

The Multiple Amidation Reactions Catalyzed by Cobyric Acid Synthetase from *Salmonella typhimurium* Are Sequential and Dissociative

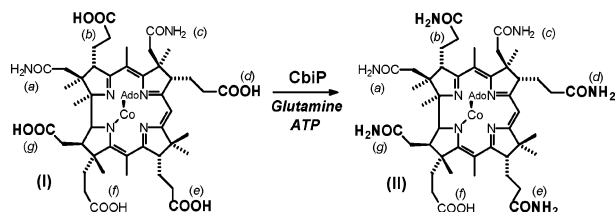
LaKenya Williams, Vicente Fresquet, Patricio J. Santander, and Frank M. Raushel*

Department of Chemistry, Texas A&M University, P.O. Box 30012, College Station, Texas 77842-3012

Received November 7, 2006; E-mail: raushel@tamu.edu

Vitamin B₁₂ is utilized as an essential cofactor in a wide range of enzyme-catalyzed reactions. Most of the enzymes involved in the biosynthesis of this complex macromolecule have now been identified and biochemically characterized.¹ Cobyric acid synthetase (CbiP) from *Salmonella typhimurium* catalyzes the ATP-dependent amidation of adenosyl-cobyric acid *a,c*-diamide (**I**) at carboxylates *b*, *d*, *e*, and *g* to produce cobyric acid (**II**) using glutamine or ammonia as the nitrogen source (Scheme 1). In the amidotransferase family of enzymes the production and utilization of ammonia takes place at physically distinct active sites. The ammonia, generated from the hydrolysis of glutamine, is translocated through the interior of the protein via an intramolecular tunnel to a separate active site for amide bond synthesis.² The reaction catalyzed by CbiP is particularly intriguing since a single synthetase active site catalyzes the amidation of four different carboxylate groups attached to the periphery of a single corrinoid substrate. It is not known whether the overall reaction mechanism is *dissociative* or *processive*. It is also unknown whether the four carboxylates are amidated in an *ordered* or *random* sequence. In this Communication we have determined the specific sequence of the four consecutive amidation reactions and the relative rates for the appearance and disappearance of the partially amidated intermediates.

Scheme 1. Reaction Catalyzed by CbiP.



The gene for cobyric acid synthetase (gi:16763390) was cloned into a pET30 expression vector. The cells were induced with IPTG and the enzyme purified by ammonium sulfate fractionation, gel filtration, and anion exchange chromatography. The identity of the purified protein was confirmed by the sequence of the first five N-terminal amino acid residues (TQAVM). CbiP elutes as a symmetric peak during gel-filtration chromatography with an estimated molecular mass of 57 kDa. CbiP is therefore a monomeric protein based upon a calculated subunit mass of 55 kDa from the DNA sequence. The substrate, adenosyl-cobyric acid *a,c*-diamide, was synthesized by enzymatic methods beginning with cobyric acid. The identity and purity of **I** was verified by mass spectrometry, UV-vis spectroscopy, and HPLC.^{3–5}

Adenosyl-cobyric acid *a,c*-diamide was incubated with CbiP in the presence of saturating concentrations of MgATP and glutamine, and the products of the enzymatic reaction were separated by HPLC as a function of time. A single compound accumulated at the end of the reaction as illustrated in Figure 1C. However, at shorter incubation times (Figure 1, parts A and B)

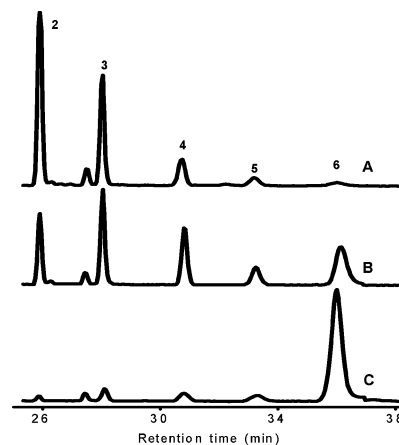


Figure 1. Time course for the production of cobyric acid and partially amidated intermediates during the reaction catalyzed by CbiA. The HPLC traces were obtained at the following times after the addition of CbiP: (A) 15 min; (B) 40 min; and (C) 80 min. The elution conditions were similar to those described previously.⁶ The number adjacent to each peak indicates the total number of amide groups in the adenosyl cobyric acid derivative: **2**, adenosyl-cobyric acid *a,c*-diamide; **3**, triamide; **4**, tetraamide; **5**, pentaamide; and **6**, cobyric acid. The small peak that immediately precedes the appearance of peak **3** is from an unidentified contaminant in the starting material.

