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

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Vitamin B<sub>12</sub> 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.<sup>1</sup> Cobyric acid synthetase (CbiP) from *Salmonella typhimurium* catalyzes the ATP-dependent amidation of adenosyl-cobyrinic 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.<sup>2</sup> 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 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-cobyrinic acid *a,c*-diamide, was synthesized by enzymatic methods beginning with cobyrinic acid 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.<sup>2</sup> 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 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-cobyrinic 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.<sup>7</sup> The correlation between the model and the experimental data is presented in Figure 2. Table 1 provides the values for *K<sub>in</sub>* and *k<sub>cat</sub>* for I and the three partially amidated derivatives.
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 acid by CbiP was determined by $^1$H, $^{15}$N HSQC NMR spectroscopy. The $^{15}$N chemical shifts for the amidated intermediates are remarkably similar to one another. The affinity and turnover numbers for the substrate and three intermediates are remarkably similar to one another.

The enzymatic reaction was quenched after 15 min, the only observable $^{15}$N resonance corresponds to that of carboxylate 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 amidated intermediates e and d, demonstrating that the second group to be amidated is carboxylate d. There is, however, a modest change in the $^{15}$N 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 proceed for 1 h the concentration of the pentamidic intermediate became significant. The NMR spectrum in Figure 3C shows an increase in the relative abundance of the resonances 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 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. The related enzyme, cobyrinic acid c,d-diamide synthetase (CbiA or CobB from the anaerobic or aerobic pathways, respectively), also has a reaction mechanism that is sequential and dissociative.

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