Mechanism of Cobyricin Acid a,c-Diamide Synthetase from Salmonella typhimurium LT2†

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ABSTRACT: Cobyricin acid a,c-diamide synthetase from Salmonella typhimurium (CbiA) is the first glutamine amidotransferase in the anaerobic biosynthetic pathway of vitamin B₁₂ and catalyzes the ATP-dependent synthesis of cobyricin acid a,c-diamide from cobyricin acid using either glutamine or ammonia as the nitrogen source. The cbiA gene was cloned, the overexpressed protein was purified to homogeneity, and the kinetic parameters were determined. CbiA is a monomer with Kₘ values of 0.74, 2.7, 53, and 26 200 μM for cobyricin acid, ATP, glutamine, and ammonia, respectively. Analysis of the glutaminase partial reaction demonstrated that the hydrolysis of glutamine and the synthesis of the cobyricin acid a,c-diamide product are uncoupled. The time course for the synthesis of the diamide product and positional isotope exchange experiments demonstrate that CbiA catalyzes the sequential amidation of the c- and a-carboxylate groups of cobyricin acid via the formation of a phosphorylated intermediate. These results support a model for the catalytic mechanism in which CbiA catalyzes the amidation of the c-carboxylate, and then the intermediate is released into solution and binds to the same catalytic site for the amidation of the a-carboxylate. Several conserved residues in the synthetase active site were mutated to address the molecular basis of the amidation order; however, no changes in the order of amidation were obtained. The mutants D45N, D48N, and E90Q have a dramatic effect on the catalytic activity, whereas no effect was found for the mutant D97N. The substitutions by alanine of L47 and Y46 residues specifically decrease the affinity of the enzyme for the c-monoamide intermediate.

The aerobic and anaerobic biosynthetic pathways for the assembly of vitamin B₁₂ are highly complex processes that require the catalytic activity of more than two dozen different enzymes (1, 2). The complete set of enzymes involved in the two reaction pathways has been identified through an extensive array of genetic and biochemical investigations. Although the aerobic and anaerobic pathways contain many homologous enzymes and common chemical transformations, there are a number of specific differences. The most notable is the timing for the insertion of cobalt and the absence of molecular oxygen during the contraction of the tetrapyrrole ring in the anaerobic pathway (3). The catalytic activity and chemical mechanisms for many of the enzymatic reactions have been established for the aerobic biosynthesis of cobalamin from Pseudomonas denitrificans. The anaerobic pathway for cobalamin biosynthesis is found in other bacteria and archaea such as Salmonella typhimurium (4), Bacillus megaterium (5), Methanococcus jannaschii (6), and Propionibacterium freudenreichii (7). Although the majority of the genes in S. typhimurium have been mapped and sequenced, some of the detailed reaction mechanisms for enzymes in this pathway remain to be elucidated.

Cobyricin acid a,c-diamide synthetase from S. typhimurium (CbiA) catalyzes the ATP-dependent amidation of the two carboxylate groups at positions a and c of cobyricin acid and utilizes glutamine or ammonia as the nitrogen source as shown in Scheme 1. This enzyme represents an intriguing example of a protein that catalyzes chemical reactions at multiple sites within the same substrate. It is not known whether the two chemical events occur in an ordered or random fashion or whether the kinetic mechanism is progressive or dissociative because this enzyme has not previously been studied in any significant detail. However, the purification of the homologous enzyme from the aerobic pathway and a partial characterization of this protein has been reported (8). CobB from P. denitrificans has been shown to catalyze the amidation of the a and c carboxylates of hydrogenobyrinic acid. This enzyme will also use cobyricin acid as a substrate, although the affinity for cobyricin acid is significantly weaker (a Kₘ of 160 μM versus 0.41 μM for hydrogenobyrinic acid) (8).

Sequence comparisons have demonstrated that CbiA and CobB are comprised of two dissimilar protein domains (9). The C-terminal domain contains the binding site for glutamine and catalyzes the hydrolysis of this substrate to glutamate and ammonia. The glutaminase domain of CbiA apparently belongs to the triad class of glutamine amidotransferases, which also includes carbamoyl phosphate synthetase (CPS) (10), cobyric acid synthetase (CobQ from P. denitrificans and CbiP from S. typhimurium LT2) (11), and CTP syn-

1 Abbreviations: CbiA, cobyricin acid a,c-diamide synthetase from Salmonella typhimurium; CobB, cobyricin acid a,c-diamide synthetase from Pseudomonas denitrificans; DNase I, deoxyribonuclease I; DTBS, dethiobiotin synthetase; DTT, dithiothreitol; MALDI, matrix-assisted laser desorption/ionization; PMSF, phenylmethylsulfonyl fluoride.

