Published in final edited form as:

Biochemistry. 2009 June 30; 48(25): 5882–5889. doi:10.1021/bi8023114.

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 is the non-orthologous 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 AdoCbi (coenzyme B₁₂). Here we show that ORF MJ1117 of the hyperthermophilic, methanogenic archaeon *Methanocaldococcus jannaschii* encodes a CobY protein that transfers the GMP moiety of GTP to AdoCbi-P to form AdoCbi-GDP. Results from isothermal titration calorimetry (ITC) experiments show that *MjCobY* binds GTP ($K_d = 5 \mu\text{M}$), but it does not bind the GTP analogs GMP-PNP (guanosine 5'-(β,γ)-imidotriphosphate) or GMP-PCP (guanylyl 5'-(β,γ)-methylenediphosphonate), nor GDP. Results from ITC experiments indicate that *MjCobY* 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 *MjCobY* function. Consistent with these findings, *MjCobY* displayed high affinity for 5'-adenosyl moiety of AdoCbi-P ($K_d = 0.76 \mu\text{M}$), but did not bind non-adenosylated Cbi-P; kinetic parameters for the *MjCobY* reaction were determined. We propose that ORF MJ1117 of *Methanocaldococcus jannaschii* be annotated as *cobY* to reflect its involvement in AdoCbi biosynthesis. Results from circular dichroism studies indicate that, in isolation, *MjCobY* denatures at 80°C with a concomitant loss of activity.

Keywords

Coenzyme B₁₂ biosynthesis; archaeal metabolism; corrinoid enzymology; nucleotide loop assembly; coenzymes of methanogenic archaea

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 pre-formed, incomplete precursors [e.g., cobinamide (Cbi), 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 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 pathway (Fig. 1). The latter can be further broken down into two sub-branches, one of which activates adenosyl-Cbi (AdoCbi) to AdoCbi-guanosine

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diphosphate (AdoCbi-GDP) (12), and a second one that activates the lower ligand base to its nucleotide (2) (Fig. 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 (Fig. 1) (14). The *cobY* gene was identified in extreme halophilic and thermophilic, methanogenic archaea as a non-orthologous 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 *E. 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 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 MgSO₄ (1 mM) was used to grow cells under nutrient-defined conditions. Solid media contained 15 g of Bacto Agar (Difco) per liter. Corrinoids [cyanocobyrinic acid (CNCby), dicyanocobinamide [(CN)₂Cbi], or cyanocobalamin [CNCbl; a.k.a. vitamin B₁₂] were added to media at 15 nM. CNCby was a gift from Paul Renz (Institut für Biologische Chemie und Ernährungswissenschaft, Universität-Hohenheim, Stuttgart, Germany); (CN)₂Cbi and CNCbl were purchased from Sigma.

Genetics and Recombinant DNA Techniques

Complementation of NTP:AdoCbi-P nucleotidyltransferase activity in

***Salmonella enterica* Δ*cobU* Δ*ycfN* strain**—Plasmid pCOBY14 (*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 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-µl 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).

Strain construction—Construction of a Δ*cobS* strain of *Salmonella 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. Deletion of the *cobS* gene was verified by DNA sequencing using BigDye® protocols (ABI PRISM); reaction mixtures were resolved by the UW-Madison Biotechnology Center.

Construction of a strain deficient in the attachment of the upper ligand. Corrinoide adenosylation was blocked by inactivation of the *cobA* gene encoding the housekeeping ATP:corrinoide adenosyltransferase (11). The *cobA363::MudJ* mutation was introduced into the chromosome of strain JE8268 ($\Delta cobU \Delta ycfN$) by bacteriophage P22-mediated transduction (22).

