Rapid Reports

In Vitro Kinetic Analysis of Substrate Specificity in Enterobactin Biosynthetic Lower Pathway Enzymes Provides Insight into the Biochemical Function of the Hot Dog-Fold Thioesterase EntH[†]

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ABSTRACT: The *Escherichia coli* siderophore enterobactin is assembled from 2,3-dihydroxybenzoate (2,3-DHB) and L-serine by the nonribosomal peptide synthetases EntB and EntF. The processive thiol-template strategy used can be sabotaged by EntB misacylation. Through in vitro kinetic analysis, we demonstrate two potential routes to EntB misacylation and provide evidence for two mechanisms by which the hot dog-fold thioesterase EntH can potentially prevent or reverse EntB misacylation.

The siderophore enterobactin is synthesized by *Escherichia coli* to function in the harvesting of exogenous iron (*1*). Because pathogenic strains must sequester iron from the human host, the enterobactin biosynthetic enzymes are ideal targets for the development of novel antibiotics (*2*). Enterobactin biosynthesis proceeds via an upper chemical pathway that forms 2,3-dihydroxybenzoate (2,3-DHB) from chorismate and a lower, iterative pathway that assembles enterobactin from L-serine and 2,3-DHB. The lower pathway (Figure 1), which is the focus of our work, employs the two nonribosomal peptide synthetases (NRPSs) EntB and EntF (*3*). The phosphopantetheinyl transferase (PPTase) "EntD" catalyzes the transfer of pantetheinephosphate from CoA to the Ser residue of the 2,3-DHB carrier domain of EntB and of the peptidyl carrier domain of EntF to generate holo-EntB and holo-EntF, respectively. EntE catalyzes the adenylation of 2,3-DHB and the subsequent aroyl transfer to the pantetheine thiol of holo-EntB. The adenylation domain of holo-EntF catalyzes the adenylation of L-Ser and subsequent transfer of an acyl group to the pantetheine thiol carrier domain of holo-EntF. The EntF condensation domain then catalyzes the transfer of the 2,3-DHB unit to the amine group of the EntF-tethered L-Ser. The 2,3-DHB-Ser unit is then transferred to the active site Ser of the EntF cyclizing thioesterase domain, thereby freeing the pantetheine thiol for acquisition of a second 2,3-DHB-Ser-DHB unit. It in turn is transferred to the Ser moiety side chain of 2,3-DHB-Ser tethered to the TE domain. Following the addition of a third 2,3-DHB-Ser unit, the chain is cyclized by the thioesterase domain to form enterobactin.

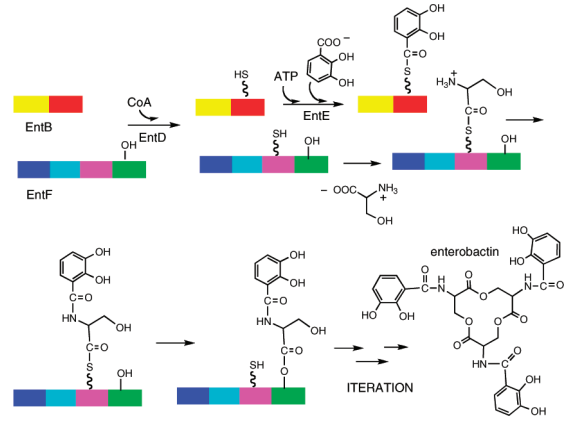

FIGURE 1: Steps of the enterobactin lower pathway.

The genes encoding the enterobactin biosynthetic enzymes are clustered within cotranscriptional units, one of which includes EntC and EntA of the upper pathway and EntE, EntB, and EntH (also known as ybdB) of the lower pathway. EntH is a member of the hot dog-fold thioesterase family (*4*). The biosynthetic pathways leading to NRPs in other bacteria possess stand-alone thioesterases from the α/β-hydrolase-fold family. Previous studies have provided evidence of the housekeeping role played by these enzymes, involving removal of non-native acyl units from the pantetheine thiols of misacylated NRPSs (*5*−*7*). Bouveret and co-workers (*8*) used in vivo two-hybrid and in vitro copurification techniques to demonstrate interaction between EntH and holo-EntB. These investigators proposed a proofreading role for EntH in which holo-EntB acylated with salicylic acid, produced by competing bacteria, is hydrolyzed.

