

Partial Randomization of the Four Sequential Amidation Reactions Catalyzed by Cobyric Acid Synthetase with a Single Point Mutation[†]

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ABSTRACT: Cobyric acid synthetase (CbiP) from *Salmonella typhimurium* catalyzes the glutamine and ATP-dependent amidation of carboxylates *b*, *d*, *e*, and *g* within adenosyl cobyrinic acid *a,c*-diamide. After each round of catalysis the partially amidated intermediates are released into solution and the four carboxylates are amidated in the sequential order of *e*, *d*, *b*, and *g* for the wild type enzyme. In the presence of [γ -¹⁸O₄]-ATP and adenosyl cobyrinic *a,c*-diamide the enzyme will catalyze the positional isotope exchange of the $\beta\gamma$ -bridge oxygen with the two β -nonbridge oxygens. These results support the proposal that ATP is used to activate the carboxylate groups via the formation of a phosphorylated intermediate. CbiP catalyzes the hydrolysis of glutamine in the absence of ATP or adenosyl cobyrinic acid *a,c*-diamide, but the rate of glutamine hydrolysis is enhanced by a factor of 60 in the presence of these two substrates together. This result suggests that the formation of the phosphorylated intermediate is coupled to the activation of the site utilized for the hydrolysis of glutamine. However, the rate of glutamine hydrolysis is approximately 2.5 times the rate of ADP formation, indicating that the two active sites are partially uncoupled from one another and that some of the ammonia from glutamine hydrolysis leaks into the bulk solution. The mutation of D146 to either alanine or asparagine results in a protein that is able to catalyze the formation of cobyrinic acid. However, the strict amidation order observed with the wild type CbiP is partially randomized with carboxylate *b* being amidated last. With the D146N mutant, the predominant pathway occurs in the sequence *d*, *e*, *g*, and *b*. It is proposed that this residue enforces the amidation order in the wild type enzyme via charge–charge repulsion between the side chain carboxylate and the carboxylates of the substrate.

The biosynthetic pathway for the assembly of vitamin B₁₂ is rather complex. There are many enzymes in this pathway that catalyze multiple methylation, decarboxylation, or amidation reactions on key reaction intermediates. For example, in the aerobic pathway from *Pseudomonas denitrificans* *S*-adenosyl-L-methionine:uroporphyrinogen III methyl transferase (SUMT) catalyzes the methylation of C2 and C7 of uroporphyrinogen III while the fusion protein CobL catalyzes the methylation of C5 and C15 of dihydro-precorrin 6 (1). The corresponding enzymes in the anaerobic pathway from *Salmonella typhimurium* are CysG and CbiE, respectively (1). Uroporphyrinogen III decarboxylase catalyzes the ordered decarboxylation of the four acetate side chains of the substrate in the heme biosynthetic pathway (2). In addition, CobB and CobQ in the aerobic pathway from *P. denitrificans* are responsible for the amidation of six different carboxylate groups on the periphery of the corrin ring system. CobB catalyzes the amidation of carboxylate groups *a* and *c* of hydrogenobyrrinic acid to produce hydrogenobyrrinic acid *a,c*-diamide (3). CobQ catalyzes the amidation of carboxylate

groups *b*, *d*, *e*, and *g* of adenosyl cobyrinic acid *a,c*-diamide (Ado-cob¹) to produce cobyrinic acid (4). The homologous enzymes in *S. typhimurium* are CbiA and CbiP, respectively (1). The multiple reactions catalyzed by cobyrinic acid synthetase (CbiP) are presented in Scheme 1.

Cobyrrinic acid *a,c*-diamide synthetase (CbiA) from *S. typhimurium* catalyzes the ATP-dependent amidation of carboxylates *a* and *c*. Carboxylate *c* is amidated first, and then this partially amidated product is released into solution prior to rebinding and formation of the final product, cobyrinic acid *a,c*-diamide (3, 5). The reaction mechanism is dissociative and sequential. Cobyrrinic acid synthetase from *S. typhimurium* catalyzes the ATP-dependent amidation of carboxylate groups *b*, *d*, *e*, and *g* to produce cobyrinic acid via a reaction mechanism that is also dissociative and sequential. Three partially amidated intermediate species have been identified, and the order of the amidation reactions have been established with ¹H–¹⁵N NMR spectroscopy (6). The specific sequence begins with the amidation of carboxylate *e*, followed in turn by the amidation of carboxylate groups *d*, *b*, and *g*. These results demonstrate that the substrate, adenosyl cobyrinic acid *a,c*-diamide, binds productively in only one of four possible orientations. Following the ami-

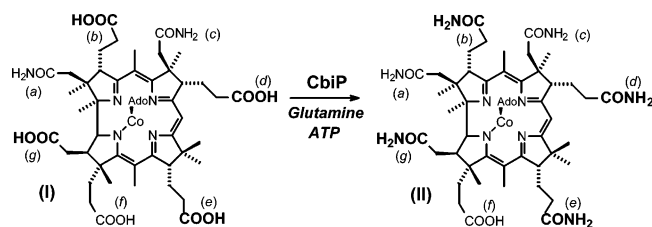
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¹ Abbreviation: CbiA, cobyrinic acid *a,c*-diamide synthetase; CbiP, cobyrinic acid synthetase; CobA, cobyrinic acid *a,c*-diamide adenosyl-transferase; PIX, positional isotope exchange; Ado-cob, adenosyl cobyrinic acid *a,c*-diamide.

Scheme 1



dation of carboxylate *e*, the partially amidated intermediate must dissociate from the active site and rebind in a different orientation for the amidation of carboxylate *d*. Amidation of carboxylate groups *b* and *g* requires similar reorientations within the active site after dissociation and rebinding to the active site. The structural or molecular basis for the strict regioselectivity as the four carboxylates are amidated is currently unknown. Having a single enzyme catalyze four separate reactions on a single substrate is biochemically more economical than the evolution of a different enzyme for each amidation reaction. An ordered dissociative mechanism also minimizes the number of intermediates that are liberated into solution.

On the basis of amino acid sequence alignments, CbiP is composed of separate glutaminase and synthetase domains (7). The N-terminal synthetase domain is proposed as the ATP and adenosyl cobyrinic acid *a,c*-diamide binding site. The amino acid sequence of the synthetase domain of CbiP shows similarity to MinD proteins and dethiobiotin synthetase (7, 8). Ammonia is predicted to be formed within the C-terminal glutaminase domain and translocated to the N-terminal domain where it reacts with an activated intermediate to form the carboxamide product. Similar glutaminase domains are found in carbamoyl phosphate synthetase and GMP synthetase (9, 10). In these enzymes an active site histidine activates a cysteine residue for nucleophilic attack on the carboxamide moiety of glutamine forming a thioester intermediate and ammonia.

We have expressed and purified the cobyrinic acid synthetase from *S. typhimurium* and determined the kinetic constants of the overall reaction as well as the hydrolysis of glutamine in the absence of the other substrates. Mutagenesis studies have identified a single amino acid residue that forces the enzyme to catalyze the partially random synthesis of amidated intermediates.

MATERIALS AND METHODS

Materials. The plasmid p278Q containing the *cbiP* gene (gi: 16763390) was kindly provided by Dr. Charles Roessner from Texas A&M University. *S. typhimurium* AR8519 strain was a gift from Dr. Evelyne Deery from Queen Mary University of London (11). The restriction enzymes, *Nde*I and *Eco*RI, and platinum *Pfx* DNA polymerase were obtained from New England Biolabs and Invitrogen, respectively. Oligonucleotide synthesis and DNA sequencing were performed by the Gene Technologies Laboratory, Texas A&M University. Nitrogen-15-labeled ammonium chloride and ^{18}O -labeled water were purchased from Cambridge Isotope Laboratories. ^{15}N -Glutamine was purchased from Sigma-Aldrich. The Microsorb 100 C18 5- μm reverse-phase chromatography column was obtained from Varian, Inc. All other chromatography columns and resins were purchased from

Amersham Biosciences. Oxygen-18-labeled potassium phosphate was prepared using the method of Risley and Van Etten (12). The synthesis of $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ was accomplished using the method of Werhli et al. from ADP-morpholidate and $\text{H}_3\text{P}^{18}\text{O}_4$ (13). The two stock solutions of $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ showed ~ 90 and 93% ^{18}O incorporation by ^{31}P NMR spectroscopy. All other materials and enzymes were purchased from Sigma.

Cloning and Site-Directed Mutagenesis of *cbiP*. The *cbiP* gene was subcloned into pET30 using the p278Q plasmid as a template. The *cbiP* gene was PCR-amplified such that the forward primer 5'-GGGAATCCCATATGACGCAG-GCAGTTATGTTGC-3' and reverse primer 5'-GGGAAT-TCTATCATCATACCGGCTCCTGATG-3' added *Nde*I and *Eco*RI sites, respectively. The resultant PCR product was purified, cut with *Nde*I and *Eco*RI, and then ligated to the expression vector pET30 (Novagen). Mutants of *cbiP* were constructed using the QuikChange site-directed mutagenesis kit from Stratagene. The entire coding region was sequenced to confirm the absence of additional mutations for all constructions.

