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Biochemical Characterization of the GTP:Adenosylcobinamide-phosphate Guanylyltransferase (CobY) Enzyme of the Hyperthermophilic Archaeon *Methanocaldococcus jannaschii*†

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ABSTRACT: The archaeal *cobY* gene encodes the nonorthologous replacement of the bacterial NTP:AdoCbi kinase (EC 2.7.7.62)/GTP:AdoCbi-P guanylyltransferase (EC 3.1.3.73) and is required for *de novo* synthesis of AdoCbl (coenzyme B_{12}). Here we show that ORF MJ1117 of the hyperthermophilic, methanogenic archaeon *Methanocaldococcus jannaschii* encodes a CobY protein (*Mj* CobY) that transfers the GMP moiety of GTP to AdoCbi-P to form AdoCbi-GDP. Results from isothermal titration calorimetry (ITC) experiments show that *Mj*CobY binds GTP (K_d = 5 μM), but it does not bind the GTP analogues GMP-PNP (guanosine 5′-(β,γ)-imidotriphosphate) or GMP-PCP (guanylyl 5′-(β,γ)-methylenediphosphonate) nor GDP. Results from ITC experiments indicate that *Mj*CobY binds one GTP per dimer. Results from *in vivo* studies support the conclusion that the 5′-deoxyadenosyl upper ligand of AdoCbi-P is required for *Mj*CobY function. Consistent with these findings, *Mj*CobY displayed high affinity for AdoCbi-P (K_d = 0.76 μM) but did not bind nonadenosylated Cbi-P. Kinetic parameters for the*Mj* CobY reaction were determined. Results from circular dichroism studies indicate that, in isolation, *Mj*CobY denatures at 80 °C with a concomitant loss of activity. We propose that ORF MJ1117 of *M. jannaschii* be annotated as *cobY* to reflect its involvement in AdoCbl biosynthesis.

Cobamides are ancient cofactors that are widely distributed in nature (*1*). With the exception of plants, cells of all domains of life have enzymes that require a cobamide as their coenzyme, yet only some bacteria and archaea synthesize cobamides *de novo* (*2*−*4*).

Although not all cobamide producers synthesize the corrin ring *de novo*, some can salvage preformed, incomplete precursors [e.g., cobinamide (Cbi),[1] cobyric acid (Cby)] from their environments using a high-affinity ATP-binding cassette transporter (*5*−*10*). Precursors such as Cbi and Cby are then converted to cobamides by two distinct branches of the AdoCbl biosynthetic pathway. One of these branches, known as the corrinoid adenosylation pathway, attaches the upper axial ligand to the corrin ring via a labile C−Co bond (*11*). The second branch assembles the nucleotide loop that tethers the lower ligand base to the corrin ring; this branch is known as the nucleotide loop assembly (NLA) pathway (Figure 1). The NLA pathway can be further broken down into two subbranches, one of which activates adenosyl-Cbi (AdoCbi) to AdoCbi-guanosine diphosphate (AdoCbi-GDP) (*12*) and a second one that activates the lower ligand base to its nucleotide (*2*) (Figure 1).

In bacteria, the activation of AdoCbi to AdoCbi-GDP is catalyzed by the bifunctional CobU enzyme (EC 2.7.1.156, EC 2.7.7.62) via an AdoCbi-P intermediate (*13*). Archaea, however, use a different strategy for the synthesis of AdoCbi-GDP (*2*). These organisms do not synthesize CobU; instead, they use CobY, a GTP:AdoCbi-P guanylyltransferase enzyme that lacks the NTP:AdoCbi-P kinase activity of CobU (Figure 1) (*14*). The *cobY* gene was identified in extreme halophilic and thermophilic, methanogenic archaea as a nonorthologous replacement for *cobU* (*14*, *15*).

In this work, we identified the locus encoding the CobY enzyme in the hyperthermophilic, methanogenic archaeon *Methanocaldococcus jannaschii*. Recombinant protein was expressed in *Escherichia coli* cells, the order of substrate binding was determined, and the interactions between substrate and enzyme were quantified.

EXPERIMENTAL PROCEDURES

Microbiological Techniques. Bacteria, Culture Media, and Growth Conditions. Bacterial strains and plasmids used in these studies are listed in Table 1. *Salmonella enterica* strains were cultured in nutrient broth (NB; Difco); *E. coli* strains were

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[1]Abbreviations: *Mj*CobY, *Methanocaldococcus jannaschii* CobY protein; Cbl, cobalamin; Cbl-P, Cbl-phosphate; AdoCbl, adenosyl-Cbl; AdoCbl-P, adenosyl-Cbl-phosphate; Cbi, cobinamide; $(CN)_2$Cbi, dicyano-Cbi; HOCbi, hydroxy-Cbi; HOCbi-P, HOCbi-phosphate; CNCby, cyanocobyric acid; α-RP, α-ribazole 5′-phosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Cba, cobamide; FPLC, fast protein liquid chromatography; RP-HPLC, reverse-phase high-performance liquid chromatography; ITC, isothermal titration calorimetry; CD, circular dichroism; NTP, nucleoside triphosphate; TCEP, tris(2-carboxyethyl)phosphine.