Biochemical Techniques

Protein overexpression and purification—CobY protein. Twenty mL 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 OD₆₀₀ of 0.5 to 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 15,000 $\times$ 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 @ 4°C) containing protease inhibitor phenylmethylsulfonyl 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 43,367 $\times$ 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 43,367 $\times$ g. Finely ground ammonium sulfate (*UltraPure*, ICN Biomedicals) was added to 10% (w/v.) Precipitates were cleared by centrifugation at 4°C for 60 min at 43,367 $\times$ 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; 50mM, pH 7.5 @ 4°C) containing (NH₄)₂SO₄ (10% w/v.) 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 @ 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 @ 4°C) to remove (NH₄)₂SO₄.

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, (50mM; pH 7.5 @ 4°C.) Protein was eluted at a flow rate of 1 mL min⁻¹ with a linear gradient to 100% HEPES buffer, (50mM; pH 7.5 @ 4°C) containing NaCl (1M.) CobY did not interact with the resin, and flow-through fractions containing CobY were concentrated and dialyzed against HEPES buffer (50mM; pH 7.5 @ 4°C). Glycerol was added to a final concentration of 10% (v/v). The CobY protein was flash-frozen by drop-wise addition into liquid N₂; frozen beads were stored at -80°C until use.

Salmonella enterica CobS protein. Cell-free membrane fractions enriched for *SeCobS* were prepared as reported (23).

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).

Size exclusion chromatography—Size exclusion chromatography was performed as described (27) except that the molecular weight 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 kDa) was linear between 25 min and 42 min elution ($r^2 = 1.000$).

Synthesis of corrinoid substrates—AdoCbi and AdoCbi-P were prepared and purified as reported (20).

***in vitro* synthesis of Adocobalamin-5'-phosphate (AdoCbi-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 μ M) (28), MgCl₂ (5 mM), GTP (2 mM), 5 μ g 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 μ l. 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 μ l 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).

***In vitro* synthesis of AdoCbi-GDP by CobY**—Reaction mixtures contained Tris-HCl (50 mM, pH 7.9), GTP (2 mM), NaCl (50 mM), MgCl₂ (10 mM), dithiothreitol (DTT; 10 mM), *MjCobY* protein (2.3 pmol), and AdoCbi-P (200 μ M) in a final volume of 40 μ l. Reaction mixtures were incubated under dim, red light for 15 min. Reactions were stopped and corrinoids converted to their cyanated forms as described above.

HPLC analysis of the *MjCobY* reaction product—Fifty-five μ l of water were added to the *MjCobY* 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 C₁₈ AQ 5 μ m 150 mm $\times$ 4.6 mm column fitted with an Alltima HP C₁₈ 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: 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]:CH₃CN (1:1)]. Corrinoids eluted from the column were detected at 367 nm with a Waters photodiode array detector. A standard curve for (CN)₂Cbi was constructed for quantification purposes; detection of (CN)₂Cbi was linear between 1 pmol and 3 nmol ($r^2 = 0.9984$).

Mass spectrometry—The product of the *MjCobY*-*SzCobS* coupled reaction was dried under vacuum in a SpeedVac® concentrator (Thermo Savant,) suspended in ddH₂O, and loaded onto a Sep-pak C18 cartridge (Waters) pre-equilibrated with ddH₂O. 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.

Kinetic analyses—Kinetic parameters were determined using the assay conditions described above, using Microsoft® Excel software.

Isothermal titration calorimetry—*MjCobY* protein was dialyzed against Tris-HCl buffer, pH 8.0 at 4°C (50mM) containing NaCl (50 mM), MgCl₂ (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 18μM 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™, Northampton, MA) and analyzed with the ORIGIN® v.7.0383 software provided with the microcalorimeter.

Circular dichroism spectroscopy—CobY protein was dialyzed against sodium phosphate buffer, pH 7.2 (50mM), containing NaCl (50mM) 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 pathlength extension spectrophotometer (C Technologies, Inc., Bridgewater, NJ) using an extinction coefficient of 14,900 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 pathlength 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 *Methanocaldococcus jannaschii*

The MJ1117 open reading frame (ORF) of *M. jannaschii* encodes a protein that has been shown to catalyze the guanylation of an intermediate in the synthesis of coenzyme F₄₂₀ in methanogenic archaea (29). Because the chemistry of the F₄₂₀ biosynthetic reaction is similar to that of the guanylation of AdoCbi-P (30), we hypothesized that the MJ1117 protein catalyzed the synthesis of AdoCbi-GDP from AdoCbi-P and GTP in *M. jannaschii*.