Cloning of Cobyrinic Acid *a,c*-Diamide Adenosyl Transferase (*cobA*) from *S. typhimurium*. The *cobA* gene (gi: 399274) was PCR-amplified using the genomic DNA from *S. typhimurium* AR8519 strain as a template. The forward primer was 5'-CGGAATTCATATGAGTGATGAACGT-TATCAGCAGC-3' and the reverse primer was 5'-GCGC-GAAGCTTTTAATAATCAATTCCTCATCTGGGC-3'. The PCR product was purified, cut, and ligated in pET30 as described above.

Expression and Purification of CbiP and CobA. The pET30 plasmid carrying the desired construction was used to transform the *E. coli* BL21(DE3) strain (Novagen), and the transformed cells were selected on LB agar with 50 $\mu\text{g}/\text{mL}$ kanamycin. Protein expression was induced by addition of 1.0 mM IPTG to a cell culture at mid-log phase. The cultures were incubated for an additional 10 h at room temperature, collected by centrifugation, frozen in liquid nitrogen, and then stored at -80°C . The cell paste was thawed into a solution containing 0.1 M Tris-HCl, pH 7.5, 1.0 mM dithiothreitol (DTT), 0.1 mg/mL PMSF, 10 μM leupeptin, 10 μM pepstatin, 1.0 $\mu\text{g}/\text{mL}$ RNAase and 1.0 $\mu\text{g}/\text{mL}$ DNAase. Cells were lysed on ice by sonication, and the mixture was clarified by centrifugation. Ammonium sulfate was added to 30% or 50% of saturation for the purification of CbiP or CobA, respectively. The solution was incubated at 4°C for 15 min and the protein pellet was collected by centrifugation. The pellet was resuspended in buffer and loaded onto a Superdex 200 column. The fractions containing the protein were pooled on the basis of SDS-PAGE and loaded onto a Resource Q ion exchange column and eluted with a linear gradient of NaCl (0–0.5 M). After concentration of the protein by ammonium sulfate precipitation, the protein solution was loaded onto a Superdex 200 column. The fractions containing the protein were pooled and stored at -80°C in the same buffer containing 10% glycerol. The concentration of protein was calculated using an extinction coefficient at 280 nm of 0.75 and 1.08 Au cm^{-1} for a 1 mg/mL solution of purified protein for CbiP and CobA, respectively. The extinction coefficients were estimated by the method of Gill and von Hippel (14) using the protein

calculator program (www.scripps.edu/cgi-bin/cdputnam/protcalc).

Synthesis of Adenosyl Cobyric Acid *a,c*-Diamide. The substrate for the CbiP reaction, adenosyl cobyrinic acid *a,c*-diamide, was synthesized from vitamin B₁₂ in three steps. First, (CN)₂-cobyrinic acid was obtained by methanolysis of vitamin B₁₂ (15). In the second step, cobyrinic acid *a,c*-diamide was synthesized enzymatically from cobyrinic acid using cobyrinic acid *a,c*-diamide synthetase (CbiA). A solution containing 80 μ M cobyrinic acid, 50 mM Tris-HCl, pH 7.7, 20 mM KCl, 1.0 mM DTT, 1.0 mM ATP, 4.0 mM MgCl₂, and 4.0 mM glutamine was incubated with 1.0 mg of CbiA for 12 h at 30 °C. The product of the CbiA reaction was degassed for 1 h by bubbling argon through the solution. To this solution were added 1.0 mM ATP, 2.0 mM MgCl₂, 0.5 mM NADPH, 50 μ M flavin mononucleotide (FMN), 10 units of ferredoxin-NADP⁺ reductase, and 1.0 mg of CobA. The reaction mixture was incubated for 12 h at 30 °C in the dark and then loaded onto a HiTrap Q HP column (Amersham) equilibrated with 20 mM Tris-HCl, pH 7.5. The adenosyl cobyrinic acid *a,c*-diamide product was eluted with a linear gradient of NaCl (0–0.5 M) and quantified using the extinction coefficients: 21 000 M⁻¹ cm⁻¹ at 303 nm, 8500 M⁻¹ cm⁻¹ at 375 nm and 9000 M⁻¹ cm⁻¹ at 456 nm (4).

Assay of Glutamine Utilization. The glutaminase activity was quantified by determination of the glutamate produced by coupling the reaction to glutamate dehydrogenase (16). The assay was carried out in a mixture (250 μ L) containing 0.1 M Tris-HCl, pH 7.5, 1.0 mM dithiothreitol, 4.0 mM MgCl₂, 10 units of glutamate dehydrogenase, 1.0 mM 3-acetylpyridine adenine nucleotide, glutamine (0–40 mM), and 3–50 μ g of protein at 30 °C. The glutamate concentration was calculated using an extinction coefficient for the reduced form of 3-acetylpyridine adenine nucleotide of 8.3 mM⁻¹ cm⁻¹ at 363 nm (5).

Assay for ATP Utilization. The consumption of ATP was measured using a pyruvate kinase/lactate dehydrogenase coupled assay by monitoring the loss of NADH spectrophotometrically at 340 nm (16). The activity was measured at 30 °C in a solution containing 0.1 M Tris-HCl, pH 7.5, 1.0 mM DTT, 4.0 mM MgCl₂, 1.0 mM phosphoenolpyruvate, 0.44 mM NADH, 28 μ g/mL pyruvate kinase, 28 μ g/mL lactic dehydrogenase and 6–30 μ g of purified CbiP. When one substrate was varied, the other substrates (ATP, glutamine, and adenosyl cobyrinic acid *a,c*-diamide) were held constant at 4.0 mM, 20 mM and 15 μ M, respectively.

Synthesis of Cobyric Acid. The production of adenosyl cobyrinic acid was quantified by HPLC (3, 17). A solution containing 0.1 M Tris-HCl, pH 7.5, 1.0 mM DTT, 4.0 mM MgCl₂, 2.0 mM ATP, 20 mM glutamine or 100 mM ammonium chloride, 20 μ M adenosyl cobyrinic acid *a,c*-diamide, and wild type or mutant CbiP was incubated at 30 °C in the dark. At various times, a 700 μ L fraction was removed, and the reaction was quenched by heating at 80 °C for 10 min. A 0.5 mL sample of the quenched reaction mixture was loaded onto a Microsorb 100 C18 column (Varian) equilibrated in 0.1 M potassium phosphate, pH 6.5, and 10 mM KCN (buffer A). The products were eluted at a flow rate of 1 mL/min at room temperature in a mobile phase using buffer A and 0.1 M potassium phosphate, pH 8.0, 10 mM KCN, and 50% acetonitrile (buffer B). The following

gradient protocol was utilized: 0 to 2% B in 5 min, 2 to 5% B in 5 min, 5 to 20% B in 10 min, followed by 10 min of isocratic elution at 20% B, 20 to 50% B in 5 min, 50 to 100% in 5 min, and 100 to 0% B in 5 min. The elution of the cobyrinic acid and derivatives was monitored by following the absorbance at 303 nm (4).

Synthesis of Adenosyl Cobyric Acid Intermediates. Adenosyl cobyrinic acid *a,c*-diamide (100 μ M) was incubated with 25 mM Tris-HCl, pH 7.5, 25 mM [¹⁵N]-NH₄Cl, 4 mM MgCl₂, 2 mM ATP, 1 mM DTT, and 200 μ g/mL wild-type CbiP at 30 °C in the dark for various lengths of time in a volume of 1.0 mL. The reactions were quenched by lowering the pH to 5. The wild-type CbiP samples were prepared for NMR analysis by adding 50 μ L of D₂O and transferring the solution to a 5 mm NMR tube. The analysis of the D146N CbiP mutant enzyme was achieved by incubating 37.5 μ M adenosyl cobyrinic acid *a,c*-diamide, 20 mM Tris-HCl, pH 7.5, 1.0 mM [¹⁵N]-glutamine, 1 mM DTT, 4 mM ATP, 8 mM MgCl₂, and 50–200 μ g/mL D146N CbiP in a volume of 1.5 mL. The reactions were quenched by reducing the pH to 5, and the unreacted glutamine was hydrolyzed by adding 10 units of glutaminase for a period of 1 h at 30 °C in the dark. The sample was concentrated and diluted to 650 μ L with the addition of 600 μ L H₂O containing 5 units of glutaminase and 50 μ L of D₂O.