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thetase (12). In these enzymes an active-site histidine activates a cysteine residue for nucleophilic attack on the carboxamide moiety of glutamine to form a thioester intermediate concomitant with the formation of ammonia. In all of the structurally characterized examples examined thus far, the ammonia is translocated to an adjacent domain via a molecular tunnel, where it reacts with an activated intermediate (13). In CbiA, the N-terminal domain is anticipated to bind ATP and cobyrinic acid and catalyze the ultimate synthesis of the diamide product. The \(\alpha\) and \(\gamma\) carboxylates of cobyrinic acid are postulated to be activated for nucleophilic attack via formation of a phosphorylated intermediate by ATP as illustrated in the reaction mechanism depicted in Scheme 2. The amino acid sequence of the N-terminal domain of CbiA shows similarity to dethiobiotin synthetase (12), MinD proteins (14), and CTP synthetase (15). These enzymes are members of the P-loop NTPase family of proteins (9, 16, 17).

We have expressed, purified, and elucidated some of the mechanistic details of the reaction catalyzed by CbiA. The catalytic coupling efficiency between the separate active sites within the N- and C-terminal domains has been determined by measurement of the synergistic relationships among the various partial reactions. The timing and sequential activation of the two carboxylate groups have been probed by positional isotope exchange (PIX) and an assessment of the individual time courses for the synthesis of amide functionality on the \(\alpha\) and \(\gamma\) carboxylates of cobyrinic acid.

**MATERIALS AND METHODS**

**Materials.** The plasmid pZT366 containing the \(cbiA\) gene (gi 16763390) and \((CN)_2\)-cobyrinic acid (18) were kindly provided by Dr. Charles Roessner and Dr. Patricio Santander, respectively, from the laboratory of Professor A. I. Scott at Texas A&M University. The restriction enzymes, \(NdeI\) and \(XhoI\), were purchased from New England Biolabs. Oxygen-18-labeled water (90%) was obtained from Cambridge Isotope Laboratories. Oxygen-18-labeled potassium phosphate was prepared using the method of Risley and Van Etten (19). The \([\gamma^{18}O_4]\)ATP was synthesized by following the procedure of Werhli et al. (20). The other materials and enzymes were obtained from either Aldrich or Sigma. Oligonucleotide synthesis and DNA sequencing reactions were performed by the Gene Technology Laboratory of Texas A&M University.

**Cloning and Mutagenesis of \(cbiA\).** The plasmid pZT366, which contains multiple genes within the vitamin B\(_{12}\) biosynthetic pathway from \(S.\ typhimurium\), was used as the template for the subcloning of the \(cbiA\) gene (21). The \(cbiA\) gene was PCR-amplified such that the forward primer 5′-CGCCGCAAGCTTGGAGGTTTAAAATCATATGGCGGCAGGCATCACGCATTTATTCTTGCAGG-3′ and reverse primer 5′-CGCGAATTCTTATAATACGCGCCTCGCCGCCAGCCAGTGCTG-3′ added \(NdeI\) and \(XhoI\) sites, respectively. The gene was amplified using \(Pfu\) turbo DNA polymerase (Stratagene) according to the instructions of the manufacturer. The PCR product was purified, cut with \(NdeI\) and \(XhoI\), and then ligated to the expression vector pET30 (Novagen). The entire coding region of \(cbiA\) was sequenced to confirm the fidelity of the PCR amplification. Mutants of CbiA were constructed using the QuikChange site-directed mutagenesis kit from Stratagene.

**Expression and Purification of CbiA.** For protein expression, \(Escherichia\ coli\ BL21(DE3)\ cells (Novagen) were transformed with pET30 carrying the \(cbiA\) gene and plated onto LB agar plates containing 50 \(\mu\)g/mL of kanamycin. Single colonies were grown overnight in 50 mL of LB media plus antibiotic and used to inoculate 6 L of LB media with kanamycin. The cells were grown at 37 °C with aeration to


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an OD$_{600}$ of 0.6–0.8. At this time, 1.0 mM IPTG was added, and the cells were allowed to grow for an additional 10 h before harvesting by centrifugation at 6000g for 10 min. The cell paste was frozen in liquid nitrogen and stored at −80 °C. The frozen cell paste was thawed in 5 volumes of 0.10 M Tris-Cl at pH 7.7, 100 mM KCl, 1.0 mM EDTA, 0.1 mg/mL PMSF, 0.1 mM leupeptin, and 0.1 mM pepstatin. Cells were lysed on ice by 5 cycles of sonication (3 min) separated by 5 min of cooling. The lysate was clarified by centrifugation at 15 000g for 15 min, and then 10 μg/mL RNase and DNase were added to the lysate. The sample was incubated for 15 min with stirring at 4 °C. Ammonium sulfate was added to 30% of saturation, and then the solution was incubated at 4 °C for 15 min before the protein pellet was collected by centrifugation. The pellet was resuspended with 5 mL of the same solution and loaded onto a Superdex-200 column. The protein fractions were pooled on the basis of SDS-PAGE and frozen in the same buffer containing 10% glycerol. The concentration of CbiA was calculated using an extinction coefficient of 280 nm of 1.21 OD for a protein concentration of 1.0 mg/mL cm$^{-1}$. The extinction coefficient was estimated by the method of Gill and von Hippel (22). The expression and purification of the mutants were similar to those described for the wild-type enzyme with the exception that after IPTG induction the cells containing the mutant plasmids were incubated at 20 °C.