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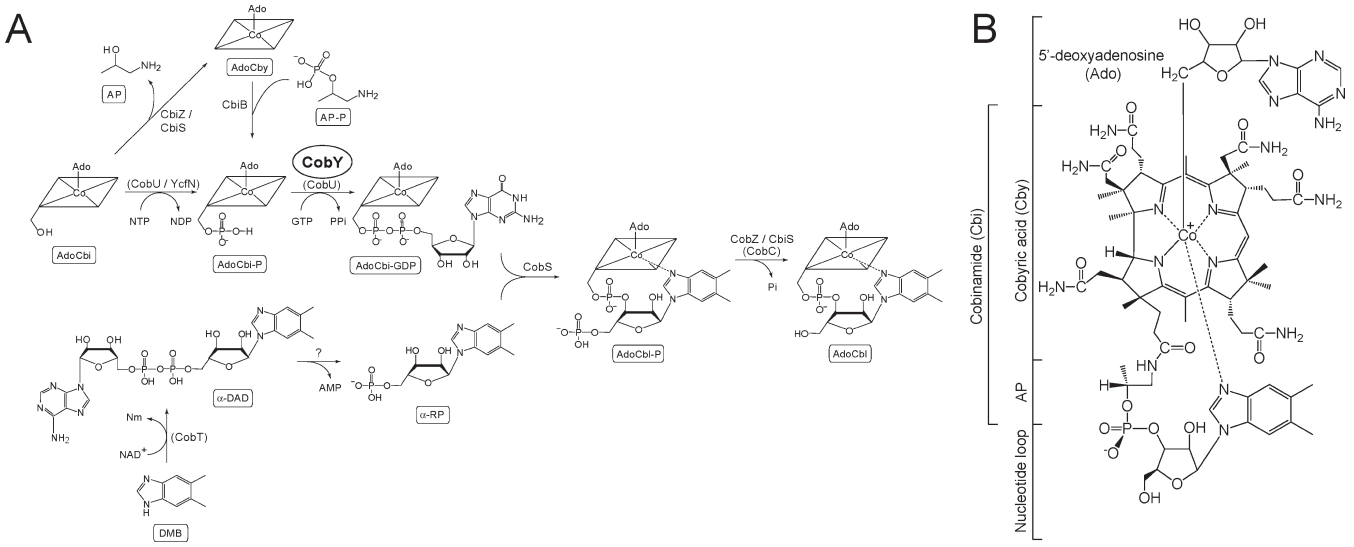

FIGURE 1: Nucleotide loop assembly pathway. (A) Enzymes, substrates, and pathway intermediates are shown. The rhomboid shape is a schematic of the corrin ring structure shown in (B). Pathway intermediates are boxed and shown below the structures; enzyme names are above or beside arrows. Archaeal enzyme names are shown; corresponding bacterial enzyme names are shown in parentheses. Abbreviations: AdoCby, adenosylcobyric acid; AP-P, aminopropanol phosphate; AdoCbi, adenosylcobinamide; AdoCbi-P, adenosylcobinamide phosphate; AdoCbi-GDP, adenosylcobinamide-guanosine diphosphate; AdoCbl-P, adenosylcobalamin phosphate; AdoCbl, adenosylcobalamin; DMB, 5,6-dimethylbenzimidazole; α-DAD, 5,6-dimethylbenzimidazole adenine dinucleotide; α-RP, α-ribazole 5′-phosphate; Nm, nicotinamide; AP, aminopropanol. Enzymes: CbiP, cobyric acid synthase; CbiB, putative AdoCbi-P synthase; CobY, GTP:AdoCbi-P guanylyltransferase; CobU, NTP:AdoCbi kinase/GTP:AdoCbi-P guanylyltransferase; CobS, AdoCbl 5′-phosphate synthase; CobZ, α-RP phosphatase; CbiS, AdoCbi amidohydrolase/α-RP phosphatase; CobC, α-RP phosphatase; CbiZ, AdoCbi amidohydrolase; YcfN, thiamin kinase/AdoCbi kinase. (B) Structure of coenzyme B_{12} (aka AdoCbl).

Table 1: Strains and Plasmids Used in These Studies

strain or plasmid	genotype and description	ref or source[b]
S. enterica[a]		
TR6583; formerly SA2929	*metE205 ara-9*	K. Sanderson via J. Roth
derivatives of TR6583		
JE8248	*cobS1313* (Δ*cobS*)	*23*
JE8249	*cobU1315* (Δ*cobU*)	*20*
JE8268	*cobU1315 ycfN112* (Δ*cobU* Δ*ycfN*)	*20*
JE8269	*cobU1315 ycfN112*/pT7-7	
JE8335	*cobU1315 ycfN112*/pCOBY14	
JE9318	*cobU1315 ycfN112 cobA343*::MudJ/pCOBY14	
E. coli		
BL21-CodonPlus (DE3)-RIL	B F^- *ompT hsdS* (r_B^-, m_B^-) *dcm*$^+$ *tet gal λ*(DE3) *endA* Hte (*argU ileY leuW cat*)	Stratagene
plasmids		
pT7-7	(*bla*$^+$); cloning vector	derivative of pT7-1 (*38*)
pCOBY14	MJ1117 *cobY*$^+$ in pT7-7 *bla*$^+$	*37*

[a] All strains are derivatives of *S. enterica* serovar Typhimurium strain LT2 and carry a null allele of the *metE* gene, which encodes the Cbl-independent methionine synthase (MetE) enzyme. In the absence of Cbl, *metE* mutants are methionine auxotrophs. When Cbl is available, *metE* mutants use the Cbl-dependent methionine synthase (MetH) enzyme to methylate homocysteine to methionine (*39*). [b] Strains were constructed for this study unless otherwise indicated.

cultured in lysogeny broth (LB) (*16*, *17*). Plasmids were maintained by the addition of ampicillin (100 μg/mL), chloramphenicol (20 μg/mL), kanamycin (50 μg/mL), or tetracycline (20 μg/mL) to NB or LB. No-carbon essential (NCE) medium (*18*) supplemented with glycerol (30 mM) and $MgSO_4$ (1 mM) was used to grow cells under nutrient-defined conditions. Solid media contained 15 g of Bacto Agar (Difco) per liter. Corrinoids [cyanocobyric acid (CNCby), dicyanocobinamide [$(CN)_2$Cbi] or cyanocobalamin (CNCbl; also known as vitamin B_{12})] were added to media at 15 nM. CNCby was a gift from Paul Renz (Institut für Biologische Chemie and Ernahrungswissenschaft, Universität—Hohenheim, Stuttgart, Germany); $(CN)_2$Cbi and CNCbl were purchased from Sigma.

