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Enterobactin Synthetase-Catalyzed Formation of P^1,P^3-Diadenosine-5′-tetraphosphate†

Alison L. Sikora, Sean M. Cahill, and John S. Blanchard*

Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT: The EntE enzyme, involved in the synthesis of the iron siderophore enterobactin, catalyzes the adenylation of 2,3-dihydroxybenzoic acid, followed by its transfer to the phosphopantetheine arm of holo-EntB, an aryl carrier protein. In the absence of EntB, EntE catalyzes the formation of Ap_4A, a molecule that is implicated in regulating cell division during oxidative stress. We propose that the expression of EntE during iron starvation produces Ap_4A to slow growth until intracellular iron stores can be restored.

Adenylylated bis(5′-nucleosidyl) tetraphosphates (Ap_4N, where N is A, G, C, or U) have been proposed to act as signaling molecules in a variety of biological systems in both prokaryotes and eukaryotes (*1*). The cellular concentration of one such molecule, Ap_4A[1] (P^1,P^3-diadenosine-5′-tetraphosphate), has been shown to rapidly increase when *Escherichia coli* cells are exposed to heat shock or oxidative stress (*1*, *2*). Subsequent studies later revealed that Ap_4A binds to several *E. coli* stress-inducible proteins, including DnaK, GroEL, E89, C45, and C40, thus suggesting Ap_4A may serve as a modulator of cellular stress (*3*). Evidence of this role has been further supported by a recent study that investigated the nature of putative interactions of Ap_4A with *E. coli* molecular chaperone GroEL (*4*). At > 37 °C, GroEL chaperone activity is known to be decreased in favor of its protein storage function (*5*). However, Tanner and co-workers showed that the binding of Ap_4A to GroEL allosterically modulates the chaperone's function, resulting in the promotion of chaperoning activities over protein storage activity at higher temperatures. This observation suggests that Ap_4A may play a role in sustaining basic cell physiology and metabolism during stress and, then immediately poststress, aiding in the restoration of normal cellular function (*4*, *6*). Although the exact role of Ap_4A has yet to be elucidated, the identification of its multiple binding partners, all of which are stress-inducible proteins, suggests that this dinucleoside polyphosphate may be an important modulator of metabolism during cellular stress.

The intracellular concentration of Ap_4A increases during heat shock (∼10-fold) and oxidative stress (∼100-fold) (*1*, *7*). Blanquet and co-workers were the first to identify *E. coli* aminoacyl-tRNA synthetases (aaRSs) as the enzymes capable of the in vivo synthesis of Ap_4N (*8*). Specifically, lysyl-, methionyl-, phenylalanyl-, and valyl-tRNA synthetases have been shown to be diverted from amino acyl-tRNA production to that of Ap_4A when in the presence of $ZnCl_2$, amino acid, and pyrophosphatase (*7*, *8*). In the 20 years since that discovery, aminoacyl-tRNA synthetases remain as the only enzymes in *E. coli* with known Ap_4N synthase activity. Ap_4A phosphorylase, an enzyme capable of synthesizing Ap_4A when acting in reverse, has been identified in yeast; however, this enzyme has yet to be identified in *E. coli* (*9*). When considering the changing, and at times quite abundant, concentration of Ap_4A in *E. coli*, it seems plausible that other *E. coli* enzymes capable of Ap_4A synthesis may exist.

When *E. coli* cells are starved for the essential nutrient iron, the bacteria respond by synthesizing and secreting enterobactin {tris[*N*-(2,3-dihydroxybenzoyl)]serine trilactone}, which chelates extracellular ferric iron and imports it back into the cell to provide iron for metabolic processes (*11*). *E. coli* enterobactin synthetase is a nonribosomal peptide synthetase composed of six genes (*entA*−*F*) of known biochemical function: EntA, -B, and -C divert the central metabolite chorismate to generate 2,3-dihydroxybenzoate (DHB), while EntB, -D, -E, and -F catalyze the ATP-dependent assembly of enterobactin from three molecules each of DHB and L-serine (*12*). Inhibition of this essential pathway represents a promising strategy for antibacterial drug development, and therefore, we began a detailed kinetic and mechanistic characterization of EntE, a key component of the synthetase system. EntE ligase catalyzes the ATP-dependent transfer of DHB onto the phosphopantothenylated cofactor that is bound to the aryl carrier protein domain (ArCP) of EntB to yield the covalently arylated EntB. This molecule then serves as the aryl donor for amide bond formation in the final assembly of enterobactin (*13*). EntE belongs to the family of aryl acid adenylating enzymes (AAAE) that are characterized by a two-step adenylation/ligation reaction (*14*) (Scheme 1). In the adenylation half-reaction, the enzyme catalyzes the condensation of DHB and ATP to form an adenylate intermediate (*15*). We sought to use pre-steady state approaches to determine the rate of adenylation for EntE; however, the results of this analysis were quite unexpected. Here we describe the EntE-catalyzed formation of Ap_4A.