three additional compounds appeared transiently, with retention times between the initial substrate and the ultimate cobyric acid product. The identities of the reaction intermediates generated by CbiP were analyzed by mass spectrometry.⁴ A molecular mass of 936.39 was obtained for the substrate **I** (Figure 1, peak 2) after the loss of the adenosyl group (expected 936.36 amu) and 932.57 (peak 6) for cobyric acid (expected 932.44 amu) using MALDI ionization. Masses of 935.40, 934.48, and 933.50 were obtained for peaks 3, 4, and 5, respectively, and identified as the tri-, tetra-, and pentaamide derivatives of adenosyl cobyric acid.

The time course for the overall reaction demonstrates that after the addition of CbiP, three partially amidated intermediates accumulate during the early stages of the enzymatic transformation and then decrease as the reaction progresses (Figure 2). These data establish that CbiP catalyzes a single amidation reaction and then releases the intermediate into solution prior to rebinding to the active site in a different orientation for the next catalytic cycle. A sequential kinetic model for CbiP was constructed to obtain the catalytic parameters for the substrate and the three partially amidated intermediates generated during the course of the enzymatic reaction. In this model the four carboxylates of adenosyl-cobyric acid *a,c*-diamide are amidated in a sequential but unspecified order, and the intermediates are liberated from the active site at the end of each catalytic cycle.⁷ The correlation between the model and the experimental data is presented in Figure 2. Table 1 provides the values for K_m and k_{cat} for **I** and the three partially amidated

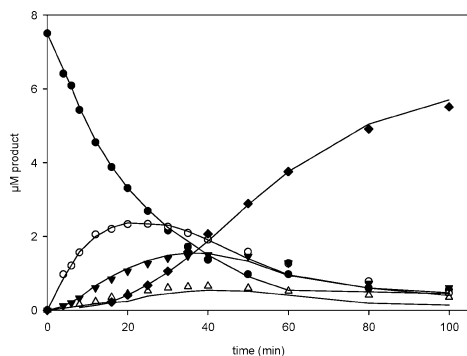


Figure 2. Time course for the CbiP reaction. The activity was assayed at 30 °C in a solution containing 50 mM Tris-HCl, pH 7.5, 1.0 mM DTT, 4.0 mM ATP, 6.0 mM MgCl₂, 20 mM glutamine, 7.5 μM of **I** and 0.15 μM CbiP. The lines represent a fit of the data to a sequential model generated using the program DNRP-RKF for changes in the concentration of **I** (●), triamide (○), tetraamide (▼), pentaamide (△), and cobyrinic acid (◆).⁷

intermediates. The affinity and turnover numbers for the substrate and three intermediates are remarkably similar to one another.

The specific order for the amidation of the four carboxylates of adenosyl cobyrinic *a,c*-diamide by CbiP was determined by ¹H, ¹⁵N HSQC NMR spectroscopy.⁸ The ¹⁵N chemical shifts for the carboxamide groups have been determined.⁹ It was therefore possible to determine the specific order that carboxylates *b*, *d*, *e*, and *g* are amidated by quenching the enzymatic reaction at time points where **I** is partially amidated. CbiP (3.6 μM) was incubated with 100 μM **I** and 2.0 mM MgATP in the presence of 25 mM ¹⁵NH₄⁺ at pH 7.5, and the reaction was quenched at various times. The product distribution of the intermediates was determined by HPLC. Shown in Figure 3D is the ¹H, ¹⁵N HSQC spectrum of the reaction mixture when the product is primarily cobyrinic acid and the last intermediate. The *b*, *d*, *e*, and *g* carboxamides resonate at 110.1, 108.2, 109.1, and 111.3 ppm downfield from an ¹⁵NH₃ standard. The ¹H resonances for the syn and anti protons of the amide groups are also shown in this figure.