Positional Isotope Exchange Reactions. The PIX reactions catalyzed by CbiP were measured using ³¹P NMR spectroscopy with [γ -¹⁸O₄]-ATP. The assays contained 100 mM Tris-HCl, pH 7.5, 1.0 mM DTT, 2.0 mM MgCl₂, 1.0 mM [γ -¹⁸O₄]-ATP, 2.0 μ M adenosyl cobyrinic acid *a,c*-diamide, and 2.0 μ M cobyrinic acid synthetase in a volume of 1.0 mL. The reaction mixture was incubated for 2 h at 30 °C. Control assays were conducted that omitted the adenosyl cobyrinic acid *a,c*-diamide or cobyrinic acid synthetase.

NMR Acquisition Parameters. ¹H–¹⁵N heteronuclear single quantum correlation (HSQC) spectra were obtained according to the parameters outlined by Kay et al. (18). All chemical shift values are reported relative to ¹⁵NH₃. The acquisition time was 80 ms with a relaxation delay of 1.1 s. The sweep width in the proton dimension was set at 7000 and 1000 Hz in the nitrogen dimension.

Data Analysis. The kinetic parameters, *k*_{cat} and *k*_{cat}/*K*_m, were determined by fitting the experimental data to eq 1 where *v* is the initial velocity, *k*_{cat} is the turnover number, *K*_m is the Michaelis constant, *E*_t is the total enzyme concentration, and *A* is the substrate concentration. The PIX rates were fit to eq 2, where *F* = fraction of equilibrium value at time *t* and *A* = concentration of ATP.

$$v/E_t = (k_{\text{cat}}A)/(K_m + A) \quad (1)$$

$$v_{\text{ex}} = -(A/t)\ln(1 - F) \quad (2)$$

RESULTS

Expression and Purification of CbiP. Cobyric acid synthetase was expressed and purified to homogeneity. Purified CbiP eluted as a symmetric peak during chromatography and migrated as a single band during SDS-PAGE with an electrophoretic mobility equivalent to 55 kDa. This value agrees with the calculated mass of 55 055 Da derived from the DNA sequence of the *cbiP* gene. The identity of the purified protein was confirmed by sequencing the first five

Table 1: Kinetic Parameters of Wild Type and Mutants of CbiP^a

protein	k_{cat} (s ⁻¹)	ATP		Ado-cob		glutamine		ammonia	
		K_m (μM)	k_{cat}/K_m (M ⁻¹ s ⁻¹)	K_m (μM)	k_{cat}/K_m (M ⁻¹ s ⁻¹)	K_m (mM)	k_{cat}/K_m (M ⁻¹ s ⁻¹)	K_m (mM)	k_{cat}/K_m (M ⁻¹ s ⁻¹)
wt ^b	0.108 ± 0.005	41 ± 6	3000 ± 400	3.5 ± 0.5	29000 ± 1000	1.2 ± 0.3	108 ± 7	29 ± 5	3.5 ± 0.4
D146A	0.013 ± 0.001	101 ± 14	104 ± 9	5.2 ± 0.2	2300 ± 150	4.0 ± 0.6	5.0 ± 0.8	36 ± 4	0.25 ± 0.02
D146N	0.024 ± 0.003	83 ± 2	330 ± 30	3.9 ± 0.5	5100 ± 800	1.4 ± 0.2	14 ± 2	33 ± 5	0.9 ± 0.1
D165A	0.033 ± 0.003	30 ± 5	1700 ± 200	3.5 ± 0.6	11000 ± 1000	2.6 ± 0.2	5.5 ± 0.3	33 ± 2	0.80 ± 0.03
D167A	0.08 ± 0.01	31 ± 6	3300 ± 500	5.8 ± 0.8	7100 ± 550	1.8 ± 0.4	50 ± 10	26 ± 4	3.1 ± 0.4
E234A	-	-	<0.05	-	<0.05	-	<0.05	-	<0.05
D235A	-	-	<0.05	-	<0.05	-	<0.05	-	<0.05
N264A ^c	0.005 ± 0.001	39 ± 5	130 ± 20	4.3 ± 0.7	930 ± 150	-	<0.05	28 ± 5	0.20 ± 0.03
D267A ^c	0.0086 ± 0.0007	48 ± 6	250 ± 30	7.7 ± 0.8	730 ± 80	-	<0.05	30 ± 4	0.27 ± 0.02

^a Catalytic activity was determined by measurement of the initial rate of ADP formation at a fixed concentration of 4 mM ATP, 15 μM Ado-cob, or 20 mM Gln. ^b The Ado-cob parameters were obtained using ammonia as the nitrogen source. ^c The ATP and Ado-cob parameters were obtained using ammonia as the nitrogen source.

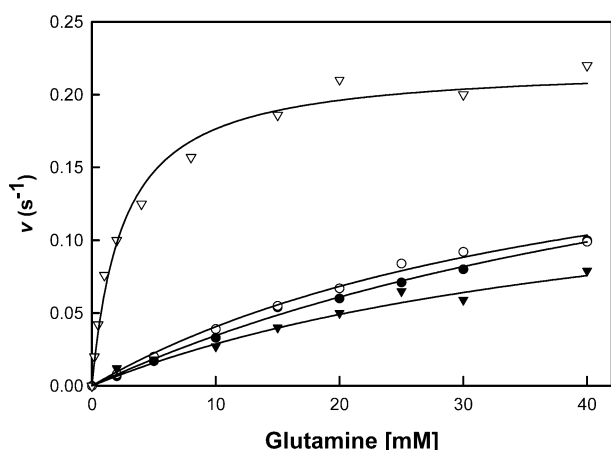


FIGURE 1: Saturation curves for the hydrolysis of glutamine by CbiP in the presence and absence of other substrates. (○) Absence of any other substrates; (●) presence of 4 mM ATP; (▼) presence of 15 μM adenosyl cobyrinic *a,c*-diamide; (▽) presence of 4 mM ATP and 15 μM adenosyl cobyrinic *a,c*-diamide. The data were fit using eq 1.

amino acid residues at the N-terminus. The sequence of the purified protein, TQAVM, corresponds with the predicted sequence for the first five amino acids of CbiP.

Initial Velocity Studies. The kinetic parameters for the substrates (ATP, adenosyl cobyrinic acid *a,c*-diamide, glutamine, and ammonia) were obtained by measuring the initial rate of ADP formation at a fixed concentration of the other two substrates. The calculated values from fits of the data to eq 1 are presented in Table 1. The k_{cat} for the utilization of ATP is similar when either glutamine or ammonia is used as the nitrogen source. However, the K_m for glutamine is significantly lower than it is for ammonia which indicates that glutamine is the preferred nitrogen source.

The majority of the characterized glutamine amidotransferases are able to hydrolyze glutamine in the absence of the other substrates (4, 19–21). Figure 1 illustrates the glutaminase activity of CbiP in the presence or absence of ATP and/or adenosyl cobyrinic acid *a,c*-diamide. CbiP hydrolyzes glutamine in the absence of the other two substrates, although the catalytic efficiency is measurably lower. The incubation of the enzyme with either ATP or adenosyl cobyrinic acid *a,c*-diamide alone has essentially no effect on the rate of glutamine hydrolysis. However, the presence of ATP and adenosyl cobyrinic acid *a,c*-diamide together dramatically increases k_{cat}/K_m for the hydrolysis of

glutamine by a factor of 60. The value of k_{cat} calculated from the production of glutamate in the presence of all the substrates is 2.5 times higher than the rate constant obtained by monitoring the rate of ADP formation or by HPLC analysis of the cobyrinic acid product. This result demonstrates that the hydrolysis of glutamine and carboxamide bond synthesis are partially uncoupled from one another. The kinetic constants for the hydrolysis of glutamine in the presence and absence of ATP and adenosyl cobyrinic acid *a,c*-diamide are presented in Table 2.

Positional Isotope Exchange Reaction. The proposed reaction mechanism for the amidation of adenosyl cobyrinic acid *a,c*-diamide involves the formation of phosphorylated substrate intermediates as presented in Scheme 2. To test whether this intermediate can form prior to the delivery of ammonia to the active site, CbiP was incubated with [γ -¹⁸O₄]-ATP in the presence and absence of the adenosyl cobyrinic acid *a,c*-diamide substrate. If the ATP is functionally able to phosphorylate the substrate and form ADP, then the ¹⁸O-label at the $\beta\gamma$ -bridging position will become torsionally equivalent with the two β -nonbridging ¹⁶O-atoms of ADP. Re-formation and dissociation of ATP from the active site will then generate ATP with ¹⁸O at either of the two β -nonbridge positions. The positional isotope exchange reaction is outlined in Scheme 3. This methodology was first developed by Midelfort and Rose for interrogation of the formation of a γ -glutamyl phosphate intermediate in the glutamine synthetase reaction (22).

