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

Vicente Fresquet, LaKenya Williams, and Frank M. Raushel*

Department of Chemistry, P.O. Box 30012, Texas A&M University, College Station, Texas 77845-3012

Received August 10, 2007; Revised Manuscript Received October 9, 2007

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-32P]ATP and adenosyl cobyrinic a,c-diamide the enzyme will catalyze the positional isotope exchange of the βγ-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 cobyric 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 hydrogenobyrinic acid to produce hydrogenobyrinic 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-cob) to produce cobyric 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.

Cobyrinic 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. Cobyrinic 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 amidation
diation 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 different enzymes 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 from S. typhimurium (8). In these enzymes an active site histidine activates a cysteine residue for nucleophilic attack on the carboxamide moiety of glutamine forming a thioester intermediate. The structural or molecular basis for the strict orientation for the amidation of carboxylate

**MATERIALS AND METHODS**

**Materials.** The plasmid p278Q containing the cbiP gene (gi: 16766390) was kindly provided by Dr. Charles Roessler 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, NdeI and EcoRI, 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 18O-labeled water were purchased from Cambridge Isotope Laboratories. 15N-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 Ettten (12). The synthesis of [γ-18O]-ATP was accomplished using the method of Werhli et al. from ADP-morpholodiate and H3P18O4 (13). The two stock solutions of [γ-18O]-ATP showed ~90 and 93% 18O incorporation by 31P 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′-GGGAATCCATATGACGCAAGCAGTTATGTTGC-3′ and reverse primer 5′-GGGAATTCTATCATCAATACGGCTTGGATG-3′ added NdeI and EcoRI sites, respectively. The resultant PCR product was purified, cut with NdeI and EcoRI, 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′-GGGAATTCTATATGATGATTAAATCATCAGCATGCAGC-3′ and the reverse primer was 5′-GGGC-GAAGCTTATTTAATAATCATCCATCACCCTTGGC-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 μg/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 μg/mL RNAase, and 1.0 μg/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...
Mechanism of Cobyric Acid Synthetase

Synthesis of Adenosyl Cobyric Acid *a,c*-Diamide. The substrate for the CbiP reaction, adenosyl cobyric acid *a,c*-diamide, was synthesized from vitamin B₁₂ in three steps. First, (CN)$_₂$-cobyric acid was obtained by methanolation of vitamin B₁₂ (15). In the second step, cobyric acid *a,c*-diamide was synthesized enzymatically from cobyric acid using cobyric acid *a,c*-diamide synthetase (Cbia). A solution containing 80 μM cobyric acid, 50 mM Tris-HCl, pH 7.5, 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 μM 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 cobyric 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 μL) 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 (0–4 μM) 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 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 mM phosphoenolpyruvate, 0.44 mM NADH, 28 μg/mL pyruvate kinase, 28 μg/mL lactic dehydrogenase and 6–30 μg of purified CbiP. When one substrate was varied, the other substrates (ATP, glutamine, and adenosyl cobyric acid *a,c*-diamide) were held constant at 4.0 mM, 20 mM and 15 μM, respectively.

Synthesis of Cobyric Acid. The production of adenosyl cobyric 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 μM adenosyl cobyric acid *a,c*-diamide, and wild type or mutant CbiP was incubated at 30 °C in the dark. At various times, a 700 μL 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 cobyric acid and derivatives was monitored by following the absorbance at 303 nm (4).

Synthesis of Adenosyl Cobyric Acid Intermediates. Adenosyl cobyric acid *a,c*-diamide (100 μM) 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 μg/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 μL 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 μM adenosyl cobyric acid *a,c*-diamide, 20 mM Tris-HCl, pH 7.5, 1.0 mM [¹⁵N]-glutamine, 1 mM DTT, 4 mM ATP, 20 mM MgCl₂, and 50–200 μg/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 μL with the addition of 600 μL H₂O containing 5 units of glutaminase and 50 μL of D₂O.