As part of a larger effort to define structural determinants of substrate recognition within the hot dog-fold thioesterase family, we have targeted EntH for structure−function analysis. Little insight into the structure of the physiological substrate can be gleaned from the structure of unliganded *E. coli* EntH (Protein Data Bank entry 1vh9; Structural Genomix, 2003). On the other hand, knowledge of the colocalization of the gene encoding EntH within the enterobactin biosynthetic gene cluster, and the reported in vivo interaction between EntH and holo-EntB, provided us with direction in designing a focused substrate screen for evaluation of EntH catalytic efficiency toward possible physi-

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ological substrates. Building on the hypothesis that EntH functions to release holo-EntB from misacylated holo-EntBs, we first identified a potential source of misacylated holo-EntBs.

Two potential routes for the formation of misacylated holo-EntB arise from EntD and EntE substrate promiscuity. Specifically, PPTases are known to catalyze the transfer of an acyl-pantetheinephosphate unit from an acyl-CoA to the protein acceptor (*6*). If CoA is scarce, an endogenous acyl-CoA might substitute as a substrate for EntD, thereby leading to a misacylated holo-EntB. Likewise, if 2,3-DHB is scarce, an endogenous carboxylate metabolite might substitute as a substrate for EntE, resulting in the formation of misacylated holo-EntB. An early study (*9*) demonstrated EntE-catalyzed [^{14}C]sialicylation of holo-EntB, thus providing evidence of EntE substrate promiscuity.The ability of EntD to utilize acyl- and aroyl-CoAs as substrates was tested by incubating reaction solutions initially containing 100 μM isobutyryl-CoA, hexanoyl-CoA, lauroyl-CoA, palmitoyl-CoA or 4-hydroxybenzoyl-CoA, 2 μM EntD, 25 μM EntB, 50 mM HEPES, 5 mM DTT, and 50 mM NaCl (pH 7.5 and 37 °C) for 30 min followed by ESI-MS analysis. The percent conversion of EntB to acylated holo-EntB was determined by calculating the ratio of the corresponding peak areas in the mass spectrum (Figure SI1 of the Supporting Information) to show 100% conversion to acyl-holo-EntB for reactions of lauroyl-CoA, palmitoyl-CoA, and 4-hydroxybenzoyl-CoA and 23 and 67% conversion for reactions of isobutyryl-CoA and hexanoyl-CoA, respectively (Table SI1 of the Supporting Information). The results show that EntD is promiscuous and suggest a potential source of misacylated holo-EntB in vivo.

Next, the specificity of EntE toward aromatic acid metabolites was tested by incubating reaction solutions initially containing 125 μM holo-EntB (generated in situ using 400 μM CoA and 2 μM EntD), 1 μM EntE, 500 μM 2,3-DHB, 2,4-DHB, 2-hydroxybenzoate, 3-hydoxybenzoate (3-HB), 4-hydroxybenzoate (4-HB), benzoate, phenylacetate, 4-hydroxyphenylacetate (4-HPA) and 3-hydroxyphenylacetate (3-HPA) in 50 mM HEPES (pH 7.5), 1 mM ATP, 10 mM $MgCl_2$, and 5 mM DTT for 2 h at 25 °C. The percent conversion of holo-EntB to acylated holo-EntB was determined by ESI-MS analysis (Figure SI2 of the Supporting Information) to be 100% for reactions of 2,3-DHB and 2,4-DHB and 47, 25, 9, 14, and 0% conversion for reactions of 4-hydroxybenzoate, 3-hydroxybenzoate, 2-hydroxybenzoate, benzoate, and phenylacetate/hydroxyphenylacetate, respectively. These results demonstrate that EntE is promiscuous in acylating holo-EntB with benzoic acids and suggest a second possible source of misacylated holo-EntB in vivo.

Having provided support for the hypothesis that misacylated holo-EntBs might be formed in vivo, we focused our attention on the possible roles that EntH might play in "prevention and rescue". We first tested EntH thioesterase activity toward acyl- and aroyl-CoAs by using a DTNB-based continuous spectrometric assay. The results, reported in Table 1, show that hydroxylated benzoyl-CoAs and hydroxylated phenyacetyl-CoA are substrates having physiologically significant activity ($k_{cat}/K_m > 1 \times 10^4$ M^{-1} s^{-1}). Thus, it is conceivable that EntH is used to harvest CoA from endogenous acyl and aroyl-CoAs, thereby facilitating EntD-catalyzed formation of holo-EntB and avoiding the formation of misacylated holo-EntB.