The PIX reaction was monitored by measuring the relative fraction of the [γ -¹⁸O₄]-ATP species by ³¹P NMR spectroscopy. The ³¹P NMR spectrum of the initial [γ -¹⁸O₄]-ATP is shown in Figure 2A. The doublet, labeled 4 in Figure 2A, represents the γ -P species with four ¹⁸O atoms. The doublet-labeled 3 in Figure 2A represents the γ -P species with three ¹⁸O atoms and one ¹⁶O atom. When the labeled ATP, adenosyl cobyrinic acid *a,c*-diamide, and CbiP were incubated together for 2 h, the fraction of the γ -¹⁸O₄ species decreased from 0.66 to 0.53 as depicted in Figure 2B. The decrease in the relative amount of the γ -¹⁸O₄ species is 28% of the value at isotopic equilibrium. The calculated PIX rate from a fit of the data to eq 2 is 0.023 s⁻¹. Incubation of the labeled ATP with adenosyl cobyrinic acid *a,c*-diamide or cobyrinic acid synthetase alone did not result in any positional isotope exchange.

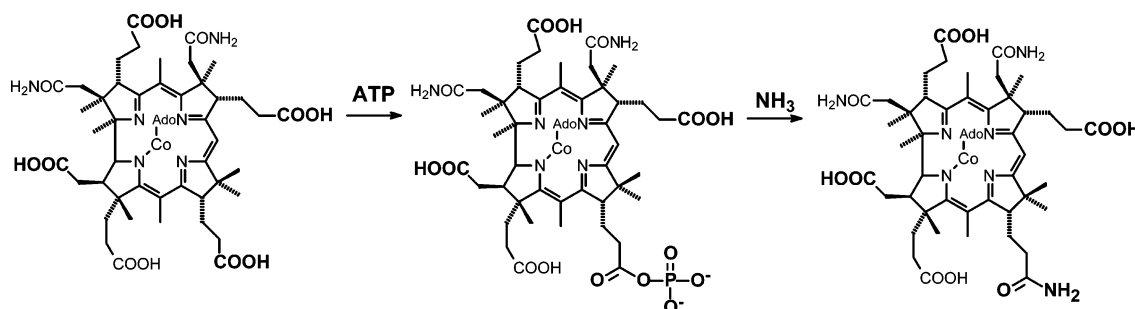
Site-Directed Mutagenesis. We have previously demonstrated that the four carboxylates of adenosyl cobyrinic acid

Table 2: Kinetic Parameters for the Hydrolysis of Glutamine by CbiP^a

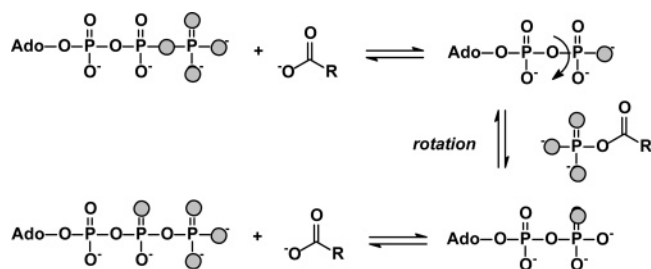
protein	none			ATP + Ado-cob		
	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ s ⁻¹)	K_a (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ s ⁻¹)
wild type	120 ± 14	0.32 ± 0.01	2.7 ± 0.1	1.8 ± 0.2	0.29 ± 0.01	160 ± 16
D146A	106 ± 22	0.15 ± 0.01	1.4 ± 0.1	4.2 ± 0.3	0.10 ± 0.01	24 ± 2.0
D146N	109 ± 12	0.13 ± 0.01	1.2 ± 0.1	1.6 ± 0.2	0.11 ± 0.01	69 ± 4.0
D165A	98 ± 24	0.11 ± 0.02	1.6 ± 0.2	2.8 ± 0.3	0.19 ± 0.01	71 ± 9.0
D167A	79 ± 15	0.25 ± 0.01	3.2 ± 0.1	1.6 ± 0.2	0.20 ± 0.01	125 ± 10
E234A	77 ± 12	0.046 ± 0.008	0.6 ± 0.1	5.4 ± 1.0	0.010 ± 0.001	1.8 ± 0.3
D235A	70 ± 5	0.175 ± 0.007	2.5 ± 0.1	18 ± 4	0.020 ± 0.002	1.1 ± 0.2
N264A	110 ± 16	0.10 ± 0.01	0.9 ± 0.1	16 ± 3	0.13 ± 0.01	8.1 ± 1.2
D267A	35 ± 3	0.018 ± 0.004	0.5 ± 0.1	17 ± 2	0.029 ± 0.001	1.7 ± 0.2

^a Kinetic parameters for the hydrolysis of glutamine in the absence or presence of 4.0 mM ATP and 15 μM Ado-cob.

Scheme 2



Scheme 3



a,c-diamide are amidated in a specific order beginning with carboxylate *e* and proceeding in turn to *d*, *b*, and finally *g* (6). The structural basis for this specific regiochemical pathway is unknown, and no crystal structure is currently available for cobyrinic acid synthetase. However, a structural model for the synthetase domain of CbiP has been proposed by Galperin and Grishin based upon the amino acid sequence identity to dethiobiotin synthetase (6). A modification of this homology model is presented in Figure 3. The carboxylate groups *b*, *d*, *e* and *f* are facing to one side of the ring, carboxylates *a* and *c* project out from the opposite face of the corrin ring system, while the *g* carboxylate is roughly in the plane of the molecule (see Scheme 1). This structure suggests that carboxylates *b*, *d*, and *e* could bind to the same location within the active site by a simple 90° rotation of the corrinoid ring about an axis perpendicular to the ring. The spacing between carboxylate *f* and the carboxylates *b*, *d*, and *e* is different than is the spacing among these three carboxylates, and this explains in part why carboxylate *f* is not amidated by CbiP. The asymmetry imposed by the spatial orientation of carboxylate *g* is proposed as the key for why the series of amidation reactions is initiated with residue *e*.

With the wild type enzyme, carboxylate *d* is not amidated until after carboxylate *e* is amidated. Therefore, the enzymatic transformation of the anionic carboxylate *e* to a neutral amide may enable the substrate to now bind in an orientation that

permits the amidation of carboxylate *d* via a rotation of ~90°. If there was a negatively charged amino acid residue within the active site that was spatially distant from the site of the amidation reaction, then charge repulsion may effectively dictate how the initial substrate must bind within the active site. Once carboxylate *e* has been amidated, the constraints to binding in an orientation that allows carboxylate *d* to be amidated are relieved. The subsequent amidation of carboxylate *d* enables carboxylate *b* to be amidated and eventually carboxylate *g* is amidated. The structural misalignment of carboxylate *g* may allow the process to be initiated at carboxylate *e*. If this scenario is correct then there should be a highly conserved glutamate or aspartate in the active site that functions as the gatekeeper for dictating the specific regiochemical pathway.

A sequence alignment of 34 bacterial cobyrinic acid synthetases shows seven residues that are universally conserved as either a glutamate or aspartate within the synthetase domain of CbiP. These residues are Glu-54, Glu-132, Glu-139, Asp-146, Glu-234, Asp-235, and Asp-267. In addition to these residues, Asp-165 and Asp-167 are essentially conserved except for a single example in each case of an asparagine substitution. Glu-54 and Glu-132 are not in the homology model of the active site nor do they appear to be in position to interact with MgADP or adenosyl cobyrinic acid *a,c*-diamide. Glu-234 and Asp-235 may coordinate the 3'-hydroxyl group of the ribose moiety of ADP and the metal ion, respectively, but a direct interaction with adenosyl cobyrinic acid *a,c*-diamide is not obvious from the model. Asp-165 and Asp-167 are apparently not in the active site nor do they appear to be in proximity to interact with MgATP or adenosyl cobyrinic acid *a,c*-diamide. Asp-146 and Glu-139 are located on a loop that is close to the adenosyl cobyrinic acid *a,c*-diamide substrate and could interact with the substrate upon binding. Glu-139 has previously been postulated to function as an axial ligand to the cobalt of the

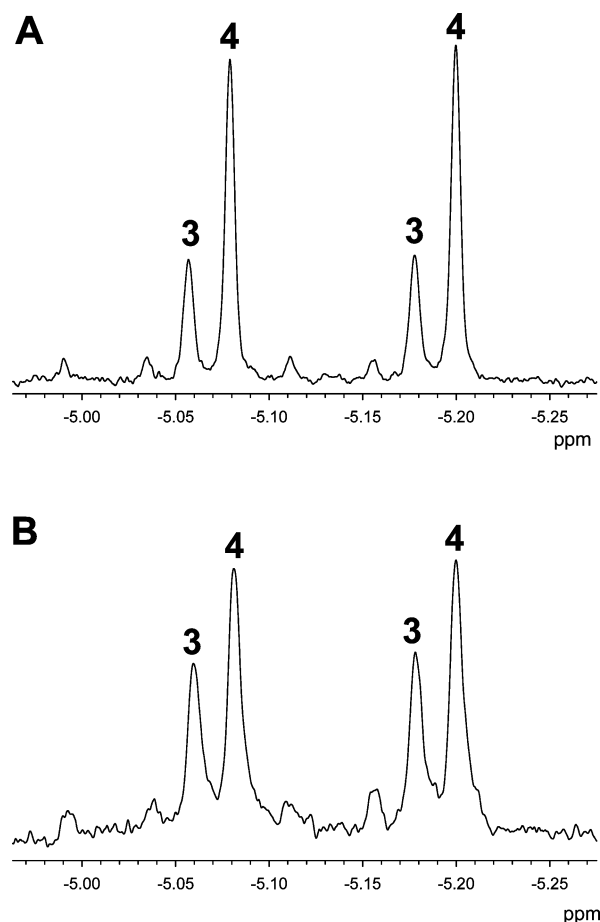


FIGURE 2: ^{31}P NMR spectra for the action of CbiP with $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$. (A) $\gamma\text{-}^{31}\text{P}$ resonance of $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ in the absence of CbiP. (B) $\gamma\text{-}^{31}\text{P}$ resonance of $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ after incubation with CbiP and 2 μM adenosyl-cobyrinic acid *a,c*-diamide for 2 h. Additional details are provided in the text.