Hydrolysis of Glutamine. The glutaminase activity of CbiA was quantified by determination of the glutamate produced by coupling the reaction products to glutamate dehydrogenase (23). The assay was carried out in a volume of 250 μL containing 0.10 M Tris-Cl at pH 7.7, 100 mM KCl, 1.0 mM dithiothreitol (DTT), 4.0 mM MgCl$_2$, 10 units of glutamate dehydrogenase, and 1.0 mM 3-acylpyrididine adenine dinucleotide (APAD), glutamate (0–40 mM), and 6–24 μg of protein at 30 °C. When the enhancement of the glutaminase activity by added nucleotide was determined, the concentration of glutamine was held at 2.0 and 12 mM in the presence or absence of cobyrinic acid, respectively. The glutamate concentration was calculated using an extinction coefficient for the reduced form of 3-acylpyridine adenine dinucleotide of 8.3 mM$^{-1}$ cm$^{-1}$ at 363 nm (23).

Utilization of ATP. The rate of ADP formation was measured using a pyruvate kinase/lactic dehydrogenase coupled assay by monitoring the loss of NADH spectrophotometrically (23). The activity was measured at 30 °C in a solution containing 0.10 M Tris-Cl at pH 7.7, 100 mM KCl, 1.0 mM DTT, 4.0 mM MgCl$_2$, 1.0 mM phosphoenolpyruvate, 0.44 mM NADH, 28 μg/mL pyruvate kinase, 28 μg/mL lactic dehydrogenase, and 6–30 μg of purified CbiA. When one of the three substrates was varied, the concentrations of glutamine, ATP, and cobyrinic acid were held constant at 4.0 mM, 2.0 mM, and 25 μM, respectively.

Enzymatic Synthesis of Cobyrinic Acid a,c-Diamide. The rate of formation of cobyrinic acid a,c-diamide was quantified by HPLC (8, 24). A solution containing 0.10 M Tris-Cl at pH 7.7, 100 mM KCl, 1.0 mM DTT, 4.0 mM MgCl$_2$, 2.0 mM ATP, 4.0 mM glutamine, 50 μM cobyrinic acid, and 30 μg/mL of CbiA was incubated at 30 °C. At various times, fractions of 900 μL were removed and added to 100 μL of 0.1 M potassium cyanide, and then the reaction was quenched by incubation at 80 °C for 10 min. A 0.50 mL sample was loaded onto a 4.6 × 150 mm Microsorb 100 C18 5-μm column (Varian) equilibrated with 0.10 M potassium phosphate at pH 6.5 and 10 mM KCl (buffer A). The products were eluted at a flow rate of 1.0 mL/min at room temperature in a mobile phase using buffer A, 0.10 M potassium phosphate at pH 8.0, 10 mM KCl, and 50% acetonitrile (buffer B). The changes in the elution profile were as follows: 0–2% buffer B over 5 min, 2–5% buffer B over 5 min, 5–20% buffer B over 10 min, followed by 10 min of isocratic elution of 20% buffer B, 20–100% buffer B over 10 min, and then re-equilibrated with 100–0% of buffer B over a period of 5 min. The elution of the cobyrinic acid and derivates was monitored by the absorption at 360 nm (8).

PIX. The PIX reaction was measured by following the relative distribution of the $^{31}$P NMR resonances for [$γ$-$^{15}$O$_2$]-ATP and [$γ$-$^{18}$O$_2$]ATP. The reaction mixtures contained 1.0 mM [$γ$-$^{15}$O$_2$]ATP, 0.2 μM cobyrinic acid, 50 mM Tris-Cl at pH 7.2, 20 mM KCl, 1.0 mM DTT, 2.0 mM MgCl$_2$, and cobyrinic a,c-diamide synthetase at a final concentration of 1.0 μM. The volume of the assay mixture was 0.5 mL, and the samples were incubated at 30 °C for up to 4 h. The reaction was quenched by adding 100 μL of CCl$_4$ with vigorous vortexing. The precipitated protein was removed by passage through a 0.45 μm Corning syringe filter and then concentrated to near dryness with the aid of a rotary evaporator. The material was then dissolved in a 750 μL solution containing 200 mM EDTA at pH 9.0 and 15% D$_2$O.