Genetics and Recombinant DNA Techniques. (A) Complementation of NTP:AdoCbi-P Nucleotidyltransferase Activity in S. enterica ΔcobU ΔycfN Strain. Plasmid pCOBY14 (*M.j. cobY*$^+$) was electroporated (*19*) into strain JE8268 (Δ*cobU* Δ*ycfN*) (*20*) to assess complementation of AdoCbi-P nucleotidyltransferase activity; the resulting strain was JE8335 (Table 1). Plasmid pT7-7 (*38*) was used as negative control in complementation experiments and was moved into strain JE8268 by electroporation, yielding strain JE8269. Isolated

colonies of strains JE8268, JE8269, JE8335, and TR6583 were inoculated into NB and incubated at 37 °C overnight (~16 h). Five microliter aliquots of the overnight cultures were diluted into 195 μL of NCE-glycerol minimal medium as described above and incubated at 37 °C with shaking in a 96-well microtiter plate using an ELx808 Ultra microplate reader (Bio-Tek Instruments).

(B) Strain Construction. (i) Construction of a ΔcobS Strain of S. enterica. A chromosomal in-frame deletion of the *cobS* gene was constructed in strain TR6583 as described (*21*), using primers OL14 (5′-ATG AGT AAG CTG TTT TGG GCC-ATG CTC GCT TTT ATT AGC CGC TTG CCC GTG GTG-TAG GCT GGA GCT GCT TC-3′) and OL24 (5′-TCA TAA–CAG AGC CAG CAG AAA GAT CAA TTC ACC AAG TTC-GAT CGC CGC GCC GAC CGT ATC GCC GGT TTG ACC-GCC CAT ATG AAT ATC CTC CTT AG-3′); the resulting strain was JE8248 (*23*). Deletion of the *cobs* gene was verified by DNA sequencing using BigDye protocols (ABI PRISM); reaction mixtures were resolved by the University of Wisconsin—Madison Biotechnology Center.

(ii) Construction of a Strain Deficient in the Attachment of the Upper Ligand. Corrinoid adenosylation was blocked by inactivation of the *cobA* gene encoding the housekeeping ATP: corrinoid adenosyltransferase (*11*). The *cobA363*::MudJ mutation was introduced into the chromosome of strain JE8268 (Δ*cobU* Δ*ycfN*) by bacteriophage P22-mediated transduction (*22*).

Biochemical Techniques. (A) Protein Overexpression and Purification. (i) CobY Protein. Twenty milliliters of LB supplemented with ampicillin and chloramphenicol were inoculated with *E. coli* strain BL21-CodonPlus (DE3)-RIL (Stratagene) harboring plasmid pCOBY14 (*M.j. cobY*$^+$) and incubated at 37 °C overnight. One liter of LB supplemented with ampicillin was inoculated with the overnight culture and incubated at 37 °C with shaking at 200 rpm until the culture density reached an OD_{600} of 0.5−0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 0.5 mM to induce expression of *cobY*$^+$. To obtain maximum yield of the recombinant protein, incubation was continued for approximately 16 h at 37 °C. Cells were harvested by centrifugation at 4 °C at 15000*g* for 15 min using a JA-25.50 rotor in an Avanti J-25I Beckman/Coulter refrigerated centrifuge and resuspended in 15 mL of tris(hydroxymethyl)aminomethane hydrochloride buffer (Tris-HCl; 100 mM, pH 8.0 at 4 °C) containing protease inhibitor phenylmethanesulfonyl fluoride (1 mM). Cells were placed on ice and lysed by sonication for 2 min (5 s pulse followed by 10 s of cooling) in a model 550 sonic dismembrator (Fisher). The extract was cleared by centrifugation at 4 °C for 30 min at 43367*g*. The cell-free extract was heated in a water bath at 75 °C for 15 min, and precipitated *E. coli* proteins were cleared by centrifugation at 4 °C for 60 min at 43367*g*. Finely ground ammonium sulfate (UltraPure; ICN Biomedicals) was added to 10% saturation. Precipitates were cleared by centrifugation at 4 °C for 60 min at 43367*g*.

CobY protein was purified in two steps from clarified extract via fast protein liquid chromatography (FPLC) using an ÄKTAprime system (GE Healthcare).

Step 1: hydrophobic interaction chromatography. Cell-free extract was applied to a 5 mL HiTrap phenyl (high-sub) FF column (GE Healthcare) equilibrated with 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid buffer (HEPES; 50 mM, pH 7.5 at 4 °C) containing $(NH_4)_2SO_4$ (10% saturation). Protein was eluted at a flow rate of 2.5 mL min^{-1} with a linear gradient to 100% HEPES buffer (50 mM, pH 7.5 at 4 °C). The CobY protein was found in the flow-through fractions, which were concentrated using a Centricon 70 centrifugal filter device (Millipore) and dialyzed against HEPES buffer (50 mM, pH 7.5 at 4 °C) to remove $(NH_4)_2SO_4$.

Step 2: ion-exchange chromatography. The concentrated protein solution was applied to a 1 mL MonoQ 5/50 column (GE Healthcare) equilibrated with HEPES buffer (50 mM, pH 7.5 at 4 °C). Protein was eluted at a flow rate of 1 mL min^{-1} with a linear gradient to 100% HEPES buffer (50 mM, pH 7.5 at 4 °C) containing NaCl (1 M). CobY did not interact with the resin, and flow-through fractions containing CobY were concentrated and dialyzed against HEPES buffer (50 mM, pH 7.5 at 4 °C). Glycerol was added to a final concentration of 10% (v/v). The CobY protein was flash-frozen by dropwise addition into liquid N_2; frozen beads were stored at −80 °C until use.

(ii) S. enterica *CobS (SeCobs) Protein.* Cell-free membrane fractions enriched for *Se*CobS were prepared as reported (*23*).

(B) Assessment of Protein Concentration and Purity. Protein concentration was determined by the Bradford method (*24*). Purity was assessed by 15% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (*25*), followed by staining with Coomassie Brilliant Blue R-250 (*26*). Protein purity was established by band densitometry using a computer-controlled Fotodyne imaging system with Foto/Analyst v.5.00 software (Fotodyne Incorporated) for image acquisition and TotalLab v.2005 software for analysis (Nonlinear Dynamics).