Positional Isotope Exchange Reactions. The PIX reactions catalyzed by CbiP were measured using 3¹⁵N NMR spectroscopy with [γ-¹⁵N]-ATP. The assays contained 100 mM Tris-HCl, pH 7.5, 1.0 mM DTT, 2.0 mM MgCl₂, 1.0 mM [γ-¹⁵N]-ATP, 2.0 μM adenosyl cobyric acid *a,c*-diamide, and 2.0 μM cobyric 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 cobyric acid *a,c*-diamide or cobyric 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 fit to eq 2, where $F = $ fraction of equilibrium value at time $t$ and $A = $ concentration of ATP.

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

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

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
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 4 mM ATP and 15 μM adenosyl cobyrinic acid a,c-diamide. The substrate (ATP, adenosyl cobyrinic acid a,c-diamide) was varied in the presence of 4 mM ATP and 15 μM adenosyl cobyrinic acid a,c-diamide. The data were fit using eq 1.

![Graph](image.png)

**Table 1: Kinetic Parameters of Wild Type and Mutants of CbiP**

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

\(^a\) 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. \(^c\) 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 acid a,c-diamide; (▲) presence of 4 mM ATP and 15 μM adenosyl cobyrinic acid a,c-diamide. 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-\(^{18}\)O\(_3\)]-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 \(^{18}\)O-label at the βγ-bridging position will become torsionally equivalent with the two β-nonbridging \(^{16}\)O-atoms of ADP. Re-formation and dissociation of ATP from the active site will then generate ATP with \(^{16}\)O 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).

**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-\(^{18}\)O\(_3\)]-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 \(^{18}\)O-label at the βγ-bridging position will become torsionally equivalent with the two β-nonbridging \(^{16}\)O-atoms of ADP. Re-formation and dissociation of ATP from the active site will then generate ATP with \(^{16}\)O 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).

**Site-Directed Mutagenesis.** We have previously demonstrated that the four carboxylates of adenosyl cobyrinic acid...
May enable the substrate to now bind in an orientation that allows carboxylate to be amidated. Therefore, the enzymatic site. Once carboxylate has been amidated, the constraints to binding in an orientation that allows carboxylate to be amidated are relieved. The subsequent amidation of carboxylate enables carboxylate to be amidated and eventually carboxylate is amidated. The structural misalignment of carboxylate may allow the process to be initiated at carboxylate. 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 cobyric 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 proximity to interact with MgATP or adenosyl cobyric acid. Glu-134 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 cobyric acid is not obvious from the model. Asp-146 and Glu-139 are located on a loop that is close to the adenosyl cobyric acid 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

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

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_m$ (mM) $k_{cat}$ (s$^{-1}$) $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>$K_m$ (mM) $k_{cat}$ (s$^{-1}$) $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>$120 \pm 14$ $0.32 \pm 0.01$ $2.7 \pm 0.1$ $1.8 \pm 0.2$ $0.29 \pm 0.01$ $160 \pm 16$</td>
<td></td>
</tr>
<tr>
<td>D146A</td>
<td>$106 \pm 22$ $0.15 \pm 0.01$ $1.4 \pm 0.1$ $4.2 \pm 0.3$ $0.10 \pm 0.01$ $24 \pm 2.0$</td>
<td></td>
</tr>
<tr>
<td>D146N</td>
<td>$109 \pm 12$ $0.13 \pm 0.01$ $1.2 \pm 0.1$ $1.6 \pm 0.2$ $0.11 \pm 0.01$ $69 \pm 4.0$</td>
<td></td>
</tr>
<tr>
<td>D165A</td>
<td>$98 \pm 24$ $0.11 \pm 0.02$ $1.6 \pm 0.2$ $2.8 \pm 0.3$ $0.19 \pm 0.01$ $71 \pm 9.0$</td>
<td></td>
</tr>
<tr>
<td>D167A</td>
<td>$79 \pm 15$ $0.25 \pm 0.01$ $3.2 \pm 0.1$ $1.6 \pm 0.2$ $0.20 \pm 0.01$ $125 \pm 10$</td>
<td></td>
</tr>
<tr>
<td>E234A</td>
<td>$77 \pm 12$ $0.046 \pm 0.008$ $0.6 \pm 0.1$ $5.4 \pm 1.0$ $0.010 \pm 0.001$ $1.8 \pm 0.3$</td>
<td></td>
</tr>
<tr>
<td>D235A</td>
<td>$70 \pm 5$ $0.175 \pm 0.007$ $2.5 \pm 0.1$ $18 \pm 4$ $0.020 \pm 0.002$ $1.1 \pm 0.2$</td>
<td></td>
</tr>
<tr>
<td>N264A</td>
<td>$110 \pm 16$ $0.10 \pm 0.01$ $0.9 \pm 0.1$ $16 \pm 3$ $0.13 \pm 0.01$ $8.1 \pm 1.2$</td>
<td></td>
</tr>
<tr>
<td>D267A</td>
<td>$35 \pm 3$ $0.018 \pm 0.004$ $0.5 \pm 0.1$ $17 \pm 2$ $0.029 \pm 0.001$ $1.7 \pm 0.2$</td>
<td></td>
</tr>
</tbody>
</table>