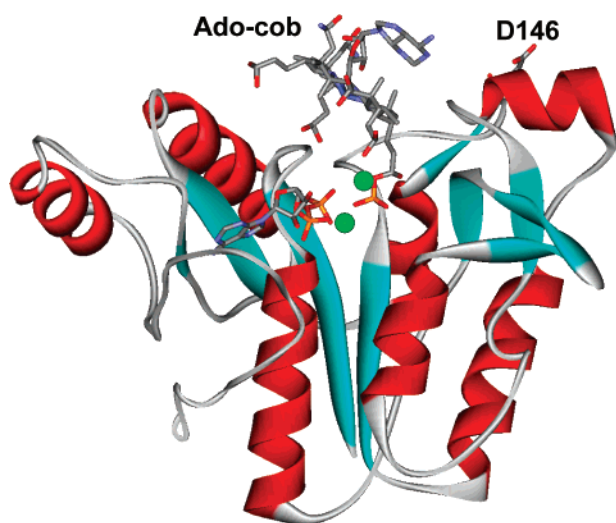


FIGURE 3: Homology model of CbiP based on the crystal structure of dethiobiotin synthetase (PDB code: 1DAK) (6). The side chain carboxylate for D146 is shown in a stick format. The position of the Ado-cob is modeled in the active site based upon the binding inhibitors to the active site of dethiobiotin synthetase (6).

substrate (7). To investigate if any of these amino acids function in the determination of the observed regiochemical pathway, the alanine mutants of the residues Asp-146, Asp-165, Asp-167, Glu-234, Asp-235, and Asp-267 were constructed and characterized. In this study the conserved Asn-

264 was mutated to an alanine and the asparagine mutant of Asp-146 was also constructed.

Synthesis of Cobyric Acid by CbiP Mutants. The relative catalytic activity of the CbiP mutants was quantified by measuring the rate of formation of the cobyrinic acid product via HPLC using both glutamine and ammonia as nitrogen sources. The alanine substitutions of Glu-234 and Asp-235 have dramatic effects on the enzymatic activity. The amidated products could not be detected when glutamine was used as the substrate, and a significant drop in catalytic activity was observed when the reaction was initiated with ammonia as the nitrogen source. The mutants D146A, D146N, D165A, and D167A have a more moderate effect on the catalytic activity that is independent of the nitrogen source. The mutant D267A has lost a substantial amount of activity with either ammonia or glutamine as the nitrogen source. The kinetic parameters for the substrates ATP, adenosyl cobyrinic acid *a,c*-diamide, glutamine and ammonia are presented in Tables 1 and 2.

Effect of CbiP Mutations on the Amidation Order. The time course of the reaction catalyzed by wild type CbiP shows three partially amidated intermediates which accumulate and then decrease as the reaction progresses (6). This distribution pattern for the product and the partially amidated intermediates is the result of the sequential and dissociative mechanism catalyzed by CbiP. The enzyme catalyzes one amidation reaction during each catalytic cycle. The intermediate is released from the active site and then rebinds to the enzyme in a different orientation for the next amidation reaction. For the mutants, only small differences in the kinetic parameters were observed with the exception of E234A and D235A, as shown in Tables 1 and 2. The HPLC chromatograms for the mutants D165A, D167A, and D267A exhibit similar distributions of the partially amidated intermediates as obtained for the wild-type CbiP (Figure 4). However, the chromatograms for the two D146 mutants are substantially different from that observed for the wild type enzyme. At early incubation times with the D146N mutant, an additional peak is observed with a retention time that is similar to that observed for the first partially amidated intermediate with the wild type enzyme. For the D146A mutant, a third peak is observed with a retention time slightly longer than the original triamide intermediate species.² A comparison of the HPLC assays for the wild type, D146N, and D146A mutants is shown in Figure 5. The exact masses of the corrinoid intermediates contained in these peaks were measured with a PE Sciex APJ Qstar Pulsar mass spectrometer. A mass of 935.5 amu was obtained for the peak labeled **a** in Figure 5. The mass is consistent with the conversion of a single carboxyl group to an amide. Therefore, this

² In the text and in the legends to the figures the substrate, product and partially amidated intermediates in the reaction catalyzed by CbiP are designated by the total number of carboxamide groups. Thus, the substrate, adenosyl cobyrinic *a,c*-diamide, is referred to as the diamide, while the final product, cobyrinic acid, is referred to as the hexamide. The three partially amidated intermediates are denoted as the triamide, tetraamide, and pentamide, respectively. For the partially amidated intermediates, the sites of amidation are labeled by the appropriate lowercase letters, as in the example, *de*-tetraamide. In the discussion of the partially amidated intermediates via ^{15}N NMR spectroscopy the specific amide whose chemical shift is being noted will be boldfaced, as in the example, *deg*-pentamide, for the resonance of the pentamide that resonates at 111.3 ppm.

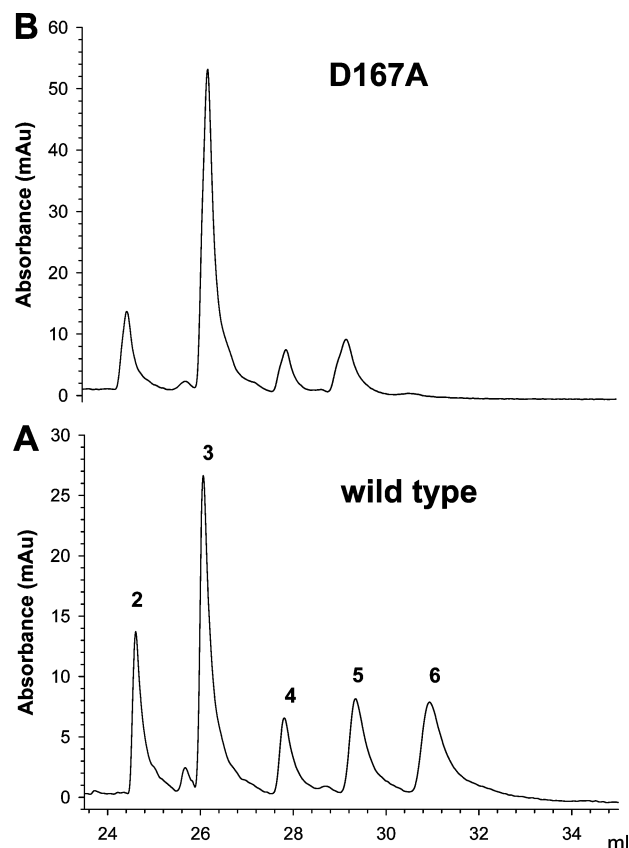


FIGURE 4: HPLC traces for the production of coobyric acid catalyzed by the wild type and D167A mutant of CbiP. (A) HPLC separation of the three partially amidated intermediates catalyzed by the wild type CbiP. (B) HPLC separation of the three partially amidated intermediates and coobyric acid catalyzed by the D167A. The peak labeled as **2** is the adenosyl coobyric acid *a,c*-diamide substrate. Peaks labeled as **3**, **4**, and **5** are the triamide, tetramide, and pentamide intermediates; peak **6** is the final product, coobyric acid.

compound is a triamide intermediate that is different than the triamide intermediate formed by the wild type enzyme since the retention time is different. An identical mass was found for the peak labeled as **b** in Figure 5 in the chromatogram for the D146A mutant. These results demonstrate that the peaks labeled as **a**, **3**, and **b** in the time courses of the mutant enzymes are triamide intermediates. This observation demonstrates that mutation of D146 to either alanine or asparagine perturbs the order of the amidation reaction and causes some randomization.