(C) Size Exclusion Chromatography. Size exclusion chromatography was performed as described (*27*) except that the molecular mass standards used were 159.5, 34.6, 11.3, and 1.9 kDa. A standard curve was created in order to calculate the molecular mass of each peak from its elution time; log molecular mass (in kilodaltons) was linear between 25 and 42 min elution (r^2 = 1.000).

(D) Synthesis of Corrinoid Substrates. AdoCbi and AdoCbi-P were prepared and purified as reported (*20*).

(E) In Vitro Synthesis of Adenosylcobalamin 5′-Phosphate (AdoCbl-P): CobY−CobS Coupled Reaction. Reactions were performed under dim, red light to avoid photolysis of the C−Co bond of the adenosylated corrinoid substrate. Reaction mixtures contained Tris-HCl (50 mM, pH 7.9), α-ribazole 5′-phosphate (α-RP; 30 μM) (*28*), $MgCl_2$ (5 mM), GTP (2 mM), 5 μg of CobS-enriched membrane extract or membrane material from a control strain in which *cobS* was not overexpressed (*23*), CobY protein (460 pmol), and AdoCbi-P (0.2 mM) in a final volume of 20 μL. Reaction mixtures were incubated at 37 °C for 1 h. Reactions were stopped and corrinoids converted to their cyanated forms by the addition of 3 μL of KCN (100 mM), heating at 80 °C for 10 min, and irradiation with a 60 W incandescent light at a distance of 6 cm for 15 min. Complete cobamides were detected using a bioassay as described (*20*) and identified by reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (see below).

(F) In Vitro Synthesis of AdoCbi-GDP by CobY. Reaction mixtures contained Tris-HCl (50 mM, pH 7.9), GTP (2 mM), NaCl (50 mM), $MgCl_2$ (10 mM), dithiothreitol (DTT; 10 mM), *Mj*CobY protein (2.3 pmol), and AdoCbi-P (200 μM) in a final volume of 40 μL. Reaction mixtures were incubated under dim, red light for 15 min. Reactions were stopped and corrinoids converted to their cyanated forms as described above.

(G) HPLC Analysis of the MjCobY Reaction Product. Fifty-five microliters of water were added to the *Mj*CobY reaction mixtures, which were then filtered using Spin-X centrifugal filters (Corning Costar). Corrinoid reactants and products were

separated by RP-HPLC on a System Gold HPLC system (Beckman Coulter) equipped with an Alltima HP C_{18} AQ 5 μm 150 mm × 4.6 mm column fitted with an Alltima HP C_{18} AQ All-Guard cartridge (Alltech). The column was equilibrated with a buffer system of 77% A:23% B (see below). A 14.4 min linear gradient was applied at 1 mL min^{-1} until the composition of the buffer system was 67% A:33% B. The solvents used were as follows: buffer A [100 mM potassium phosphate buffer (pH 6.5) containing KCN (10 mM)]; buffer B [100 mM potassium phosphate buffer (pH 8.0) containing 10 mM KCN]:CH_3CN (1:1). Corrinoids eluted from the column were detected at 367 nm with a photodiode array detector. A standard curve for $(CN)_2$Cbi was constructed for quantification purposes; detection of $(CN)_2$Cbi was linear between 1 pmol and 3 nmol (r^2 = 0.9984).

(H) Mass Spectrometry. The product of the *Mj*CobY−*Se*CobS coupled reaction was dried under vacuum in a SpeedVac concentrator (Thermo Savant), suspended in ddH_2O, and loaded onto a Sep-Pak C_{18} cartridge (Waters) preequilibrated with ddH_2O. Corrinoids were eluted from the resin with 2.5 mL of 100% (v/v) methanol, and the sample was dried under vacuum. The dried sample was analyzed at the Mass Spectrometry Facility at the University of Wisconsin—Madison Biotechnology Center. Mass spectra were obtained using a Bruker Daltonics (Billerica, MA) BILFLEX III matrix-assisted laser desorption ionization−time-of-flight (MALDI-TOF) mass spectrometer.

(I) Kinetic Analyses. Kinetic parameters were determined using the assay conditions described above, using Microsoft Excel software.

(J) Isothermal Titration Calorimetry. *Mj*CobY protein was dialyzed against Tris-HCl buffer, pH 8.0 at 4 °C (50 mM), containing NaCl (50 mM), $MgCl_2$ (10 mM), and tris(2-carboxyethyl)phosphine hydrochloride (4 mM); GTP and AdoCbi-P were dissolved in spent dialysis buffer. Experiments were conducted at 27 °C and consisted of 27 10-μL injections of a 1 mM stock of ligand into a 1.45 mL sample cell containing 18 μM CobY dimers. Injections were made over a period of 20 s with a 3 min interval between injections. The sample cell was stirred at 307 rpm. Data were acquired on a VP-ITC microcalorimeter (Microcal, Northhampton, MA) and analyzed with the ORIGIN v.7.0383 software provided with the microcalorimeter.

(K) Circular Dichroism Spectroscopy. CobY protein was dialyzed against sodium phosphate buffer, pH 7.2 (50 mM), containing NaCl (50 mM) and submitted for circular dichroism (CD) spectroscopy to the Biophysics Instrumentation Facility, Department of Biochemistry, University of Wisconsin—Madison. Protein concentration for CD spectroscopy was measured on a SoloVPE variable path length extension spectrophotometer (C Technologies, Inc., Bridgewater, NJ) using an extinction coefficient of 14900 M^{-1} cm^{-1} and determined to be 0.2 mg/mL. CD spectra were recorded on a Model 202SF circular dichroism spectrometer (Aviv Biomedical, Lakewood, NJ). The cell path length was 0.1 cm for CD measurements. CD spectra were recorded from 300 to 200 nm at 1 nm steps with an averaging time of 5 s for baselines and 3 s for protein-containing samples. Baselines were recorded at 20, 37, and 80 °C and used as baselines for the respective temperatures.