**a** Kinetic parameters for the hydrolysis of glutamine in the absence or presence of 4.0 mM ATP and 15 μM Ado-cob.
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 cobyric 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 cobyric 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 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 cobyric acid a,c-diamide, is referred to as the diamide, while the final product, cobyric acid, is referred to as the hexamide. The three partially amidated intermediates are denoted as the triamide, tetramide, and pentamide, respectively. For the partially amidated intermediates, the sites of amidation are labeled by the appropriate lowercase letters, as in the example, de-tetrameride. In the discussion of the partially amidated intermediates via 13N 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.
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 D146 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 15N-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-15N 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 15N 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 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
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 6**: HPLC traces for the formation of reaction intermediates by the D146N mutant of CbiP. (A) Sample was incubated with 50 μg/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 μg/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.

**Figure 7**: 1H–15N HSQC 2D NMR spectra for cobyric acid and the various intermediates catalyzed by the D146N mutant of CbiP in the presence of 15N-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, tetramide, 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, tetramide, pentamide, and cobyric acid are approximately 4.7:2.3:1.8:1.0. (D) NMR spectrum of the product, cobyric acid. Additional details are provided in the text.
pentamide intermediate. Changes in chemical shift for carboxylamides \( d \) 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 affect 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 \( \approx \)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 \( \approx \)8% of the total NMR integration (20% - 12%). Therefore, based on the NMR data, the contribution of the \( de \)-tetramide species is \( \approx \)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 \( \approx \)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, cobyric acid (\( bdeg \)-hexamidine), 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 cobyric acid in the vitamin B\( _{12} \) biosynthetic pathway is completed by adenosylation and amidation of six carboxylic acid groups in cobyric acid. Cobyric acid \( a,c \)-diamide synthetase (CobB from \( P. \) denitrificans and CbiA from \( S. \) typhimurium) catalyzes the amidation of carboxylate groups \( a \) and \( c \) of cobyric acid which is followed by cobalt reduction and adenosylation. Cobyric acid synthetase (CobQ from \( P. \) denitrificans and CbiP from \( S. \) typhimurium) catalyzes the amidation of carboxylate groups \( b \), \( d \), \( e \), and \( g \) on adenosyl cobyric 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 cobyric acid \( a,c \)-diamide and ATP. However, the presence of ATP and adenosyl cobyric 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 cobyric 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 cobyric acid 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 [\( \gamma \)-\( ^{18} \)O\(_4 \)]-ATP. This exchange reaction occurs only in the presence of CbiP, labeled ATP, and the substrate, adenosyl cobyric acid \( a,c \)-diamide. This exchange reaction cannot occur unless the bond between the \( \gamma \)-phosphoryl group and the \( \beta y \)-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 [\( \gamma \)-\( ^{18} \)O\(_4 \)]-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
D267A do not form cobyric 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 carboxamid 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. 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 triamide intermediates converge to a common deg-pentamide intermediate prior to amidation at carboxylate b to form the ultimate product, cobyric acid. There are 24 possible pathways for the amidation of carboxylate groups b, d, e, and g during the formation of cobyric 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 cobyric 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.

ACKNOWLEDGMENT

We thank Drs. Charles Roessner and Howard Williams for a critical reading of this manuscript and the generous help of Dr. Patricio Santander throughout the course of this investigation. We are indebted to Dr. Xiangming Kong for assistance with the NMR measurements.

REFERENCES