The identity of the partially amidated intermediates present in the reaction catalyzed by the D146N mutant was investigated by utilizing ^{15}N -glutamine as the nitrogen source. At various times, samples of the reaction mixture catalyzed by the D146N mutant were prepared and the corresponding 2D ^1H - ^{15}N HSQC NMR spectra were obtained. The first sample was quenched early in the reaction cycle, and two triamide species were identified by HPLC as shown in Figure 6A. The NMR spectrum confirms the presence of two distinct triamide species as illustrated in Figure 7A. The ^{15}N chemical shift values of these two species are 108.6 and 109.3 ppm for the upfield and downfield resonances, respectively. The previous NMR experiments with the wild-type CbiP established a chemical shift of 109.2 ppm for the *e*-triamide (**5**). Thus, the resonance at 109.3 ppm must represent the *e*-triamide species labeled as **3** in Figure 6A. The resonance

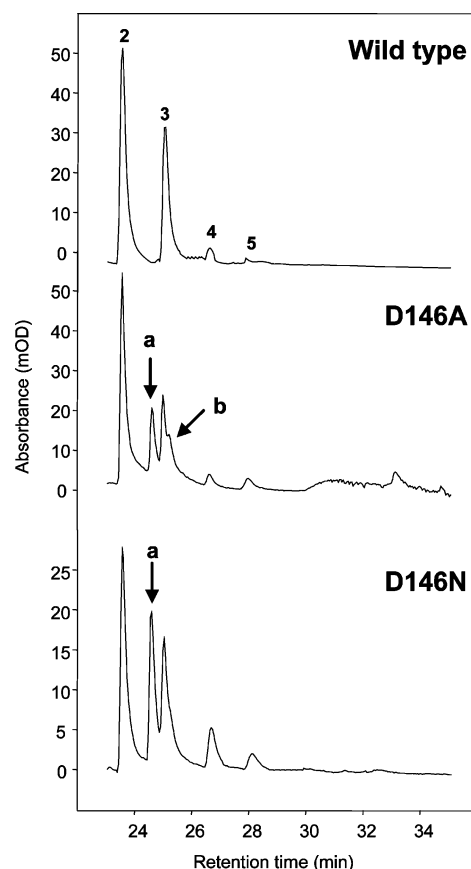


FIGURE 5: HPLC traces showing the variation in the identity of the reaction intermediate catalyzed by the wild type CbiP and two mutants, D146N and D146A. The labels **2**, **3**, **4**, and **5** are the same as found in the legend to Figure 4. The labels **a** and **b** represent the *d*-triamide and *g*-triamide intermediates, respectively.

at 108.6 ppm is in the range of reported values for intermediates amidated at carboxylate *d* but differs from the chemical shift of carboxamide *d* of the *de*-tetramide intermediate species observed in the wild-type CbiP reaction which resonates at 108.2 ppm (**6**). Therefore, the resonance at 108.6 ppm must represent the *d*-triamide intermediate formed by the D146N mutant which is amidated at the *d* carboxylate. This NMR spectrum confirms the formation of multiple triamide species catalyzed by the D146N mutant.

Formation of two different triamide species at carboxylates *e* and *d* suggests that either multiple species will be observed for the tetramide and pentamide intermediates or, alternatively, the two triamide species will be amidated so that a common tetramide or pentamide intermediate is produced. A sample prepared at a later incubation time is shown in Figure 6B. Multiple peaks for the tetramide or pentamide intermediates are not observed. However, another peak (**b** in Figure 6B) with a retention time longer than the *e*-triamide intermediate is evident. The lack of multiple peaks in the HPLC for the tetramide and pentamide species suggests that the two triamide species converge to a common tetramide species or that the retention times for the multiple tetramide species are essentially identical. The NMR spectrum of this sample is shown in Figure 7B. The spectrum shows five resonances in the regions normally observed for the carboxamides *d*, *e*, and *g*. The resonances at 108.6 and 108.3 ppm are in the range where the *d* carboxamides resonate. The peak at 108.6 ppm has been assigned as the *d*-triamide

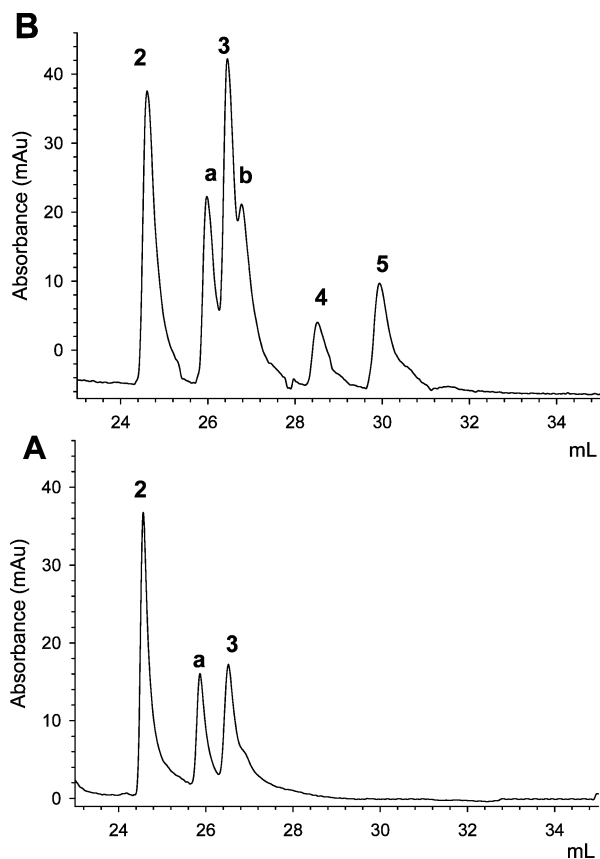


FIGURE 6: HPLC traces for the formation of reaction intermediates by the D146N mutant of CbiP. (A) Sample was incubated with 50 $\mu\text{g/mL}$ of D146N for 60 min. The *d*-triamide (a) and *e*-triamide (3) species are formed in a ratio of approximately 1:1. (B) Sample incubated with 200 $\mu\text{g/mL}$ of D146N for 30 min showing the triamide (a, 3, and b), tetraamide (4) and pentamide (5) species in the ratio of approximately 74:9:17.

species from the previous NMR spectrum in Figure 7A. The resonance at 108.3 ppm is identical to the resonance observed for the *de*-tetramide species in the wild-type CbiP reaction (6) and would also include the *d* carboxamide of the *deg*-pentamide intermediate. The chemical shift range of 109–110 ppm is the region where the *e* carboxamides resonate. The resonance at 109.3 ppm was observed in the NMR spectrum shown in Figure 7A. This resonance has previously been assigned to the *e*-triamide species. The resonance at 109.2 ppm is the *e* amide of the *de*-tetramide and *deg*-pentamide intermediate species. The chemical shift range for the *g* amides is between 111 and 112 ppm. Thus, the resonance(s) at 111.3 ppm in Figure 7B potentially represents the *g*-triamide, *dg*- and *eg*-tetramides, and *deg*-pentamide intermediates that are amidated at position *g*. The absence of any observable resonances in the region for species amidated at position *b* establishes that the pentamide intermediate is exclusively amidated at positions *d*, *e*, and *g* and that carboxylate *b* is amidated last in the reaction catalyzed by the D146N mutant.

The small chemical shift change among the amides in the different intermediate species is caused by amidation of the adjacent carboxylic acid. In the reaction catalyzed by wild-type CbiP, the chemical shift of the *e*-triamide species changes from 109.2 to 109.1 ppm in the *de*-tetramide species (6). There is a small chemical shift change for carboxamide *d* in the *de*-tetramide species compared with the *bde*-