RESULTS AND DISCUSSION

Identification of the Gene Encoding CobY in M. jannaschii. The MJ1117 open reading frame (ORF) of *M. jannaschii* encodes a protein that has been shown to catalyze the guanylylation of an intermediate in the synthesis of coenzyme F_{420} in methanogenic archaea (*29*). Because the chemistry of the F_{420} biosynthetic reaction is similar to that of the guanylylation of AdoCbi-P (*30*), we hypothesized that the MJ1117 protein catalyzed the synthesis of AdoCbi-GDP from AdoCbi-P and GTP in *M. jannaschii*.

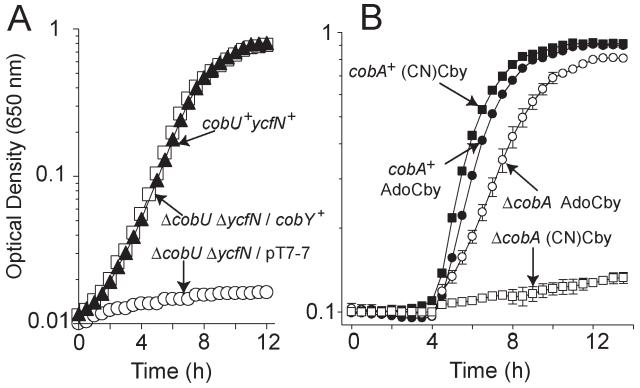

FIGURE 2: Cbl-dependent growth of *S. enterica* strains. Growth in minimal medium (*18*) containing glycerol (30 mM), $MgSO_4$ (1 mM), and corrinoid (15 nM) at 37 °C is reported as optical density at 650 nm as a function of time. All strains were methionine auxotrophs whose growth required Cbl for the synthesis of methionine. (A) *Mj*CobY allows a *S. enterica* Δ*cobU* Δ*ycfN* strain to salvage Cby. *S. enterica* strains used were TR6583 (*cobU*$^+$ *ycfN*$^+$, filled triangles), JE8335 [*cobU ycfN*/pCOBY14 (M.j. *cobY*$^+$), open squares], and JE8269 (Δ*cobU* Δ*ycfN*/pT7-7, empty vector control, open circles). (B) *Mj*CobY requires an adenosylated corrinoid substrate. Medium contained either (CN)Cby or AdoCby. Cultures were incubated in the dark at 37 °C with shaking. *S. enterica* strains used were JE8335 (*cobA*$^+$, filled squares and circles) and JE9318 (Δ*cobA*, open squares and circles).

Bioinformatics analyses support the assignment of the MJ1117 gene product as the NTP:AdoCbi-P nucleotidyltransferase enzyme in *M. jannaschii*. Using the protein Basic Local Alignment Search Tool (BLASTP) program from the National Center for Biotechnology Information (*31*), we searched the *M. jannaschii* proteome for homologues of CobY proteins from *Methanothermobacter thermautotrophicus* (*Mth*; formerly *Methanobacterium thermoautotrophicum*) strain ΔH (*14*) and *Halobacterium* sp. strain NRC-1 (*15*). ORF MJ1117 encoded a putative protein that shared 34% identity and 58% similarity to *Mth* CobY (expect = 6e−26) and 31% identity and 55% similarity to the CobY protein of *Halobacterium* sp. NRC-1 (expect = 6e−08). Although the level of identity was low, we suspected that ORF MJ1117 encoded the *M. jannaschii* CobY protein; hence, hereafter we refer to ORF MJ1117 as *M.j. cobY*.

In Vivo Evidence That MjCobY Has NTP:AdoCbi-P Nucleotidyltransferase Activity. The CobY proteins studied to date lack AdoCbi kinase activity. To determine whether *Mj*CobY had AdoCbi kinase and/or AdoCbi-P nucleotidyltransferase activity, we introduced plasmid pCOBY14 (*M.j. cobY*$^+$) into *S. enterica* strain JE8268 (Δ*cobU* Δ*ycfN*) (*20*). This strain lacks both AdoCbi kinase and AdoCbi-P nucleotidyltransferase activity; thus, it can neither synthesize AdoCbl *de novo* nor convert AdoCbi or AdoCby to AdoCbl. The ability of strain JE8268 to convert AdoCby to AdoCbl was restored by the addition of the *M.j. cobY*$^+$ gene *in trans* (Figure 2A), indicating that *Mj*CobY functioned as an NTP:AdoCbi-P nucleotidyltransferase *in vivo*. Growth was not restored in medium supplemented with $(CN)_2$Cbi (data not shown), indicating that *Mj*CobY lacked NTP:AdoCbi kinase activity.

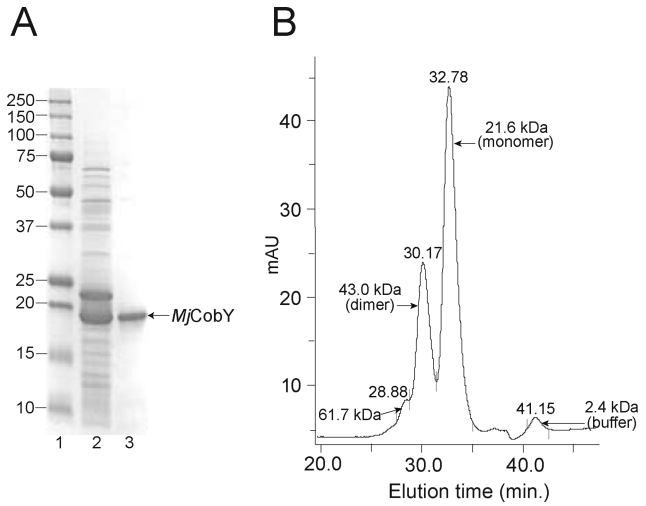

FIGURE 3: Isolation of MjCobY. (A) SDS−PAGE analysis of recombinant *Mj*CobY protein isolated as described under Experimental Procedures. Lanes: 1, molecular mass standards; numbers on the left indicate molecular mass in kiloDaltons; 2, lysate of *Mj*CobY-overexpressing *E. coli*; 3, purified recombinant *Mj*CobY protein. (B) FPLC gel permeation analysis of the protein preparation shown in panel A shows a mixed population of dimers and monomers of *Mj*CobY under the conditions used for the analysis.