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 *MjCobY* 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 (Fig. 2A), indicating that *MjCobY* functioned as an NTP:AdoCbi-P nucleotidyltransferase *in vivo*. Growth was not restored in medium supplemented with (CN)₂Cbi (data not shown,) indicating that *MjCobY* lacked NTP:AdoCbi kinase activity.

CobY activity requires an adenosylated corrinoid substrate

To determine whether *MjCobY* could use non-adenosylated 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 ($\Delta cobU \Delta ycfN$) 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 *MjCobY* would use non-adenosylated Cby. Only AdoCby supported growth of the transductants, suggesting that *MjCobY* required adenosylated corrinoid substrate *in vivo* (Fig. 2B).

In vitro evidence that CobY has GTP:AdoCbi-P guanylyltransferase activity

The $\Delta cobU \Delta ycfN$ strain failed to salvage exogenous AdoCbi-GDP (the product of the *MjCobY* reaction) (data not shown), leaving unanswered the question of whether *MjCobY* had GTP:AdoCbi-P guanylyltransferase activity. To address this question we isolated recombinant *MjCobY* protein (Fig. 3A) and took three *in vitro* approaches to determine whether *MjCobY* made AdoCbi-GDP. First, we coupled the *MjCobY* reaction to the *SeCobS* enzyme (cobalamin-5'-phosphate synthase; EC 2.7.8.26) and used a bioassay to look for formation of AdoCbl-P (the product of *SeCobS*) (23). We used strain JE8248 ($\Delta cobS$) (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 *MjCobY-SeCobS* reaction supported growth of strain JE8248, while the reaction mixture that did not contain CobY did not (data not shown.) Secondly, we converted the corrinoid product of the *MjCobY* reaction and detected it by RP-HPLC (Fig. 4A). Thirdly, the purified, cyanated corrinoid product of the *MjCobY-SeCobS* 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-P]⁺ ion. Additional signals for [Cbi-GDP]⁺ and [(CN)Cbi-GDP]⁺ ions (*m/z* = 1436.4041 and 1452.3751, respectively) were also observed (Fig. 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 *MjCobY*, to determine the order of substrate binding, and to quantify the thermodynamic parameters of the interactions between *MjCobY* and its substrates.

CobY functions as a dimmer—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 ± .01) (Fig. 5.) Size exclusion chromatography revealed that the purified protein was actually a mix of monomers and dimers (Fig. 3B). Oligomers were separated and each species found to be stable when passed separately over the size exclusion column, i.e., dimers did not separate to monomers or vice versa. *MjCobY* dimers were able to bind both GTP and AdoCbi-P (see below); dimers were used for all subsequent *in vitro* experiments.

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 *MjCobY* bound GTP (Fig. 5, Table 2) but failed to bind other NTPs, the GTP analogs GMP-PNP (5'-guanylylimidetriphosphate) 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 *MjCobY*. 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 $5.0 \pm .02$ μ M. Table 2 also shows the thermodynamic parameters for association of GTP-bound *MjCobY* and AdoCbi-P.

The adenosyl upper ligand of the corrinoid substrate is required for binding—Although we showed that *MjCobY* 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 *MjCobY* to corrinoids with different upper ligands, including AdoCbi-P, (CN)₂Cbi-P, and hydroxycobinamide-phosphate [(HO)Cbi-P]. *MjCobY* did not bind either (CN)₂Cbi-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.