$^{31}$P NMR spectra were acquired using a Varian Inova-400 multinuclear NMR spectrometer operating at a frequency of 162 MHz. Acquisition parameters were 5000-Hz sweep width, 6.0-s acquisition time, and 2.0-s delay between pulses.

Data Analysis. The kinetic parameters, $k_{cat}$ and $K_m$, 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, and $S$ is the substrate concentration. The kinetic parameters for cobyrinic acid and cobyrinic acid c-monoamide from the time-course investigations were calculated using DNR-P-RKF (25) that was kindly provided by Professor Ronald Duggleby (University of Queensland, Australia) based on eqs 2–4, where $S_o$ is the initial concentration of cobyrinic acid, while $P$ and $Q$ represent the concentrations of cobyrinic acid c-monoamide and cobyrinic acid a,c-diamide, respectively, at any time $t$. $E_i$ is the concentration of enzyme, $k_i$ and $k_p$ are the turnover numbers for the reaction of each substrate, and $K_i$ is the Michaelis or inhibition constant for each of the three possible corrinoids. The data for the enhancement of glutamine hydrolysis in the presence of other ligands were fitted to eq 5 (26), where $V_o$ is the initial velocity in the absence of ligand, $K_i$ is the apparent activation constant, and $α$ is the ratio of the velocities at saturating concentrations of added ligand. The rate of the PIX reactions were fit to eq 6, where $F = $ fraction of equilibrium value at time $t$ and $A = $ concentration of ATP (27).

$$v/E_i = k_{cat} S/(K_m + S)$$

(1)

$$S_o = S + P + Q$$

(2)

$$dS/dt = -k_v E_i / (1 + K_v)S(1 + P/K_p + Q/K_q)$$

(3)

$$dP/dt = dS/dt - (k_p E_i )/(1 + K_p)(1 + S/K_s + Q/K_q)$$

(4)
RESULTS

Expression and Purification of CbiA. Cobiamic acid a-c-diamide synthetase was overexpressed in E. coli and purified to homogeneity. CbiA elutes as a symmetric peak during chromatography and migrates as a single band on SDS-PAGE with an electrophoretic mobility of \( \sim 50 \text{kDa} \), which agrees with the value of 50 039 Da calculated from the DNA sequence. The molecular weight of the native enzyme was estimated to be 55 \pm 3 \text{kDa} \) by gel filtration, and thus CbiA is a monomer. The identity of the purified protein was confirmed by measurement of the N-terminal amino acid sequence (Protein Chemistry Laboratory, Texas A&M University). Two sequences, AARHHA and MAARHHA (20%), were found, which correspond with the predicted sequence for the first six amino acids of CbiA, although a small fraction of the isolated enzyme remains with an unprocessed methionine. The catalytic activity exhibited by CbiA was stable for a period longer than 6 months in the storage conditions described above. However, the exclusion of glycerol in the storage buffer promotes precipitation of the protein. When DTT was removed from the reaction mixture, no activity was detected.

Initial Velocity Studies. Initial velocity studies were conducted by measuring the rate of ADP formation while varying the concentration of glutamine, ammonia, ATP, and cobiamic acid. The catalytic activity is essentially constant between pH 6.8 and 8.0. CbiA uses either glutamine or ammonia as the nitrogen source with very similar kinetic parameters. Two sequences, AARHHA and MAARHHA (20%), were found, which correspond with the predicted sequence for the first six amino acids of CbiA, although a small fraction of the isolated enzyme remains with an unprocessed methionine. The catalytic activity exhibited by CbiA was stable for a period longer than 6 months in the storage conditions described above. However, the exclusion of glycerol in the storage buffer promotes precipitation of the protein. When DTT was removed from the reaction mixture, no activity was detected.

Hydrolysis of Glutamine. CbiA is able to hydrolyze glutamine in the absence of the other substrates. The addition of ATP alone decreases the \( K_m \) for glutamine by a factor of 2. A more substantial decrease in the \( K_m \) for glutamine is observed when the activity is determined in the presence of ATP and cobiamic acid. The catalytic activity of CbiA was stable for a period longer than 6 months in the storage conditions described above. However, the exclusion of glycerol in the storage buffer promotes precipitation of the protein. When DTT was removed from the reaction mixture, no activity was detected.

The enhancement of the glutaminase activity upon the binding of other substrates was exploited to obtain the apparent activation constants for the addition of various nucleotides and cobiamic acid to the enzyme. The kinetic constants are shown in Table 3 from fits of the data to eq 5. The activation constant for cobiamic acid is similar to the \( K_m \) value measured by monitoring the rate of ATP turnover in the presence of all substrates (Table 1). The addition of ATP induces a 3-fold decrease in the apparent \( K_a \) for cobiamic acid. These experiments also show that ADP, AMP, GTP, and UTP are able to bind to the enzyme with varying degrees of affinity.