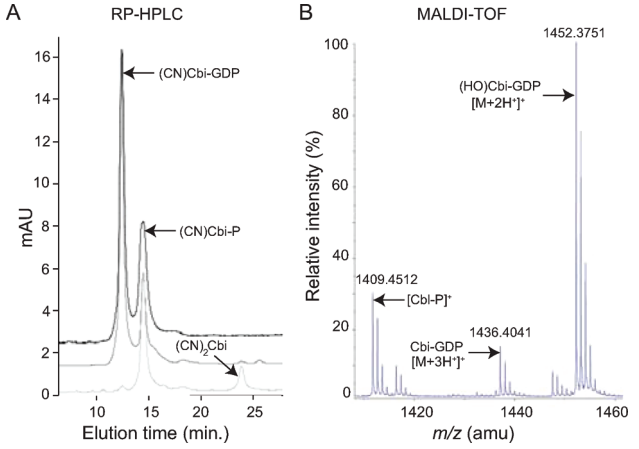

FIGURE 4: The product of the MjCobY reaction is AdoCbi-GDP. (A) RP-HPLC resolution of the cyanated *Mj*CobY reaction product (black), authentic (CN)Cbi-GDP prepared using CobU reaction as described (*14*) (dark gray), and authentic (CN)Cbi-P prepared using CobU as described (*14*) (light gray). *Mj*CobY reaction conditions were as described under Experimental Procedures. mAU, milliabsorbance units. (B) Positive MALDI-TOF spectrum of the product generated by coupling *Mj*CobY to *Se*CobS. Signals with *m*/*z* values of 1409.4512, 1436.4041, and 1452.3751 are consistent with the molecular masses of $[Cbl\text{-}P]^+$ (1408.3), $[Cbi\text{-}GDP + 3H^+]^+$ (1432.3), and $[(OH)Cbi\text{-}GDP + 2H^+]^+$ (1449.3), respectively. amu, atomic mass units.

CobY Activity Requires an Adenosylated Corrinoid Substrate. To determine whether *Mj*CobY could use nonadenosylated corrinoid substrate *in vivo*, we blocked the conversion of Cby to AdoCby by CobA, the housekeeping ATP:corrinoid adenosyltransferase (*11*). A null allele of the *cobA* gene (*cobA363*::MudJ) was introduced into the chromosome of *S. enterica* strain JE8268 (Δ*cobU* Δ*ycfN*) by bacteriophage P22-mediated transduction (*22*). Kanamycin-resistant transductants were freed of phage (*22*), and plasmid pCOBY14 (*M.j. cobY*$^+$) was introduced by electroporation (*19*). Cbl-dependent growth of the resulting strain was assessed to determine whether *Mj*CobY would use nonadenosylated Cby. Only AdoCby supported growth of the transductants (Figure 2B), suggesting that *Mj*CobY required adenosylated corrinoid substrate *in vivo*.

In Vitro Evidence That CobY Has GTP:AdoCbi-P Guanylyltransferase Activity. The Δ*cobU* Δ*ycfN* strain failed to salvage exogenous AdoCbi-GDP (the product of the *Mj*CobY reaction) (data not shown), leaving unanswered the question of whether *Mj*CobY had GTP:AdoCbi-P guanylyltransferase activity. To address this question, we isolated recombinant *Mj*CobY protein (Figure 3A) and took three *in vitro* approaches to determine whether *Mj*CobY made AdoCbi-GDP. First, we coupled the *Mj*CobY reaction to the *S. enterica* CobS (*Se*CobS) enzyme (cobalamin-5′-phosphate synthase; EC 2.7.8.26) and used a bioassay to look for formation of AdoCbl-P (the product of *Se*CobS) (*23*). We used strain JE8248 (Δ*cobS*) (*23*) as the indicator strain to detect the presence of AdoCbl-P in the reaction mixture. Strain JE8248 is a Cbl auxotroph whose growth requires Cbl or Cbl-P. Conditions for the bioassay have been described (*20*). The product of the coupled *Mj*CobY−*Se*CobS reaction supported growth of strain JE8248, while the reaction mixture that did not contain CobY did not (data not shown). Second, we converted the corrinoid product of the *Mj*CobY reaction to its cyanated form and detected it by RP-HPLC (Figure 4A). Third, the HPLC-purified, cyanated corrinoid product of the *Mj*CobY−*Se*CobS reaction was analyzed by mass spectrometry. The MALDI-TOF mass spectrum of the reaction product contained a molecular ion signal with an *m*/*z* of 1409.4512 amu, consistent with a $[Cbl\text{-}P]^+$ ion. Additional $[Cbi\text{-}GDP + 3H^+]^+$ and $[(OH)Cbi\text{-}GDP + 2H^+]^+$ ions (*m*/*z* = 1436.4041 and 1452.3751, respectively) were also observed (Figure 4B).

Interactions of CobY with Its Substrates. We used isothermal titration calorimetry (ITC) to obtain information about enzyme:substrate binding stoichiometry, to identify the preferred nucleotide and corrinoid substrates for *Mj*CobY, to determine the order of substrate binding, and to quantify the thermodynamic parameters of the interactions between *Mj*CobY and its substrates.

(i) CobY Functions as a Dimer. Stoichiometric binding to GTP was observed when the protein concentration used in the calculation of thermodynamic parameters was adjusted to reflect dimeric, rather than monomeric, protein ($n = 0.99 \pm 0.01$) (Figure 5). Size exclusion chromatography revealed that the purified protein was actually a mix of monomers and dimers (Figure 3B). Oligomers were separated, and each species was found to be stable when passed separately over the size exclusion column; i.e., dimers did not separate to monomers or *vice-versa*. *Mj*CobY dimers were able to bind both GTP and AdoCbi-P (see below); dimers were used for all subsequent *in vitro* experiments.