Table 1: Steady-State Kinetic Constants for EntH-Catalyzed Hydrolysis of Acyl-CoA and Acyl-S-holo-EntB Thioesters Measured at pH 7.5 and 25 °C[a]

substrate	k_{cat} (s^{-1})	K_m (μM)
acetyl-CoA	$(4.4 \pm 0.2) \times 10^{-3}$	800 ± 90
propionyl-CoA	$(1.25 \pm 0.06) \times 10^{-2}$	400 ± 40
hexanoyl-CoA	$(5.8 \pm 0.1) \times 10^{-2}$	350 ± 90
decanoyl-CoA	$(2.67 \pm 0.04) \times 10^{-2}$	49 ± 3
lauroyl-CoA	$(2.81 \pm 0.03) \times 10^{-2}$	45 ± 2
palmitoyl-CoA	$(8.5 \pm 0.4) \times 10^{-2}$	55 ± 9
3-HPA-CoA	2.1 ± 0.5	37 ± 1
3-HB-CoA	1.21 ± 0.01	35 ± 1
4-HB-CoA	1.55 ± 0.05	21 ± 2
2,4-DHB-EntB	3.7 ± 0.1	25 ± 2
2,3-DHB-EntB	2.8 ± 0.1	16 ± 1
lauroyl-EntB	$(1.9 \pm 0.2) \times 10^{-2}$	32 ± 2

[a] Experimental details are provided as Supporting Information.

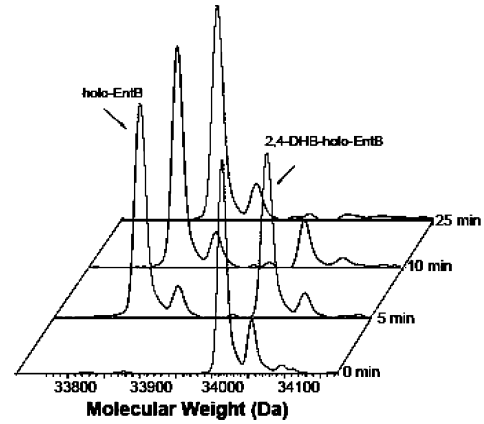

FIGURE 2: Time course for EntH-catalyzed hydrolysis of 2,4-DHB-holo-EntB monitored by ESI-MS. See the Supporting Information for details.

Next, EntH-catalyzed hydrolysis of acyl- and aroyl-holo-EntBs was examined. First, reaction of 98 μM 2,4-DHB-holo-EntB and 2 μM EntH in 50 mM HEPES buffer (pH 7.5 and 25 °C) containing 50 mM NaCl and 1 mM DTT was monitored by ESI-MS over a 25 min period to demonstrate the time-dependent consumption of 2,4-DHB-holo-EntB and formation of holo-EntB (Figure 2). The control reaction not including EntH showed <5% hydrolysis at 25 min. The rate of 2,4-DHB-holo-EntB was measured by ESI-MS as a function of EntH concentration to demonstrate linearity (Figure SI3 of the Supporting Information). In addition, reaction of 250 μM 2,4-DHB-holo-EntB and 2 μM EntH in 50 mM HEPES buffer (pH 7.5) containing 50 mM NaCl was monitored by HPLC to demonstrate the time-dependent formation of 2,4-DHB (Figure SI4 of the Supporting Information).

Having demonstrated EntH-catalyzed conversion of 2,4-DHB-holo-EntB to holo-EntB and 2,4-DHB, we employed a DTNB-based spectrophotometric assay to determine the steady-state kinetic constants for the hydrolysis of lauroyl-holo-EntB, 2,4-DHB-holo-EntB, and 2,3-DHB-holo-EntB (Table 1). The k_{cat} and K_m values measured for lauroyl-holo-EntB hydrolysis are equivalent to those measured for lauroyl-CoA. Because repeated attempts to synthesize 2,3-DHB-CoA and 2,4-DHB-CoA chemically failed, we were unable to make a direct comparison. Nevertheless, 2,3-DHB-holo-EntB and 2,4-DHB-holo-EntB proved to be only 2−4-fold better substrates than 3-HB-CoA and 4-HB-CoA. The results suggest that EntH binds the phosphopantetheine arm of the