CobY must bind GTP before binding AdoCbi-P—ITC experiments showed that *MjCobY* would not bind AdoCbi-P in the absence of GTP (Fig. 6,) indicating that the enzyme binds GTP first, before binding 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 *MjCobY* reaction at 37°C. Under conditions of saturating GTP concentration (2 mM) and varying AdoCbi-P levels, $K_m^{app} = 18.4 \pm 1.1$ μ M, $k_{cat} = 4.7 \pm 0.5$ s⁻¹, and $k_{cat}/K_m^{app} = 2.6 \times 10^5$ M⁻¹ s⁻¹. Under conditions of saturating corrinoid concentration (100 μ M) and varying GTP levels, the apparent K_m (K_m^{app}) = 2.4 ± 0.3 μ M, $k_{cat} = 1.8 \pm 0.2$ 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 sub-saturating levels suggested that the enzyme must be fully saturated with GTP for maximum turnover. To calculate the maximal reaction rate (V_{max}), the reaction mixture contained *MjCobY* (2.3 pmol), AdoCbi-P (100 μ M), and GTP (2 mM). Under the assay conditions used, $V_{max} = 1.36 \pm 0.1$ μ mol min⁻¹ mg⁻¹.

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 *MjCobY* protein. A 40-min incubation at 80°C resulted in a time-dependent decrease in intensity, indicating that the protein denatured at 80°C (Fig. 7A). In contrast, a 30-min incubation at 37°C did not affect the conformation of the protein. Notably, a shift from 80 °C to 20°C did not reverse the conformational change back to the one observed for the protein at 37 °C (Fig.7B). This suggests that the lack of increase in enzyme activity at 80°C was due to partial denaturation of the *MjCobY* protein at the elevated temperature. It is possible that, *in vivo*, the *MjCobY* 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 *MjCobY* catalyzed the condensation of GTP and 2-phospho-L-lactate (LP) during coenzyme F₄₂₀ biosynthesis, in a reaction analogous to the guanylation of AdoCbi-P (37). However, *MjCobY*'s high K_m^{app} for LP (6 mM), as compared to that for AdoCbi-P (18.4 μ M), is inconsistent with its role in coenzyme F₄₂₀ 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 *MjCobY* 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*. Based on the data presented, we propose a change in gene nomenclature, from MJ1117 to *cobY*, to reflect the role of the *MjCobY* protein in AdoCbi biosynthesis.

Acknowledgments

This work was supported by funds from the NIH to J.C.E.-S. (GM40313). We thank Robert H. White (Virginia Polytechnic Institute and State University) for providing the *M.j. cobY*⁺ plasmid for over-expression. We also thank Paul Renz for his gift of cyanocobyrinic acid.

Abbreviations

MjCobY, *Methanocaldococcus jannaschii* CobY protein
 Cbl, cobalamin
 Cbl-P, Cbl-phosphate
 AdoCbl, adenosyl-Cbl
 AdoCbl-P, adenosyl-Cbl-phosphate
 Cbi, cobinamide
 (CN)₂Cbi, dicyano-Cbi
 HOCbi, hydroxy-Cbi
 HOCbi-P, HOCbi-phosphate
 CNCby, cyanocobyrinic 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, adenosylcobyrinic acid; AP-P, aminopropanol phosphate; AdoCbi, adenosylcobinamide; AdoCbi-P, adenosylcobinamide-phosphate; AdoCbi-GDP, adenosylcobinamide-GDP; 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, cobyrinic 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, thiamine kinase/AdoCbi kinase. **B.** Structure of coenzyme B₁₂ (a.k.a. AdoCbl).

Figure 2. Cbl-dependent growth of *S. enterica* strains

Growth in minimal medium (18) medium 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.

AMjCobY allows a *S. enterica* $\Delta \text{cobU} \Delta \text{ycfN}$ strain to salvage Cby. *S. enterica* strains used were TR6583 ($\text{cobU}^+ \text{ycfN}^+$; filled triangles), JE8335 [$\Delta \text{cobU} \Delta \text{ycfN} / \text{pCOBY14} (\text{cobY}^+)$; open squares], and JE8269 ($\Delta \text{cobU} \Delta \text{ycfN} / \text{pT7-7}$; empty vector control; open circles).

BMjCobY 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 (ΔcobA ; open squares and circles).