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The formation of a phosphorylated reaction intermediate in the chemical mechanism of CbiA was assessed by measurement of the PIX reaction within \(^{18}\text{O}\)-labeled ATP.

\[
v \ln(1 - F) = \frac{A}{(1 - I)} - \left( \frac{(1 - I)}{(1 - I)} \right) \ln(1 - F)
\]

\[
v = \frac{A}{(1 - I)} - \ln(1 - F)
\]

\[
\frac{v}{E} = V_o (K_a + \Delta I)(K_a + I)
\]

\[
v = \frac{A}{(1 - I)} \ln(1 - F)
\]
in the presence of cobyrinic acid. The overall mechanism for the migration of the $\beta\gamma$-bridge oxygen to the $\beta$-nonbridge position within the labeled ATP is illustrated in Scheme 3. The $^{31}$P NMR spectrum for the $\gamma$-P of the original $[\gamma^{-18O_4}]$-ATP is presented in Figure 2A and shows two pairs of doublets. The larger pair of doublets is for the $\gamma$-P with 4 atoms of $^{18}$O, while the smaller doublet is for the fraction of the original ATP that was made with 3 atoms of $^{18}$O and 1 atom of $^{16}$O. Incorporation of $^{18}$O into the $\beta$-nonbridge position of $[\gamma^{-18O_4}]$ATP was observed after incubation with cobyrinic acid and enzyme (Figure 2B). The fraction of the $[\gamma^{-18O_4}]$ATP species decreased from 0.75 to 0.61 after an incubation period of 4 h. The rate of the PIX reaction, calculated from eq 6, is $0.023 \text{ s}^{-1}$.

**Synthesis of Cobyrinic Acid a,c-Diamide.** The synthesis of the ultimate product, cobyrinic acid $a,c$-diamide, was monitored by HPLC. This methodology quantifies the relative amounts of the substrate, product, and the two possible reaction intermediates, cobyrinic acid $a$-monoamide and the $c$-monoamide, as a function of time (8, 24). The observed retention times for cobyrinic acid and cobyrinic acid $a,c$-diamide were 4 and 25 min, respectively. The intermediate monoamide product eluted at 22 min. The estimated elution times for cobyrinic $a$-monoamide and cobyrinic $c$-monoamide were 12 and 22 min, respectively, based on the relative elution profile for cobyrinic acid, cobyrinic $c$-monoamide, cobyrinic $c$-monoamide, and cobyrinic $a,c$-diamide (8). The exact mass of cobyrinic acid and the monoamide and diamide products were measured with a PE Sciex APJ Qstar Pulsar mass spectrometer by the Laboratory for Biological Mass Spectrometry at Texas A&M University. A mass of 938.41 amu for cobyrinic acid (expected 938.32 amu) was obtained using MALDI ionization. The isolated product that accumulates at the end of the reaction cycle has a molecular mass of 936.49 amu and corresponds to the theoretical value predicted for cobyrinic acid $a,c$-diamide (936.36 amu). The intermediate, cobyrinic acid $c$-monoamide, was found to have a molecular mass of 937.64 amu (expected 937.34 amu). As the reaction proceeds, the intensity of the peak for cobyrinic acid decreases and the peak for cobyrinic acid $c$-monoamide increases as a function of time (data not shown). At later stages of the reaction, the intensity of the peak for cobyrinic acid $c$-monoamide decreases, while the cobyrinic acid $a,c$-diamide increases. The time course of the reaction is shown in Figure 3. The intermediate accumulates until approximately half of the initial concentration of substrate is utilized and then decreases, indicating that the product of the first amidation step is released from the enzyme. There was no indication for the formation of the cobyrinic $a$-monoamide.

![Scheme 3](image-url)

**Figure 2:** $^{31}$P NMR spectra of the $\gamma$-P of $[\gamma^{-18O_4}]$ATP. (A) Resonances for the doublet of the $\gamma$-P of $[\gamma^{-18O_4}]$ATP prior to the addition of CbiA and cobyrinic acid with 4, 3, and 2 atoms of $^{18}$O. (B) Resonance for the $\gamma$-P of the labeled ATP after incubation with CbiA and cobyrinic acid. The most upfield doublet in each spectrum is the resonance for the $\gamma$-P of ATP that contains 4 atoms of $^{18}$O. The small doublet is from the $\gamma$-P of ATP that contains 3 atom of $^{18}$O. Additional details are provided in the text.

**Figure 3:** Time course for the synthesis of cobyrinic $a,c$-diamide (■) and cobyrinic $c$-monoamide (▲) as quantified by HPLC using 50 $\mu$M cobyrinic acid (●) and 30 $\mu$g/mL of CbiA. The lines represent fits of the data using DNRP-RKF.