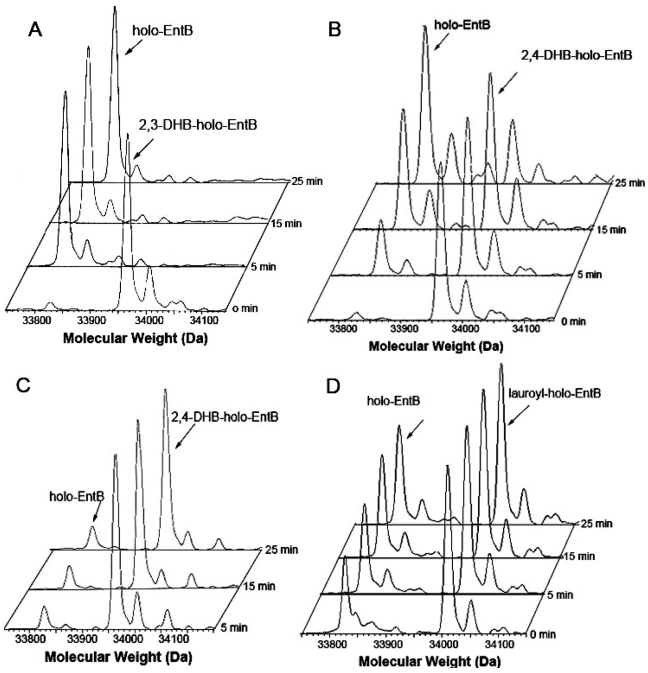

FIGURE 3: ESI-MS spectra measured as a function of incubation time for reactions of EntF with (A) 2,3-DHB-holo-EntB, (B) 2,4-DHB-holo-EntB, (C) 2,4-DHB-holo-EntB without ATP and L-Ser, and (D) lauroyl-holo-EntB.

thiol unit and does not bind the nucleotide or EntB moieties. This kinetics-based finding is consistent with the report that EntH binds holo-EntB but not apo-EntB (*8*). We conclude that EntH recognizes the acyl-pantetheinephosphate and aroyl-pantetheinephosphate of the corresponding CoA or EntB thioester substrate and that it discriminates potential substrates on the basis of the structure of the acyl/aroyl unit, as indicated by the distinct preference observed for aromatic substrates versus aliphatic substrates (Table 1).

The observation that the lower pathway intermediate 2,3-DHB-holo-EntB is efficiently hydrolyzed by EntH raises the important question of how EntH might selectively remove misacylated holo-EntB. One possible scenario is that EntF removes the 2,3-DHB unit from 2,3-DHB-holo-EntB more effectively than does EntH, whereas the reverse might be true for misacylated substrates. To explore this idea, we measured the comparative rate at which L-Ser-holo-EntF abstracts the 2,3-DHB, 2,4-DHB, or lauroyl group from the corresponding charged holo-EntB by using ESI-MS to monitor the reactions (see the Supporting Information for details). The results (Figure 3) show that 2,3-DHB-holo-EntB (Figure 3A) is processed significantly faster than 2,4-DHB-holo-EntB (Figure 3B; control reaction in Figure 3C) and that the rate of formation of holo-EntB from lauroyl-holo-EntB (Figure 3D) does not exceed the rate of spontaneous hydrolysis.

The slower reaction rate with misacylated holo-EntB might provide EntH with the opportunity to hydrolyze the misacylated holo-EntB, thus avoiding precursor misincorporation and/or premature termination of the assembly process. Overproduction of EntH, however, impairs enterobactin synthesis (*8*).

In summary, we have demonstrated two possible routes for formation of misacylated holo-EntB that are made possible by the low substrate specificity of EntD and EntE. We have also shown by kinetic analysis that EntH can potentially prevent EntD-catalyzed mischarging of EntB through hydrolysis of endogenous acyl- or aroyl-CoAs and that EntH can release the holo-EntB from misacylated holo-EntB. Future work in this area will focus on the X-ray crystal structure determination of EntH in complex with inert substrate analogues so that the structural determinants of substrate recognition may be determined.

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SUPPORTING INFORMATION AVAILABLE

Detailed experimental protocol, tables, and figures reporting experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

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