity of CbiS. The x axes show times of elution after injection.

CbiS-dependent conversion of Cbi to Cby. The CbiS protein was isolated by using a C-terminal chitin-binding protein tag, which was subsequently cleaved (data not shown). Purified protein (95% homogeneous) (data not shown) was tested for Cbi amidohydrolase activity by incubation of the protein with Cbi under anoxic conditions and by the monitoring of the formation of the product Cby by HPLC. Cby was clearly detectable in the complete reaction mixture (retention time, 14.7 min) (Fig. 4B) but was absent when the CbiS protein was not added to the mixture (Fig. 4A). Mass spectrometry was used to confirm the identity of the enzyme product. The signal with an m/z of 932.6 was consistent with the expected molecular mass of Cby without ligands (932.0 atomic mass units [amu]) (Fig. 5A). Under the conditions used, a specific activity of 18 ± 0.7 nmol per min per mg of protein was calculated. When the same reactions were performed in the presence of atmospheric O_2 , however, no product was detected (<0.1 nmol per min per mg of protein). The addition of 10 mM dithiothreitol did not reverse this effect (data not shown). By calculating specific activities of the CbiS protein amidohydrolase reaction across a pH range from 6.0 to 9.5 in 1.5 M KP_i buffer, we measured the highest amidohydrolase activity at a pH of 9 (Fig. 6A). Under the initial conditions, a temperature range of 90°C to 100°C exhibited the highest specific activity rates (Fig. 6B). At pH 8.0, replacing KP_i buffer with 50 mM Na-HEPES buffer reduced the activity 2.3-fold, but the activity was raised 2.7-fold upon addition of 10 mM $MgCl_2$ (Fig. 6C). The addition of $MgCl_2$ to

FIG. 5. Mass spectrometry analyses of the reaction products. Panel A shows the matrix-assisted laser desorption/ionization-time-of-flight mass spectrum of the HPLC-purified corrinoid product of the CbiS-like activity of the CbiS enzyme. The signal with the m/z of 932.6 was consistent with the molecular mass of Cby (without ligands) where z equals +1. No significant signals were detected above an m/z of 1,200. Panel B shows the electrospray ionization mass spectrum of the HPLC-purified ribazole product of CobZ-like α -ribose-P phosphatase activity of the CbiS enzyme. The signal with the m/z of 279.6 was consistent with the molecular mass of α -ribose where z equals +1. No significant signals were detected above an m/z of 400. cps, counts per second; Co, cobalt-containing corrin ring.

reaction mixtures containing KP_i buffer did not significantly increase activity (data not shown). Increases in the KP_i concentration at pH 8.0 showed that 500 mM KP_i resulted in the highest level of activity (Fig. 6C). The addition of 190 μ M α -RP (the proposed substrate of the C-terminal half of CbiS) did not significantly alter the amidohydrolase activity of the protein when in KP_i buffer or HEPES and $MgCl_2$ (data not shown). Under all the conditions tested, activity rates remained constant for at least one hour.

The ability of CbiS to use different substrates was also tested. As expected, CbiS hydrolyzed AdoCbi to Cby at a rate similar to that of $(CN)_2$ -Cbi (20 ± 2 nmol per min per mg protein) (Fig. 6D). Unexpectedly, however, CbiS hydrolyzed $(CN)_2$ -Cbi-P, $(CN)_2$ -Cbi-GDP, and CNB_{12} to Cby at rates of 27 ± 1.8 , 9 ± 1.1 , and 9 ± 0.3 nmol per min per mg of protein, respectively (Fig. 6D). The HPLC-purified products of these reactions were verified to be Cby by mass spectrometry.

FIG. 6. CbiS cobinamide amidohydrolase activity profiles. Shown are the profiles of CbiS enzyme activity (expressed as nanomoles of Cby product min⁻¹ mg⁻¹ protein). Panel A. Activity profile as a function of pH at 90°C. Panel B. Activity profile as a function of temperature at pH 9.0. Panel C. Activity profile as a function of reaction mixture composition at pH 8.0. Panel D. Enzyme activity as a function of species of corrinoid in the reaction mixture (90°C, pH 9.0). The variable conditions are indicated under each column. Unless indicated, reactions contained 1.5 M KP_i. When indicated, Na-HEPES (pH 8.0) was added at 50 mM; MgCl₂ was added to 10 mM.

etry analysis. A single 932.6-*m/z* signal corresponding to Cby was observed in all cases (data not shown).

CbiS-dependent dephosphorylation of α -RP. The nutritional analyses of *S. enterica* cells harboring *cbiS* on a plasmid strongly suggested that the CbiS protein had an α -RP phosphatase activity. We measured this activity of the CbiS protein in vitro. Purified CbiS protein was incubated under anoxic conditions with α -RP and the conversion to α -R was monitored by HPLC. A signal for α -R was observed in the complete reaction mixture (Fig. 4D), but was absent when CbiS protein was not added to the reaction mixture (Fig. 4C). Under these conditions, a specific activity of 28 ± 1.1 nmol per min per mg protein was calculated. When KP_i buffer was substituted for HEPES buffer or when MgCl₂ was excluded from the reaction mixture, no product was observed (<1.3 pmol per min per mg of protein). Performing the reaction with atmospheric levels of O₂, activity was reduced only 3.5-fold to 8 ± 1.2 nmol per min per mg of protein. The addition of Cbi (5 or 340 μ M) did not significantly alter the activity (data not shown). The HPLC-purified reaction product was analyzed by mass spectrometry. The observed signal at *m/z* 279.6 was consistent with an *m/z* equal to the *M* + 1 value of the expected atomic mass of α -ribazole (278.3 amu) (Fig. 5B). These data strongly supported the conclusion that the CbiS protein had α -RP phosphatase activity.