Figure 3. Isolation of MjCobY

ASDS-PAGE analysis of recombinant *MjCobY* protein isolated as described under Experimental Procedures. Lane 1, molecular mass standards; numbers on the left indicate molecular mass in kilodaltons; Lane 2, lysate of *MjCobY*-overexpressing *E. coli*; Lane 3, purified recombinant *MjCobY* protein. **B** FPLC gel permeation analysis of the protein preparation shown in panel **A** shows a mixed population of dimers and monomers of *MjCobY* under the conditions used for the analysis.

Figure 4. The product of the *MjCobY* reaction is AdoCbi-GDP

ARP-HPLC resolution of the *MjCobY* 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). *MjCobY* reaction conditions were as described under Experimental Procedures. mAU, milliabsorbance units. **B.** Positive MALDI-TOF spectrum of the product generated by coupling *MjCobY* to *SeCobS*. Signals with m/z values of 1409.4512, 1436.4041, and 1452.3751 are consistent with the molecular masses of $[Cbl-P]^+$ (1408.3), $[Cbi-GDP]^+$ (1432.3), and $[(CN)Cbi-GDP]^+$ (1458.3), respectively. amu, atomic mass units.

Figure 5. CobY binds GTP

Calorimetric titration of CobY with GTP. Twenty-seven injections of 1 mM GTP were added to 18 μ M *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.

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 μ M *Mj*CobY dimers (circles) or 18 μ M *Mj*CobY dimers + 100 μ M 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.

Figure 7. Temperature-induced changes in MjCobY conformation

A The conformational change of MjCobY at 80°C is not reversible. CD spectra of MjCobY 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): down shifted from 80°C to 20°C and scanned after 10 min at 20°C. **B.** Stability of the MjCobY conformation. Shown are difference CD spectra of MjCobY at different temperatures relative to the spectrum obtained at 20°C. Spectra were acquired under conditions described in panel A.

Table 1
Strains and plasmids used in these studies.

Strain or plasmid	Genotype and description	Reference or source [†]
<i>Salmonella enterica</i> [*]		
TR6583; formerly SA2929	<i>metE205 ara-9</i>	K. Sanderson via J. Roth
Derivatives of TR6583:		
JE8248	<i>cobS1313</i> ($\Delta cobS$)	(23)
JE8249	<i>cobU1315</i> ($\Delta cobU$)	(20)
JE8268	<i>cobU1315 ycfN112</i> ($\Delta cobU \Delta ycfN$)	(20)
JE8269	<i>cobU1315 ycfN112</i> / pT7-7	
JE8335	<i>cobU1315 ycfN112</i> / pCOBY14	
JE9318	<i>cobU1315 ycfN112 cobA343::MudJ</i> / pCOBY14	
<i>Escherichia coli</i>		
BL21-CodonPlus® (DE3)-RIL	B F <i>ompT hsdS</i> (r_B^- , m_B^-) <i>dcm</i> ⁺ <i>tet gal</i> λ (DE3) <i>endA Hte</i> (<i>argU ileY leuW cat</i>)	Stratagene
Plasmids		
pT7-7	(36) ⁺ ; cloning vector	Derivative of pT7-1 (38)
pCOBY14	MJ1117 <i>cobY</i> ⁺ in pT7-7 <i>bla</i> ⁺	(37)

^{*} 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).

[†] Strains were constructed for this study unless otherwise indicated.

Table 2

Thermodynamic parameters of the interactions of CobY with its substrates.

Ligand	K_b (M^{-1})	K_d (μM)	ΔH (kcal/mol)	ΔS (kcal mol ⁻¹ K ⁻¹)
GTP	$(2.00 \pm 11) \times 10^5$	5.0 ± 0.2	$(-1.70 \pm 0.02) \times 10^4$	-32.5
AdoCbi-P	$(1.32 \pm 97) \times 10^6$	$0.76 \pm .44$	$(-0.37 \pm 0.04) \times 10^4$	-16.2

Protein and substrates were dissolved in HEPES buffer (50 mM, pH7.5 @ 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.