As described for other enzymes (e.g., *Mycobacterium smegmatis* cysteine ligase and *Mycobacterium tuberculosis* pantothenate synthetase), the rate of adenylation in the first half-reaction can be determined by single-turnover experiments using rapid-quench flow techniques with [α-^{33}P]ATP in the absence of the third substrate (*16*, *17*). The expected products of this reaction, radiolabeled adenylate and/or AMP, can be resolved from ATP on a PEI-TLC plate after separation with 0.9 M guanidine as the mobile phase. Reaction mixtures containing either DHB or

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*To whom correspondence should be addressed. Phone: (718) 430-3096. Fax: (718) 430-8565. E-mail: blanchar@aecom.yu.edu.

[1]Abbreviations: AAAE, aryl acid adenylating enzyme; aaRSs, aminoacyl-tRNA synthetases; AMP, adenosine monophosphate; Ap_4A, P^1,P^3-diadenosine-5′-tetraphosphate; ArCP, aryl carrier protein; DHB, 2,3-dihydroxybenzoate; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PEI-TLC, polyethyleneimine cellulose thin layer chromatography.

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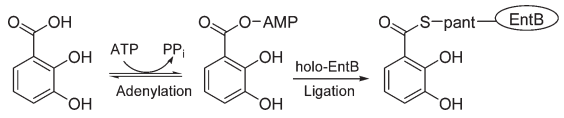
Scheme 1: Adenylation/Ligation Reaction Catalyzed by EntE



salicylic acid, ATP, and EntE resulted in a variety of radiolabeled spots on the TLC plate, including one which did not correspond to the location of expected products, adenylate or AMP (Figure S1, Supporting Information). To determine the identity of the radiolabeled products, we followed the adenylation reaction using ^{31}P NMR (see the Supporting Information for experimental details). Comparison of the ^{31}P NMR spectrum from reaction mixtures with and without added EntE revealed that an unexpected product was indeed formed (Figure 1). Analysis of the chemical shift positions and peak integrations allowed us to hypothesize that the identity of this product might be Ap_4A. The ^{31}P NMR spectrum is identical to that of commercially available Ap_4A.

To confirm that EntE, in the absence of the third substrate (phosphopantothenylated EntB, ArCP), was catalytically producing Ap_4A, we sought to isolate the product and confirm its identity via ESI-MS and ^{31}P NMR. The products of the reaction were separated by HPLC using a Mono Q ion exchange column with a gradient from 0.01 to 0.8 M ammonium bicarbonate (see the Supporting Information for details). The peak corresponding to Ap_4A was collected, lyophilized overnight, and dissolved in water for ESI-MS or 10% D_2O for ^{31}P NMR analysis. Using commercial Ap_4A as a standard, ESI-MS unambiguously confirmed the identity of the EntE reaction product to be Ap_4A (Figure S2, Supporting Information; see the Supporting Information for experimental details). Furthermore, ^{31}P NMR analysis was repeated on the purified product and resulted in chemical shifts and splitting patterns corresponding to the resonances of the unique peaks we initially observed (Figure 1C). Validation by both ^{31}P NMR and ESI-MS allowed us to confirm the EntE-catalyzed formation of Ap_4A.