DISCUSSION

The *cbiS* gene encodes at least two distinct, separable activities involved in different branches of the cobamide biosynthetic pathway. The genetic and biochemical evidence re-

ported in this paper strongly supports the conclusion that the *cbiS* gene from *M. kandleri* encodes an enzyme with at least two activities involved in different branches of the cobamide biosynthetic pathway.

The N-terminal portion of CbiS has CbiZ-like cobinamide amidohydrolase activity used by archaea to salvage cobinamide from the environment, allowing the product of the reaction (i.e., cobyric acid) to serve as a substrate for the enzyme catalyzing the last step of the de novo corrin ring biosynthetic branch of the pathway (38). The C-terminal portion of CbiS has α -RP phosphatase activity involved in the assembly of the nucleotide loop of cobalamin.

CbiS activities seem insulated from one another. The evidence reported here indicates that neither segment of the CbiS protein is required for the activity of the other. Although O₂ inhibited amidohydrolase activity, it caused only a threefold reduction of phosphatase activity. Although the removal of MgCl₂ reduced phosphatase activity 2,000-fold, it reduced amidohydrolase activity only threefold. Also, the addition of the substrate for one half of the enzyme did not affect the activity of the other half.

The specific activities for CbiS amidohydrolase activity reported here are very similar to the reported activity of the CbiZ protein from *M. mazei* (6.4 pmol per min per mg protein) (35). The inhibitory effect of O₂ on the amidohydrolase activity of CbiS was not due to instability of the protein since the phosphatase activity of CbiS remained relatively unchanged in the presence of O₂. Although CbiS contains five cysteine residues within the N-terminal portion, it is unlikely that the redox state of the Cys residues is responsible for the sensitivity of the amidohydrolase activity to oxygen. First, these residues are not conserved in archaeal CbiZ amidohydrolases that are not fused to CobZ, and second, the addition of dithiothreitol did not restore amidohydrolase activity in the presence of O₂. The relevance of the Cys residues in the N-terminal portion of CbiS remains unknown.

Is there a physiological reason for the lack of corrinoid specificity of CbiS? We were surprised to learn about the lack of specificity of the amidohydrolase activity of CbiS for its corrinoid substrate, i.e., CbiS cleaved Cbi-P, Cbi-GDP, and B₁₂ into Cby (Fig. 6D). From these data, we predict that the corrinoid substrate must bind to CbiS in a way that renders the presence of a complete nucleotide loop (as in B₁₂) irrelevant. This lack of specificity, however, is potentially deleterious, unless the amidohydrolase activity is controlled by interactions of CbiS with other proteins or B₁₂ biosynthetic enzymes or by differences in CbiS affinity for the substrates mentioned above. A more detailed kinetic analysis of CbiS function is needed to address this possibility. In vivo, however, CbiS did not noticeably affect the ability of *S. enterica* to synthesize and use corrinoids to grow (data not shown).

The ability of CbiS to dephosphorylate α -RP both in vivo and in vitro suggests that the enzyme may be able to dephosphorylate a variety of activated bases. *S. enterica* synthesizes α -RP of α -AMP (12, 13), both of which were substrates for CbiS. As far as we know, the lower ligand base of the cobamide synthesized by *M. kandleri* is not known. Cobamides with adenine as the lower ligand and 5-hydroxybenzimidazole have been isolated from other methanogens (15, 22, 27). Most

likely, α -RP is not the true substrate of CbiS or its orthologs but rather the activated form of structurally similar bases.

Why are CbiZ and CobZ fused in extremely thermophilic archaea? Although every archaeal genome sequenced to date is predicted to contain a CbiZ ortholog, only three species (two crenarchaeotes and one euryarchaeote) have the *cbiZ* and *cobZ* genes fused (Fig. 2). The gene fusion with the same pair of functions in distant species suggests that there is a positive selection for the multidomain architecture of the CbiS protein (39). From a catalytic point of view, there is no obvious selective advantage to associating Cbi amidohydrolase activity with α -RP phosphatase activity. These two steps are not sequential in the biosynthetic pathway, and the amidohydrolase activity is required only for Cbi salvaging (38), whereas the phosphatase activity is required for de novo synthesis (21).

Because the organisms whose genomes encode CbiS orthologs have optimal growth temperatures above 95°C (10, 25, 33), it is possible that the fusion of these two activities was an adaptation to extremely high temperatures. Although the two halves can function separately at 37°C, it is possible that this is not the case at 90 to 100°C, at which the fused enzymes may be thermostable. Thermostability may not be the sole reason for fusing CobZ and CbiZ, since these proteins are encoded separately in *Pyrococcus* species. It is possible that pyrococci have evolved a different strategy for stabilizing CobZ and CbiZ or that other environmental factors that exacerbate the thermal instability of CobZ and CbiZ in *M. kandleri*, *A. pernix*, and *P. aerophilum* are absent in the environment of pyrococci. It may be, however, that the protein fusion with these two activities achieves something entirely different.

ACKNOWLEDGMENTS

This work was supported by NIH grant GM40313 to J.C.E.-S.; J.D.W. was supported in part by the Ira L. Baldwin and Jerome Stefaniak Predoctoral Fellowships.

We thank P. Renz for his gift of cobyrinic acid and M. Thomm for his gift of *M. kandleri* chromosomal DNA.

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