A model was constructed to facilitate the determination of the kinetic parameters for the substrate and for the intermediate $c$-monoamide. In this model, the enzyme...
catalyzes the amidation of cobyrinic acid exclusively at the c-carboxylate. This product is released and rebounds in a different orientation, and then the second amide group is introduced to the a-carboxylate. Using this model, $K_m$ values of $0.20 \pm 0.01$ and $0.41 \pm 0.01 \, \mu M$ were obtained for cobyrinic acid and cobyrinic c-monoamide, respectively, with $k_{cat}$ values of $0.11 \pm 0.01$ and $0.20 \pm 0.03 \, s^{-1}$, respectively. Figure 3 illustrates the correlation between the experimental data and a fit of the data to eqs 2–4.

**Nucleotide Specificity.** CbiA belongs to the P-loop family of NTPases (17), and ATP has been shown to be a substrate for CbiA. However, it has been reported that members of the P-loop family of GPTases can use other nucleotide triphosphates as phosphoryl donors. The binding of GTP and UTP to CbiA was studied by measuring the enhancement of these nucleotides on the glutaminase activity, and the results are presented in Table 3. CbiA has a $K_m$ for GTP in the micromolar range, whereas the apparent $K_m$ for UTP is 3 orders of magnitude higher than that observed for ATP. To confirm that both nucleotides are competent as substrates, the production of cobyrinic acid $a,c$-diamide was monitored by HPLC. In either case, CbiA is able to synthesize the final product using GTP or UTP. However, at a concentration of 2.0 mM nucleotide, the rate of product formation is 50–60% slower than that measured with ATP. These results demonstrate that CbiA is able to use other nucleotide triphosphates, although less efficiently than ATP.

**Site-Directed Mutagenesis.** The structural basis for the strict order of the amidation products was addressed through site-directed mutagenesis of several residues located in the putative binding pocket for cobyrinic acid. There is currently no crystal structure available for cobyrinic acid $a,c$-diamide synthetase from any source. However, a model for the synthetase domain of CbiA has been proposed based on homology to dethiobiotin synthetase (9). Coordinates of cobyrinic acid were taken from the Cambridge database of small molecules (http://www.ccdc.cam.ac.uk) accession VASTUE. Cobyrinic acid is shown in a tube format, and the parts of cobyrinic acid that are modeled on the carboxylic acid substrate for dethiobiotin synthetase are shown in a darker gray. Carbon, oxygen, nitrogen, and cobalt atoms are shown in gray, red, blue, and pink, respectively. ADP, $P_i$, and the magnesium ions found in the DTBS structure are shown. The side chains of the mutated residues are indicated. The figure was prepared using WebLab ViewerPro.

**Glutaminase Activity of CbiA Mutants.** The glutaminase active site is located in the C-terminal domain of the protein. To determine if the mutations produced conformational changes that were transmitted throughout the protein, the overall effect on the glutaminase activity was assessed. None of the mutations affected the glutaminase activity in the absence of the other two substrates (ATP and cobyrinic acid) (Table 4). However, differences in the activation of the glutaminase reaction by ATP and cobyrinic acid were obtained. In the wild-type enzyme, the binding of cobyrinic acid in the absence of ATP produces a 20-fold decrease in the $K_m$ for glutamine. This decrease is not observed in the mutants Y46A, L47A, and D97N, suggesting that these residues may be involved in the communication between the two active sites in CbiA or the binding of cobyrinic acid. Despite the low synthetase activity of the mutants D45N, D48N, and E90Q, the effect of binding cobyrinic acid in the absence of ATP was similar to the wild-type CbiA, suggesting that the main effect of these mutations is a decrease in the affinity for ATP (Table 4) and not by changes in the cobyrinic acid binding.

**Cobyrinic Acid $a,c$-Diamide Synthetase Activity of CbiA Mutants.** The mutants D48N and E90Q exhibit dramatic
c, d, e, and g carboxylates. In the anaerobic and aerobic pathways, the six amidation steps are carried out by two separate enzymes. Cobyric acid a,c-diamide synthetase (CbiA from S. typhimurium and CobB from P. denitrificans) is responsible for the introduction of the two amide groups at the a and c positions in either cobyric acid or hydrogenobyrinic acid. The amidation of the b, d, e, and g carboxylate groups in adenosyl-Cob(I)yrinic acid a,c-diamide is catalyzed by Cbp (Salmonella) and CobQ (Pseudomonas) (8, 11).

The enzyme CbiA was expressed and purified to homogeneity. Gel-filtration experiments are consistent with the conclusion that this enzyme functions as a monomer in solution, whereas the corresponding enzyme (CobB) from P. denitrificans oligomerizes to a dimer. CbiA has a high affinity for its substrates with $K_m$ values in the micromolar range. In comparison, CobB has a 500-fold higher affinity for cobyric acid than CobB from P. denitrificans. This property is a direct consequence of the differences in the two pathways in which they are involved. In the aerobic pathway hydrogenobyrinic acid is the physiological substrate for CobB. The insertion of cobalt takes place after the amidations of the a and c carboxylate groups. The affinity of CbiA for its substrate is similar to that reported for CobB when hydrogenobyrinic acid is used as the substrate (8).