Considering the rapid increase in the intracellular Ap_4A concentration during conditions of stress, it was essential to evaluate the kinetics of this reaction as well as the amount of Ap_4A formed catalytically (*1*, *2*). The concentration of Ap_4A produced, using either 0.1 mM salicylic acid or DHB as the aryl acid substrate, was determined by HPLC separation of Ap_4A and ATP and quantitation by peak integration and comparison to a standard curve (see the Supporting Information for experimental details). The rate of Ap_4A formation is nearly identical when either salicylic acid or DHB is used in the presence of 1 mM ATP (Figure 2). The maximal amount of Ap_4A produced appears to be ~80 μM after 16 h (Figure 2). The initial rate, calculated from 0 to 30 min, was 7.2×10^{-4} s^{-1} compared to the k_{cat} for the overall reaction of 2.8 s^{-1}. The maximal amount of Ap_4A formed was not equal to the concentration ATP (1 mM), suggesting that Ap_4A may act as an inhibitor of the reaction. When Ap_4A was tested as an inhibitor versus ATP, a competitive inhibition pattern was observed, yielding a K_i value of 1.2 ± 0.1 μM (Figure S3, Supporting Information). The much tighter binding of Ap_4A than of ATP ($K_m \sim 400$ μM) suggests that this molecule may bind to both the ATP and aromatic acid sites on the enzyme.

To determine if the formation of Ap_4A occurs while the DHB-adenylate is enzyme-bound or whether the reaction occurs in solution after release of the adenylate, we performed competition studies with the normal arylation substrate EntB. In the presence

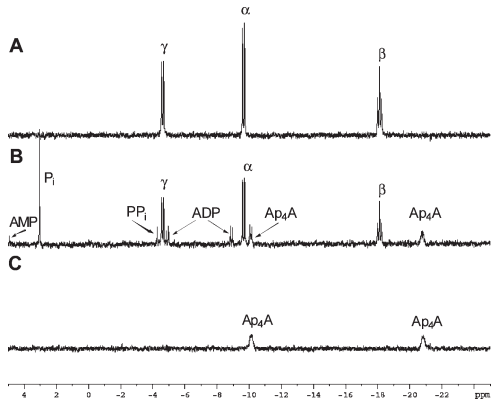


FIGURE 1: (A) ^{31}P NMR spectrum of a reaction mixture [100 mM HEPES (pH 7.8), 10 mM $MgCl_2$, 1 mM ATP, and 0.1 mM DHB] without EntE. Resonances at −4.62, −9.66, and −18.11 ppm correspond to γ-, α-, and β-phosphates, respectively, of ATP. (B) Reaction mixture including 10 μM EntE after reaction for 16 h. Resonances corresponding to ATP are still present, in addition to the formation of new products that were identified by use of commercial standards: AMP (4.65 ppm), inorganic phosphate (3.05 ppm), inorganic pyrophosphate (−4.29 ppm), ADP (−4.91 and −8.92 ppm), and Ap_4A (−10.11 and −20.75 ppm). (C) Ap_4A purified from the EntE reaction mix (B) after reaction for 16 h.

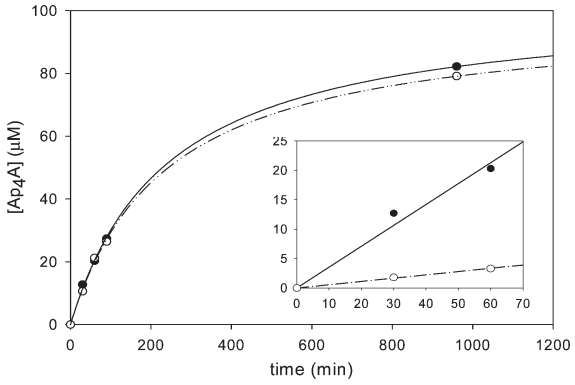
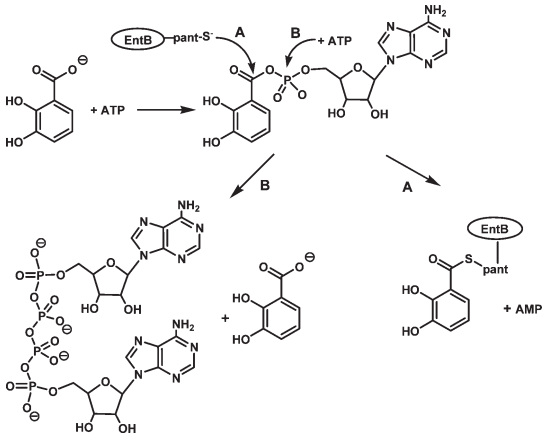