(ii) GTP Is the Nucleotide Substrate of MjCobY. Previous to this work, the preferred nucleotide substrate of CobY was not known. Results from ITC experiments revealed that *Mj*CobY bound GTP (Figure 5, Table 2) but failed to bind other NTPs, the GTP analogues GMP-PNP (guanosine 5′-(β,γ)-imidotriphosphate) or GMP-PCP (guanylyl 5′-(β,γ)-methylenediphosphonate), or GDP (data not shown), strongly suggesting that GTP is the nucleotide substrate for the enzyme *in vivo*. Favorable enthalpy changes [$\Delta H = (-1.70 \pm 0.02) \times 10^4$ kcal/mol] drove the binding of GTP to *Mj*CobY. The binding constant (K_b) for GTP was $(2.00 \pm 0.11) \times 10^5$ M^{-1}, which corresponded to a dissociation constant (K_d) of 5.0 ± 0.02 μM. Table 2 also shows the thermodynamic parameters for association of GTP-bound *Mj*CobY and AdoCbi-P.

Table 2: Thermodynamic Parameters of the Interactions of CobY with Its Substrates[a]

ligand	K_b (M^{-1})	K_d (μM)	ΔH (kcal/mol)	ΔS (kcal mol^{-1} K^{-1})
GTP	$(2.00 \pm 0.11) \times 10^5$	5.0 ± 0.02	$(-1.70 \pm 0.02) \times 10^4$	−32.5
AdoCbi-P	$(1.32 \pm 0.97) \times 10^6$	0.76 ± 0.44	$(-0.37 \pm 0.04) \times 10^4$	−16.2

[a] Protein and substrates were dissolved in HEPES buffer (50 mM, pH 7.5 at 4°C). ITC experiments were performed at 27 °C. Data collected were analyzed with the ORIGIN v.7.0383 software provided with the microcalorimeter and fit to the one-site binding model. K_b, binding constant; K_d, dissociation constant; ΔH, enthalpy change; ΔS, entropy change.

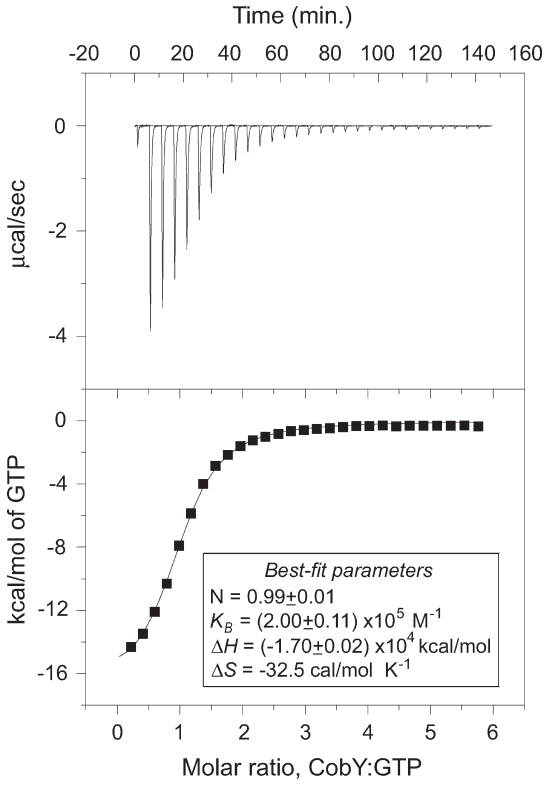

FIGURE 5: CobY binds GTP. Calorimetric titration of CobY with GTP. Twenty-seven injections of 1 mM GTP were added to 18 μM *Mj*CobY dimers in the ITC cell. The area under each injection peak (top panel) equals the total heat released for that injection. The heat of dilution of GTP into buffer has been subtracted. A binding isotherm for the interaction between *Mj*CobY and GTP was obtained from the integrated heat (bottom panel). Shown is the enthalpy per mole of GTP injected as a function of the molar ratio of *Mj*CobY to GTP. The best-fit values for stoichiometry (N), binding constant (K_b), enthalpy (ΔH), and entropy (ΔS) are shown in the inset and listed in Table 2.

(iii) The Adenosyl Upper Ligand of the Corrinoid Substrate Is Required for Binding. Although we showed that *Mj*CobY required an adenosylated corrinoid substrate *in vivo* and *in vitro*, it was unclear whether the adenosyl moiety was required for binding to the enzyme, for catalysis, or for both. To examine the role of the upper ligand, we used ITC to determine the enthalpic change of binding of *Mj*CobY to corrinoids with different upper ligands, including AdoCbi-P, $(CN)_2$Cbi-P, and hydroxycobinamide phosphate [(HO)Cbi-P]. *Mj*CobY did not bind either $(CN)_2$Cbi-P or (HO)Cbi-P (data not shown), indicating that the upper ligand was important for binding. At present it is unclear whether the adenosyl group is involved in catalysis.

(iv) CobY Must Bind GTP before Binding AdoCbi-P. ITC experiments showed that *Mj*CobY would not bind AdoCbi-P in the absence of GTP (Figure 6), indicating that the enzyme binds GTP first, before binding AdoCbi-P.

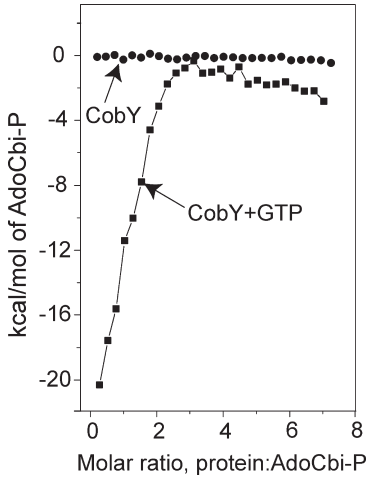

FIGURE 6: CobY binds GTP before binding AdoCbi-P. Thermodynamics of binding to AdoCbi-P. Twenty-seven injections of 1 mM AdoCbi-P were added to 18 μM *Mj*CobY dimers (circles) or 18 μM *Mj*CobY dimers + 100 μM GTP (squares) in the ITC cell. Shown is the enthalpy per mole of AdoCbi-P injected as a function of the molar ratio of protein solution to AdoCbi-P.