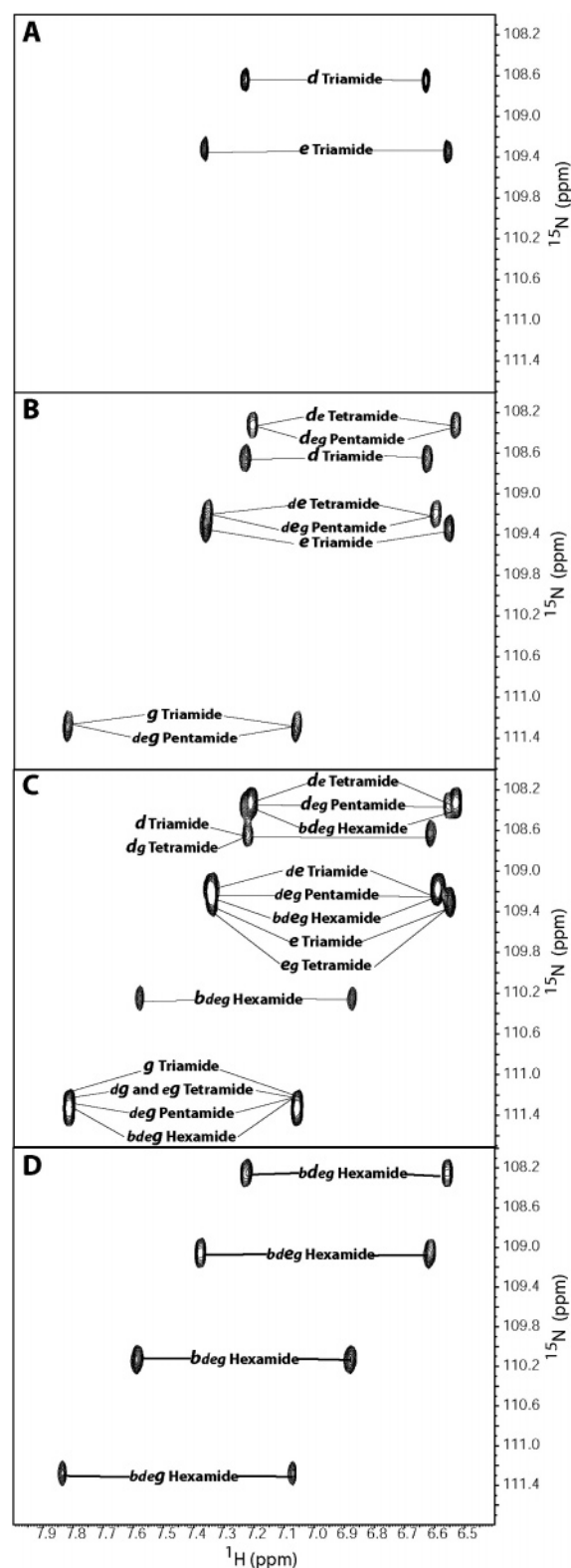


FIGURE 7: ^1H – ^{15}N HSQC 2D NMR spectra for cohyric acid and the various intermediates catalyzed by the D146N mutant of CbiP in the presence of ^{15}N -glutamine. (A) The *d*- and *e*-triamide intermediates in a ratio of approximately 1:1 (see Figure 6A). (B) The NMR spectrum for the reaction mixture when the ratio of the total triamide, tetraamide, and pentamide intermediates are approximately 74:9:17 (see Figure 6B). (C) The NMR spectrum for the reaction mixture when the ratio of the total triamide, tetraamide, pentamide, and cohyric acid are approximately 4.7:2.3:1.8:1.0. (D) NMR spectrum of the product, cohyric acid. Additional details are provided in the text.

pentamide intermediate. Changes in chemical shift for carboxamides *d* or *e* were not observed upon amidation of carboxylate *g* in the *bdeg*-cobyric acid product, relative to the chemical shifts in the *bde*-pentamide intermediate. This result suggests that amidation of carboxylate *g* has no effect on the chemical shift value for either carboxamide *d* or *e*. Therefore, the resonance at 108.3 ppm represents *de*-tetramide and *deg*-pentamide species. Likewise, the resonance at 109.2 ppm represents the *de*-tetramide and *deg*-pentamide species.

Integration of the chromatogram in Figure 6B for the sum of the triamide, tetramide, and pentamide intermediates gives the percentages of these species as 74, 9, and 17%, respectively. From these percentages, the approximate contributions of these intermediates to the NMR spectrum can be calculated. The triamide, tetramide, and pentamide intermediates will therefore contribute approximately 52, 13, and 36%, respectively, to the total NMR integration. The peak volumes for each of the five NMR signals at 108.3, 108.6, 109.2, 109.3, and 111.3 ppm are 20, 21, 20, 19, and 20% of the total integration.

The resonance at 111.3 ppm contains all of the signals for any species amidated at position *g*. This potentially includes the *g*-triamide, *dg*- and *eg*-tetramide, and *deg*-pentamide species. It has previously been concluded that amidation at position *g* has no effect on the ^{15}N chemical shift observed for carboxamides *d* or *e* (6). Thus the *dg*- and *eg*-tetramide species will appear together with the resonance for the *d*-triamide (108.6 ppm) and the *e*-triamide (109.3 ppm), respectively. Similarly, the resonances for the *deg*-pentamide intermediate will contribute to the signal at 108.3 ppm with the *de*-tetramide signal and at 109.2 ppm with the *de*-tetramide. The *deg*-pentamide species also contributes to the resonance observed at 111.3 ppm. Since the *deg*-pentamide intermediate contributes ~36% of the total NMR signal, then 12% each of this intermediate is contributing to the NMR resonances at 108.3, 109.2, and 111.3 ppm. The NMR resonances at 108.3 and 109.2 are also contributed by the *de*- and *de*-tetramide species and thus each of these intermediates will contribute to ~8% of the total NMR integration (20% - 12%). Therefore, based on the NMR data, the contribution of the *de*-tetramide species is ~16% (8% + 8%) of the total NMR signal. From the HPLC integration the expected sum of the NMR integration for all of the possible tetramide species ~13%, and thus it can be concluded that essentially none of the *eg*- or *dg*-tetramide species are present in the sample corresponding to the HPLC shown in Figure 6B. The *deg*-pentamide intermediate contributes 12% of the total NMR integration at 111.3 ppm and thus the remaining 8% (20% to 12%) must originate from the *g*-triamide intermediate. The *g*-triamide must be the identity of the peak labeled as **b** in Figure 6B. Therefore, the *dg*- and *eg*-tetramide species must be formed later in the reaction but are not represented in the NMR spectrum shown in Figure 7B.

Carboxylate *b* is the last position to be amidated. An NMR spectrum is shown in Figure 7C for a sample that was quenched near the end of the reaction cycle. This spectrum confirms the amidation of carboxylate *b* as the last step in the reaction catalyzed by D146N CbiP. Figure 7D displays the NMR spectrum of the final product, cobyrinic acid (*bdeg*-hexamide), obtained with wild-type CbiP showing the

resonance positions for the carboxamides *d*, *e*, *b*, and *g* at 108.2, 109.1, 110.1, and 111.3 ppm, respectively (6).

DISCUSSION

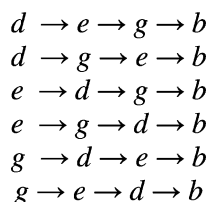
The formation of cobyrinic acid in the vitamin B₁₂ biosynthetic pathway is completed by adenosylation and amidation of six carboxylic acid groups in cobyrinic acid. Cobyrinic acid *a,c*-diamide synthetase (CobB from *P. denitrificans* and CbiA from *S. typhimurium*) catalyzes the amidation of carboxylate groups *a* and *c* of cobyrinic acid which is followed by cobalt reduction and adenosylation. Cobyrinic acid synthetase (CobQ from *P. denitrificans* and CbiP from *S. typhimurium*) catalyzes the amidation of carboxylate groups *b*, *d*, *e*, and *g* on adenosyl cobyrinic acid *a,c*-diamide (1). Previous experiments from this laboratory have shown that the four carboxylates are amidated in the specific order of *e*, *d*, *b*, and *g* in a dissociative process (6). Carboxylate group *f* is ultimately amidated with aminopropanol.

Glutamine Hydrolysis. CbiP is capable of hydrolyzing glutamine in the absence of adenosyl cobyrinic acid *a,c*-diamide and ATP. However, the presence of ATP and adenosyl cobyrinic acid *a,c*-diamide enhances the catalytic efficiency for glutamine hydrolysis by a factor of 60. The formation of glutamate is 2.5 times higher than the rate of ADP formation, and thus the hydrolysis of glutamine is partially uncoupled from the amidation of the carboxylic acid groups. This observation is not uncommon for some of the other amidotransferase enzymes that utilize glutamine as a direct precursor for the generation of ammonia. Previously, partially uncoupled reactions were observed for cobyrinic acid *a,c*-diamide synthetase (CbiA) from *S. typhimurium* and asparagine synthetase B from *E. coli* (5, 23). In contrast, fully coupled reactions were observed for carbamoyl phosphate synthetase and glutamine phosphoribosylpyrophosphate amidotransferase from *E. coli* (24, 25).

Substrate Activation by ATP. A phosphorylated carboxylate intermediate is proposed as the functional group attacked by the ammonia during amide bond formation (Scheme 3). Experimental support for the phosphorylated intermediate in the CbiP reaction has been obtained by the observation of a positional isotope exchange within $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$. This exchange reaction occurs only in the presence of CbiP, labeled ATP, and the substrate, adenosyl cobyrinic acid *a,c*-diamide. This exchange reaction cannot occur unless the bond between the γ -phosphoryl group and the $\beta\gamma$ -bridge oxygen is broken. In addition, the terminal phosphoryl group of the resulting ADP must be free to rotate and the ATP must reform and dissociate from the active site. No PIX activity was observed when CbiP was incubated with $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ alone which demonstrates that a phosphorylated intermediate can only form in the presence of substrate. These results also demonstrate that a phosphorylated enzyme intermediate does not form and that the proposed phosphorylated substrate intermediate can form prior to the binding of ammonia or glutamine.