FIGURE 2: EntE-catalyzed Ap_4A production as a function of reaction time. Reaction mixtures included 100 mM HEPES (pH 7.8), 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM DHB (●) or salicylic acid (○), and 10 μM EntE and were analyzed via HPLC as described. For the inset, 10 μM holo-EntB ArCP was added to the standard reaction mix, resulting in inhibition of Ap_4A formation (○) when compared to the standard reaction mix containing DHB (●).

of 10 μM holo-EntB-ArCP, the rate of formation of Ap_4A is significantly inhibited (Figure 2, inset), even after all the holo-EntB-ArCP is arylated at these high EntE concentrations. This is most consistent with the DHB-adenylate being tightly bound, and reacting with ATP while bound in the absence of holo-EntB-ArCP.

We propose that, after aryl acid-adenylate formation in the first half-reaction, the γ-phosphate of ATP acts as a nucleophile to attack the α-phosphate group of the enzyme-bound adenylate, resulting in production of Ap_4A and regeneration of the free aryl acid (Scheme 2). In contrast, the normal reaction occurs between the thiol of the pantothenylated (holo) EntB-ArCP and the carbonyl group of the mixed carboxyl-phosphoric anhydride to generate the thioester. This differing regioselectivity of reactions between the common adenylate substrate and either ATP or holo-EntB-ArCP, that is, the P−O versus C−O chemistry shift, is also known for the formation of Ap_4A by aminoacyl tRNA synthetases (*7*).

Scheme 2: Proposed Chemical Mechanism of the Reaction Catalyzed by EntE in the Presence (A) and Absence (B) of Holo-EntB



Previous studies have shown that the in vivo Ap_4A concentration in unstressed wild-type *E. coli* cells is ~1−3 μM (*3*). This concentration of Ap_4A quickly increases to ~100−160 μM when the cells experience environmental stress, such as heat shock or oxidative stress (*18*). Conversely, cellular Ap_4A concentrations have not been shown to increase in cells experiencing a variety of metabolic conditions, including deficiencies in nucleic acids, amino acids, fatty acids, carbon, nitrogen, phosphate, or oxygen. Interestingly, iron is absent from this list (*7*). Our data, as described in this report, suggest that Ap_4A may be catalytically produced in *E. coli* under iron limiting conditions when the *ent* genes are expressed. Furthermore, we show that at least 80 μM Ap_4A is catalytically produced by EntE, a concentration that is significantly increased from that of the unstressed state and in the concentration range proposed to elicit biological responses in cells (*1*, *2*, *19*).

Until now, aminoacyl-tRNA synthetases were the only known enzymes capable of Ap_4A formation in *E. coli* (*10*). However, how these constitutively expressed enzymes would be able to rapidly respond catalytically to oxidative stress and heat shock is unclear. In this report, we demonstrate that the *E. coli* enzyme EntE catalytically produces Ap_4A in the absence of its third substrate. The synthesis of Ap_4A catalyzed by EntE provides new implications for the *E. coli* enterobactin synthetase system. Under iron-limiting conditions, the genes that produce both DHB and the enzymes that generate enterobactin will be expressed. Once produced, and once EntB is posttranslationally modified, they will initiate the synthesis and export of enterobactin will begin. If pantothenylation is slow compared to DHB synthesis and EntE production, then the system is perfectly poised to synthesize Ap_4A. This product will signal the cell of the iron limiting condition and slow growth while iron is scavenged from the surroundings. Thus, the synthesis of Ap_4A by EntE in concert with enterobactin assembly may provide additional aid to iron-depleted cells such that, while enterobactin is scavenging ferric iron, Ap_4A is acting to moderate cellular activities until sustainable iron concentrations are restored (*4*, *6*). The in vivo demonstration that Ap_4A is produced under iron limiting conditions is presently under investigation.

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SUPPORTING INFORMATION AVAILABLE

Materials, detailed experimental procedures, and figures of a TLC plate, ESI-MS data, and inhibition data. This material is available free of charge via the Internet at http://pubs.acs.org.

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