Kinetic Parameters of the MjCobY Reaction. We used RP-HPLC to quantify the *in vitro* formation of AdoCbi-GDP and to determine pseudo-first-order kinetic parameters of the *Mj*CobY reaction at 37 °C. Under conditions of saturating GTP concentration (2 mM) and varying AdoCbi-P levels, the apparent *K*m (K_m^{app}) = 18.4 ± 1.1 μM, k_{cat} = 4.7 ± 0.5 s^{-1}, and k_{cat}/K_m^{app} = 2.6 × 10^5 M^{-1} s^{-1}. Under conditions of saturating corrinoid concentration (100 μM) and varying GTP levels, K_m^{app} = 2.4 ± 0.3 μM, k_{cat} = 1.8 ± 0.2 s^{-1}, and the catalytic efficiency (k_{cat}/K_m^{app}) = 7.5 × 10^5 M^{-1} s^{-1}. The lower k_{cat} when GTP was at subsaturating levels suggested that the enzyme must be fully saturated with GTP for maximum turnover.

When experiments were repeated at 80 °C, we found that neither the K_m^{app} nor the k_{cat} differed appreciably from those obtained when the experiments were conducted at 37 °C (data not shown). This result was surprising, given that (i) *M. jannaschii* grows optimally at 85 °C and (ii) its rapid doubling time of 26 min at that temperature (*32*) would require substantial amounts of Cba for energy generation, thus demanding high efficiency of Cba biosynthetic enzymes. We used circular dichroism (CD) spectroscopy to examine whether temperature influenced the secondary structure of the *Mj*CobY protein. A 40 min incubation at 80 °C resulted in a time-dependent decrease in intensity, indicating that the protein denatured at 80 °C (Figure 7A). In contrast, a 30 min incubation at 37 °C did not affect the conformation of the protein. Notably, a shift from 80 to 20 °C did not reverse the conformational change back to the one observed for the protein at 37 °C (Figure 7B). This suggests that the lack of increase in enzyme activity at 80 °C was due to partial denaturation of the *Mj*CobY protein at the elevated

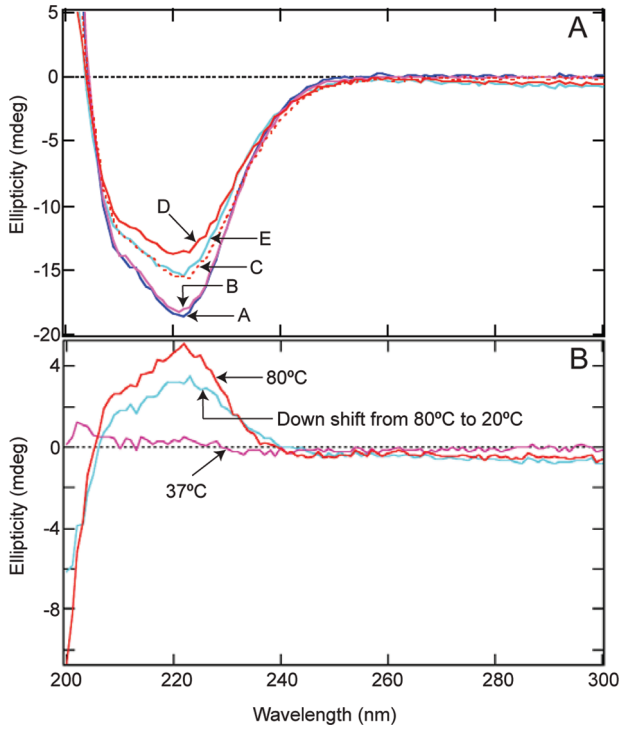

FIGURE 7: Temperature-induced changes in *Mj*CobY conformation. (A) The conformational change of *Mj*CobY at 80 °C is not reversible. CD spectra of *Mj*CobY protein were obtained at four different temperatures. Scan A (dark blue line) at 20 °C for 10 min; scan B (purple line) at 37 °C for 30 min; scan C (red dotted line) at 80 °C for 10 min; scan D (red line) at 80 °C for 40 min; scan E (light blue line) downshifted from 80 to 20 °C and scanned after 10 min at 20 °C. (B) Stability of the *Mj*CobY conformation. Shown are difference CD spectra of *Mj*CobY at different temperatures relative to the spectrum obtained at 20 °C. Spectra were acquired under conditions described in panel A.

temperature. It is possible that, *in vivo*, the *Mj*CobY protein is stabilized by interactions with other proteins or cell structures, examples of which have been reported in the literature (*33*−*36*).

Grochowski et al. recently showed that *Mj*CobY catalyzed the condensation of GTP and 2-phospho-L-lactate (LP) during coenzyme F_{420} biosynthesis, in a reaction analogous to the guanylylation of AdoCbi-P (*37*). However, *Mj*CobY's high K_m^{app} for LP (6 mM), as compared to that for AdoCbi-P (18.4 μM), is inconsistent with its role in coenzyme F_{420} biosynthesis. In addition, the authors identified locus MJ0887 as encoding the kinetically competent lactoyl-phosphate guanylyltransferase (CofC) enzyme. Nevertheless, structural data would help us understand how *Mj*CobY binds GTP and is able to use both AdoCbi-P and LP as substrates *in vitro*. This work is currently in progress.

We have shown here that the *M. jannaschii* MJ1117 gene product is a GTP:AdoCbi-P guanylyltransferase *in vivo* and *in vitro*. On the basis of the data presented, we propose a change in gene nomenclature, from MJ1117 to *cobY*, to reflect the role of the *Mj*CobY protein in AdoCbl biosynthesis.

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