**Activation of Glutamine Hydrolysis.** The proposed chemical mechanism for the reaction catalyzed by CbiA is presented in Scheme 2. During this transformation, the two carboxylate groups must be activated for nucleophilic attack through phosphorylation with ATP and the ammonia must be liberated by hydrolysis of glutamine. In all of the glutamine amidotransferases examined structurally thus far, these two chemical reactions occur within spatially distinct active sites and are connected by a molecular tunnel for the passage of ammonia. CbiA is able to catalyze the hydrolysis of glutamine in the absence of any other substrates. However, the addition of either ATP or cobyric acid alone enhances the rate of glutamine hydrolysis by lowering the $K_m$ for glutamine by a factor of 270.

**DISCUSSION**

The synthesis of vitamin B$_{12}$ is a complex process that requires approximately 25 different enzymes. During this transformation, six amide groups are introduced at the $a$, $b$, and $g$ positions in either cobyric acid or hydrogenobyrinic acid. The introduction of each amide group is catalyzed by a different enzyme.

**Table 4: Kinetic Parameters for the Hydrolysis of Glutamine by Mutants of CbiA**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>25 ± 2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>D45N</td>
<td>32 ± 4</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Y46A</td>
<td>75 ± 7</td>
<td>0.75 ± 0.1</td>
</tr>
<tr>
<td>L47A</td>
<td>33 ± 2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>D48N</td>
<td>45 ± 6</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>E90Q</td>
<td>20 ± 3</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>D97N</td>
<td>46 ± 2</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

* Activity was determined by HPLC and assayed using 4.0 mM ATP, 12.5 uM cobyric acid, and 20 mM of glutamine or 70 mM of ammonia. The kinetic constants were obtained by a fit of the data to eqs 2-4.

**Table 5: Relative Amidation Activity of Wild Type and Mutants of CbiA**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Glutamine</th>
<th>Ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100 ± 2</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>D45N</td>
<td>&lt;0.02</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Y46A</td>
<td>3.8 ± 0.2</td>
<td>2.0 ± 0.01</td>
</tr>
<tr>
<td>L47A</td>
<td>10.6 ± 0.05</td>
<td>16.5 ± 1</td>
</tr>
<tr>
<td>D48N</td>
<td>0.2 ± 0.01</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>E90Q</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>D97N</td>
<td>32.4 ± 2</td>
<td>44 ± 0.3</td>
</tr>
</tbody>
</table>

* Activity was determined by HPLC and assayed using 4.0 mM ATP, 12.5 uM cobyric acid, and 20 mM of glutamine or 70 mM of ammonia. 10% glycerol was added to the reaction buffer.
The degree of uncoupling between the two active sites is somewhat surprising because there is no uncoupling in the analogous set of reactions catalyzed by carbamoyl phosphate synthetase from E. coli (28). In glutamine phosphoribosylpyrophosphate amidotransferase, the binding of the phosphoribosyl pyrophosphate decreases the $K_m$ for glutamine about 100-fold (29). The catalytic activities of all glutamine amidotransferases are coupled to one another with the exception of asparagine synthetase B. In this enzyme, the glutaminase activity is 1.5-fold faster than the synthetase activity (30).

**Activation of Carboxylates.** In the absence of a nitrogen source, there is no turnover of ATP. The activation of the cobyrinic acid by ATP was addressed by the utilization of $[\gamma^{-18}O]$ATP. When cobyrinic acid and $[\gamma^{-18}O]$ATP are incubated with CbiA, there is a migration of $^{18}O$ from the $\beta\gamma$-bridge position to the $\beta$-nonbridge position. This result is consistent with the formation of a phosphorylated cobyrinic acid derivative that is formed in the absence of glutamine or ammonia. Because a PIX reaction is not observed in the absence of cobyrinic acid, it is unlikely that this reaction represents the formation of a phosphorylated-enzyme intermediate.

**Reaction Processivity.** The time-course analysis for the formation of the reaction products shows that a single intermediate species accumulates during the early stages of the reaction and is subsequently utilized as a substrate for the formation of the ultimate $a,c$-diamide product. This intermediate corresponds to the cobyrinic acid $c$-monoamide derivative (24). This result clearly indicates that this enzyme catalyzes the amidation of the two carboxylates in a specific order; the $c$-carboxylate is activated and amidated prior to the $a$-carboxylate. Moreover, the cobyrinic $c$-monoamide intermediate is released into solution prior to the formation of the amide at the $a$-carboxylate position. Therefore, the reaction chemistry at the $a$- and $c$-carboxylates of cobyrinic acid is sequential, and the kinetic order is dissociative. A fit of the time-course data indicates that the kinetic constants for the amidation of cobyrinic acid and the cobyrinic $c$-amide are nearly the same. This behavior is similar to that reported for CobB.