Cobyric Acid Synthesis by CbiP Mutants. The functional role of conserved aspartate and glutamate residues within the N-terminal domain of CbiP was assessed by site-directed mutagenesis. The mutation of D146, D165, and D167 with alanine substitutions did not result in significant changes in the catalytic activity of CbiP. The mutants N264A and

Scheme 4



D267A do not form cobyrinic acid when glutamine is used as a source of ammonia. Mutating E234 or D235 resulted in enzymes that are unable to form amidated products using either glutamine or ammonia. However, all of the mutants were able to hydrolyze glutamine, and thus these changes have not disrupted the ability of CbiP to generate ammonia at sufficient rates. The lack of carboxamide formation with the N264A and D267A mutants with glutamine as the nitrogen source indicates that the synthetase domain and the hydrolase domain have become completely uncoupled from one another.

Amidation Order of Mutants. The wild type enzyme catalyzes the amidation of four carboxylates from the substrate in the order of *e*, *d*, *b*, and *g*, and three partially amidated intermediates can be identified by HPLC as the *e*-triamide, *de*-tetramide, and *bde*-pentamide. The strict amidation order can be disrupted by the mutation of the conserved residue D146 and multiple intermediates are identified using HPLC and NMR spectroscopy. In the first catalytic cycle the *e*-, *d*-, and *g*-triamides have been identified in significant amounts and these three triamide intermediates converge to a common *deg*-pentamide intermediate prior to amidation at carboxylate *b* to form the ultimate product, cobyrinic acid. There are 24 possible pathways for the amidation of carboxylate groups *b*, *d*, *e*, and *g* during the formation of cobyrinic acid. The evidence presented here establishes that carboxylate *b* is amidated last. This observation eliminates 18 of the 24 possible pathways, and the remaining pathways are shown in Scheme 4. The predominant amidation pathway for the D146N mutant is *d*, *e*, *g*, and *b*. A structural model of CbiP was constructed based on homology to dethiobiotin synthetase (7). In this model, D146 is located on a loop together with E139 above the ATP and adenosyl cobyrinic acid *a,c*-diamide binding pocket. These residues may dictate how the initial substrate binds within the active site and determine the order of the subsequent amidation reactions.

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REFERENCES

- Scott, A. I., and Roessner, C. A. (2002) Biosynthesis of cobalamin (vitamin B₁₂), *Biochem. Soc. Trans.* 30, 613–620.
- Luo, J., and Lim, C. K. (1993) Order of uroporphyrinogen III decarboxylation on incubation of porphobilinogen and uroporphyrinogen III with erythrocyte uroporphyrinogen decarboxylase, *Biochem. J.*, 289, 529–532.
- Debussche, L., Thibaut, D., Cameron, B., Crouzet, J., and Blanche, F. (1990) Purification and characterization of cobyrinic acid *a,c*-diamide synthase from *Pseudomonas denitrificans*, *J. Bacteriol.* 172, 6239–6244.
- Blanche, F., Couder, M., Debussche, L., Thibaut, D., Cameron, B., and Crouzet, J. (1991) Biosynthesis of vitamin B₁₂: stepwise amidation of carboxyl groups *b*, *d*, *e*, and *g* of cobyrinic acid *a,c*-diamide is catalyzed by one enzyme in *Pseudomonas denitrificans*, *J. Bacteriol.* 173, 6046–6051.
- Fresquet, V., Raushel, F. M., and Williams, L. (2004) Mechanism of cobyrinic acid *a,c*-diamide synthetase from *Salmonella typhimurium* LT2, *Biochemistry* 43, 10619–10627.
- Williams, L., Fresquet, V., Santander, P. J., and Raushel, F. M. (2007) The multiple amidation reactions catalyzed by cobyrinic acid synthetase from *Salmonella typhimurium* are sequential and dissociative, *J. Am. Chem. Soc.* 129, 294–295.
- Galperin, M. Y., and Grishin, N. V. (2000) The synthetase domains of cobalamin biosynthesis amidotransferases cobB and cobQ belong to a new family of ATP-dependent amidoligases, related to dethiobiotin synthetase, *Proteins* 41, 238–247.
- deBoer, P. A., Crossley, R. E., Hand, A. R., and Rothfield, L. I. (1991) The MinD protein is a membrane ATPase required for the correct placement of the *Escherichia coli* division site., *EMBO J.* 10, 4371–4380.
- Thoden, J. B., Holden, H. M., Wesenberg, G., Raushel, F. M., and Rayment, I. (1997) Structure of carbamoyl phosphate synthetase: a journey of 96 Ångströms from substrate to product, *Biochemistry* 36, 6305–6316.
- Tesmer, J. G., Klem, T. J., Deras, M. L., Davisson, V. J., and Smith, J. L. (1996) The crystal structure of GMP synthetase reveals a novel catalytic triad and is a structural paradigm for two enzyme families, *Nature* 3, 74–86.
- Raux, E., Lanois, A., Levillayer, F., Warren, M., Brody, E., Rambach, A., and Thermes, C. (1996) *Salmonella typhimurium* cobalamin (vitamin B₁₂) biosynthetic genes: functional studies in *S. typhimurium* and *Escherichia coli*, *J. Bacteriol.* 178, 753–767.
- Risley, J. M., and Van Etten, R. L. (1978) A convenient synthesis of crystalline potassium phosphate-¹⁸O₄ (monobasic) of high isotopic purity, *J. Labelled Compd. Radiopharm.* 15, 533–538.
- Werhli, W. E., Verheyden, D. L. M., and Moffatt, J. G. (1965) Dismutation reactions of nucleoside polyphosphates. II. Specific chemical syntheses of α -, β -, and γ -³²P-nucleoside 5'-triphosphates, *J. Am. Chem. Soc.* 87, 2265–2277.
- Gill, S. C., and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data, *Anal. Biochem.* 182, 319–326.
- Schlingmann, G., Dresow, B., Koppenhagen, V., and Ernst L. (1980) Preparation and structure determination of dicyanocobyrinic methyl ester amides and correlation of their ¹³C NMR data, *Liebigs Ann. Chem.* 1186–1197.
- Miles, B. W., Thoden, J. B., Holden, H. M., and Raushel, F. M. (2002) Inactivation of the amidotransferase activity of carbamoyl phosphate synthetase by the antibiotic acivicin, *J. Biol. Chem.* 277, 4368–4373.
- Blanche, F., Thibaut, D., Couder, M., and Muller, J.-C. (1990) Identification and quantitation of corrinoid precursors of cobalamin from *Pseudomonas denitrificans* by high-performance liquid chromatography, *Anal. Biochem.* 189, 24–29.
- Kay, L., Keifer, P., and Saarinen, T. (1992) Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity, *J. Am. Chem. Soc.* 114, 10663–10665.
- Fresquet, V., Thoden, J. B., Holden, H. M., and Raushel, F. M. (2004) Kinetic mechanism of asparagine synthetase from *Vibrio cholerae*, *Bioorg. Chem.* 32, 63–75.
- Miles, B. W., Banzon, J. A., and Raushel, F. M. (1998) Regulatory control of the amidotransferase domain of carbamoyl phosphate synthetase, *Biochemistry* 37, 16773–16779.
- Boehlein, S. K., Stewart, J. D., Walworth, E. S., Thirumoorthy, R., Richards, N. G. J., and Schuster, S. M. (1998) Kinetic mechanism of *Escherichia coli* asparagine synthetase B, *Biochemistry* 37, 13230–13238.
- Midelfort, C. F., and Rose, I. A. (1976) A stereochemical method for detection of ATP terminal phosphate transfer in enzymatic reactions, *J. Biol. Chem.* 251, 5881–5887.
- Li, K. K., Beeson, W. T., Ghiviriga, I., and Richards, N. G. J. (2007) A Convenient gHMQC-based NMR assay for investigating ammonia channeling in glutamine-dependent amidotransferases: studies of *Escherichia coli* asparagine synthetase B, *Biochemistry* 46, 4840–4849.

24. Kim, J. H., Krahn, J. M., Tomchick, D. R., Smith, J. L., and Zalkin, H. (1996) Structure and function of the glutamine phosphoribosylpyrophosphate amidotransferase glutamine site and communication with the phosphoribosylpyrophosphate site, *J. Biol. Chem.* **271**, 15549–15557.
25. Raushel, F. M., Rawding, C. J., Anderson, P. M., and Villafranca, J. J. (1979) Paramagnetic probes for carbamoyl-phosphate synthetase: metal ion binding studies and preparation of nitroxide spin-labeled derivatives, *Biochemistry*. **18**, 5562–5566.
26. Bauer, C. B., Fonseca, M. V., Holden, H. M., Thoden, J. B., Thompson, T. B., Escalante-Semerena, J. C., and Rayment, I. (2001) Three-dimensional structure of ATP:corrinoid adenosyltransferase from *Salmonella typhimurium* in its free state, complexed with MgATP, or complexed with hydroxycobalamin and MgATP, *Biochemistry* **40**, 361–374.

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