When the enzymatic reaction was quenched after 15 min, the only observable ¹⁵N resonance corresponds to that of carboxamide *e* at 109.2 ppm (Figure 3A). This result establishes that carboxylate *e* is the first group to be amidated by CbiP. Shown in Figure 3B is the NMR spectrum after the reaction was allowed to proceed for 30 min. In this spectrum the most predominant resonances originate from carboxamides *e* and *d*, demonstrating that the second group to be amidated is carboxylate *d*. There is, however, a modest change in the ¹⁵N chemical shift for the amide of carboxylate *e* in the triamide (109.2 ppm) and tetraamide (109.1 ppm) intermediates. When the reaction was allowed to occur for 1 h the concentration of the pentaamide intermediate became significant. The NMR spectrum in Figure 3C shows an increase in the relative abundance of the resonance for the amide of carboxylate *b* at 110.2 ppm and essentially no labeling of carboxylate *g*. Therefore, carboxylate *b* is the penultimate group to be amidated and carboxylate *g* participates last in the series of amidation reactions. The NMR results clearly establish the order of the amidation reactions as *e*, *d*, *b*, and *g*. The structural basis for the precise order of the amidation reactions is presently unknown.

The kinetic analysis of the reaction catalyzed by CbiP demonstrates that four carboxylates attached to the corrinoid ring are amidated in a dissociative fashion. The four carboxylates are amidated in a specific sequence beginning with carboxylate *e* and followed in turn by carboxylates *d*, *b*, and *g*. These results

Table 1. Kinetic Parameters for Reaction Catalyzed by CbiP

compound	K_m (μM)	k_{cat} (min ⁻¹)	K_i (μM)
ado-cobyrinic acid diamide	2.7 ± 0.06	3.6 ± 0.2	
ado-cobyrinic acid triamide	1.0 ± 0.06	2.4 ± 0.1	
ado-cobyrinic acid tetraamide	0.8 ± 0.02	3.6 ± 0.2	
ado-cobyrinic acid pentaamide	0.7 ± 0.08	8.4 ± 0.1	
cobyrinic acid			5.9 ± 0.2

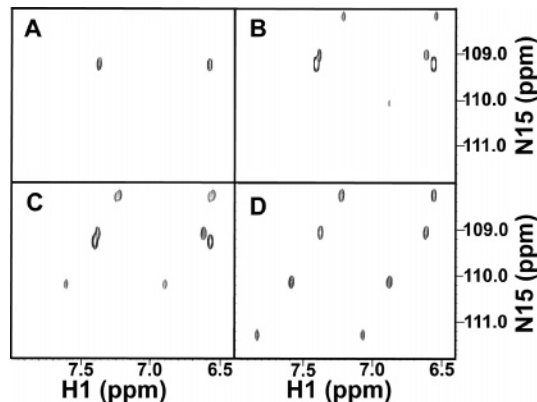


Figure 3. ¹H, ¹⁵N NMR spectra for the appearance of labeled cobyrinic and three partially amidated intermediates (triamide/tetraamide/pentaamide/cobyrinic acid): (A) 1:0:0:0, (B) 6:1:0:0, (C) 13:7:4:0, (D) 0:0:1:1.

demonstrate that the initial substrate can bind productively in only one of four possible orientations. After the amidation of carboxylate *e*, the first amidated intermediate must dissociate from the enzyme and rebind in an orientation that is rotated by ~90°. Similar events must follow after the amidation of carboxylates *d* and *b*. The homologous enzyme in the aerobic pathway from *Pseudomonas denitrificans* (CobQ) has previously been shown to catalyze multiple amidation reactions of **I** with a dissociative mechanism, but the specific order for the amidation of the four carboxylate groups has not been experimentally addressed.⁵ The related enzyme, cobyrinic acid *a,c*-diamide synthetase (CbiA or CobB from the anaerobic or aerobic pathways, respectively), also has a reaction mechanism that is sequential and dissociative.^{6,10}

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