In this model, the $a$- and $c$-carboxylate groups of the cobyrinic acid adopt a similar position within the binding pocket. Unfortunately, there are no structural data available for CbiA. However, a model of CobB for the synthetase domain has been proposed by Galperin and Grishin (9) based on the sequence identity between CobB and dethiobiotin synthetase. The structure of cobyrinic acid shows that the $a$- and $c$-carboxylate groups are located on one side of the molecule, the $g$-carboxylate is positioned in the plane, and the remaining carboxylate groups are orientated toward the opposite face of the macrocycle. The sequential amidation of the $c$- and $a$-carboxylates can be accommodated by a simple rotation of cobyrinic acid within the active site that requires the dissociation of the intermediate into solution. This structural model is consistent with a single catalytic site that is able to amidate both carboxylate groups but it does not explain the order of the two amidation steps. Apparently, the binding of cobyrinic acid within the active site does not permit the $a$-carboxylate to be positioned near the bound ATP unless the $c$-carboxylate has been amidated. This binding orientation may be prevented via charge repulsion by an active-site carboxylate from either aspartate or glutamate. A sequence comparison of several cobyrinic $a,c$-diamide synthetases, including CobB from P. denitrificans and CbiA from S. typhimurium reveals that 5 negatively charged amino acids are conserved in the synthetase domain of these proteins. On the basis of this structural model, Asp-48 and Glu-90 of CbiA are implicated in the coordination of the magnesium ion (9). However, Asp-45 and Asp-97 are located in the binding site, while Asp-64 is positioned outside of the active site.

The results with D45N mutant show that this amino acid is not involved in the amidation order in CbiA. In DTBS, the homologous residue is a serine that has been proposed to play a role in the catalysis and binding of the substrate (12). Kinetic and crystallographic studies show that the substitution of this amino acid by alanine has only a modest effect on the binding of the substrate (31). The structural model of CbiA shows that the side chain of D45 is orientated away from the active site. The lack of an effect on cobyrinic acid binding in the absence of ATP suggests that this residue does not play an important role in substrate binding. The kinetic constants demonstrate that the $K_m$ for cobyrinic acid decreases by an order of magnitude in the presence of ATP, suggesting possible changes in the interaction of cobyrinic acid with the protein in the absence or presence of the nucleotide. The selective effect of the mutant Y46A in the binding of both corrinoids suggests a different rearrangement in the active site for these substrates. In this model, the side chain of Y46 is oriented toward the active site and it can interact with the carbonyl oxygen of both substrates. However, the results obtained with the Y46A mutant demonstrate that this amino acid is not important for the correct orientation of the $c$-carboxylate, but it could be involved in the binding of the $a$-carboxylate.

The structural basis for the order of the amidation remains to be clarified. In the proposed model, the substrate binds on the surface of the enzyme with most of the charged groups facing outside of the binding pocket. Previous studies with CobB using cobyrinic acid $a$- or $g$-monoamide substrates reveal that the absence of a negative charge at either of these positions has no effect on the amidation order or activity of the enzyme (8). Sequence comparisons among different cobyrinic acid $a,c$-diamide synthetases show no conservation of residues that can interact with the substrate, with the exception of the amino acids included in our study, suggesting that these functional groups are not implicated in the amidation order. CbiA is organized into two functional domains, glutaminase and synthetase. Each of these domains contains an independent active site responsible for the hydrolysis of glutamine and the synthesis of the amidated corrinoid, respectively. Our results show that the presence of the substrates in the synthetase active site decreases the $K_m$ for glutamate by 2 orders of magnitude. The activation of the glutaminase activity is a consequence of a conformational change transmitted through the interaction of residues of both domains or by a direct interaction of amino acids from the glutaminase domain in the binding of the synthetase substrates.
CONCLUSIONS
The reaction mechanism for the amidation of the α- and ω-carboxylate of cobyrinic acid has been addressed. The enzyme from S. typhimurium has been shown to catalyze the sequential amidation of the ω- and α-carboxylate groups of cobyrinic acid. The cobyrinic acid ω-monooamide is released into solution prior to the formation of the α,ω-diamide product. The enzyme catalyzes the formation of a phosphorylated intermediate in the absence of a nitrogen source. The enzyme catalyzes the hydrolysis of glutamine in the absence of cobyrinic acid and/or ATP, but the rate of hydrolysis is enhanced in the presence of these two substrates via a reduction in the Michaelis constant for glutamine. At saturating concentrations of all substrates, the hydrolysis of glutamine is approximately 8 times faster than the rate of amidation.

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REFERENCES