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Suppression of Linear Side Products by Macromolecular Crowding in Nonribosomal Enterobactin Biosynthesis

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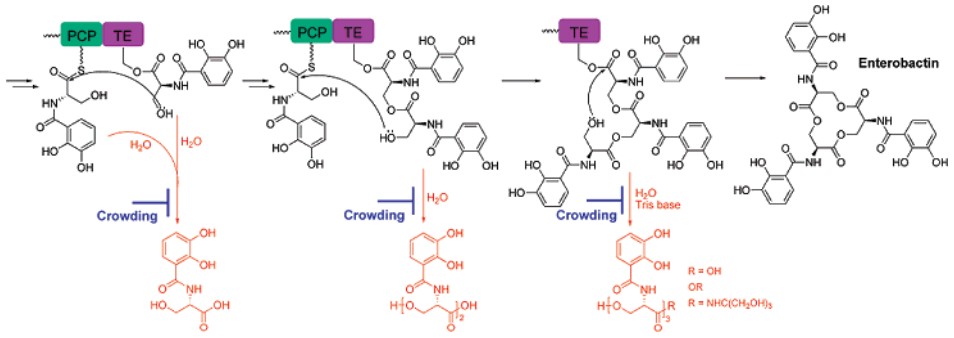
ABSTRACT



Nonribosomal enterobactin synthetase of *Escherichia coli* was found to prematurely release a large amount of linear precursors in an in vitro reconstitution. However, these side products are suppressed to negligible levels by polymeric cosolvents that create macromolecular crowding, a prominent feature of the intracellular environment. These findings show that macromolecular crowding is essential to normal functioning of the nonribosomal peptide synthetase and suggest that it may be crucial to biotechnological utilization of similar enzyme systems.

Nonribosomal peptide synthetases (NRPSs) are large, modular proteins responsible for biosynthesis of many medicinally important peptide natural products.[1] There is intense interest in using these synthetases in biomedical research because of their ability to assemble complex natural peptide products and analogues from simple amino acids. For example, the cyclosporine synthetase has been used in isotope-labeling of cyclosporine A and synthesis of various analogues of the natural product.[2] Enniatin synthetase has also been used for analogue synthesis.[3] However, the cyclosporine synthetase has been found to prematurely release precursors in the cell-free reconstitution.[4] In addition, the stability and synthetic efficiency of the synthetase are less than desirable.[2] Similar problems were found in cell-free reconstitution of dozens of other NRPSs.[5]

As a step to overcome these problems, we chose enterobactin synthetase as a model to investigate the factors influencing the activities of these multifunctional enzymes. We noticed that the previous cell-free reconstitution of NRPSs was exclusively carried out in dilute solution with a

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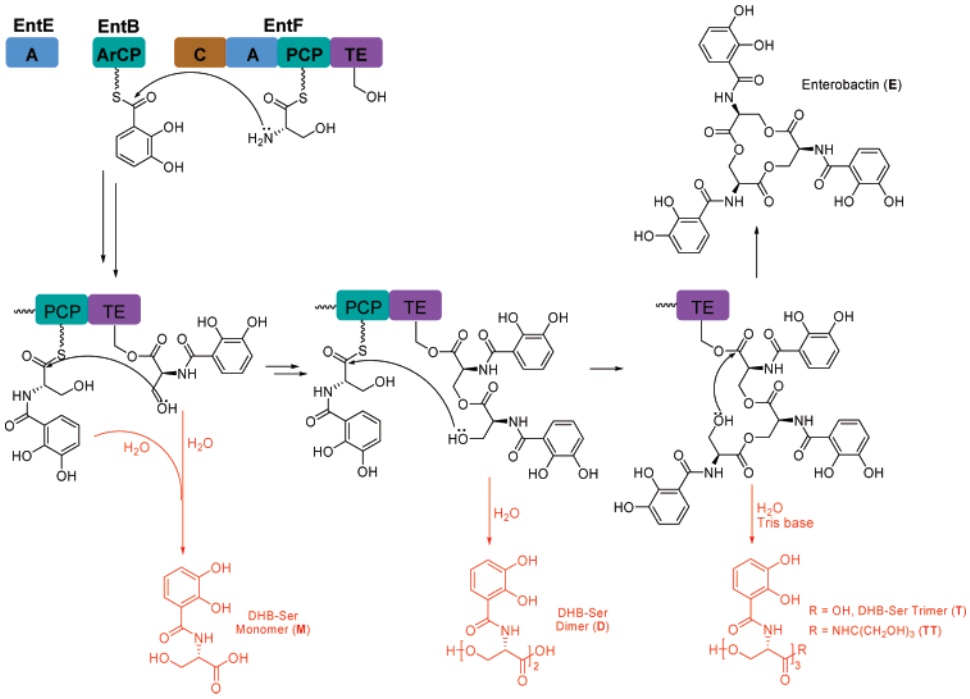
Scheme 1. Non-ribosomal Enterobactin Biosynthesis and the Premature Release of the Precursors[a]



[a] A, adenylation domain; C, condensation domain; ArCP, aryl carrier protein; PCP, peptidyl carrier protein; TE, thioesterase domain; vertical squiggle line, phosphopantetheinyl.

low macromolecular content. This in vitro condition differs significantly from the highly crowded intracellular environment,[6] where biomacromolecules occupy a large proportion of volume and cause the excluded volume effect or macromolecular crowding.[7] We created in vitro crowding conditions to mimic the intracellular environment and studied the effects of macromolecular crowding on the activities of the enterobactin NRPS. It was found that macromolecular crowding suppresses premature release of precursors from the synthetase. This finding provides the first experimental evidence for the importance of the crowded intracellular environment to catalytic properties of a nonribosomal peptide synthetase.[8]

The enterobactin synthetase is a two-module NRPS responsible for siderophore biosynthesis in *E. coli* under iron-deficient conditions (Scheme 1).[9] Its protein components EntB, EntE, and EntF were determined by rabbit polyclonal antibodies to be 1.4, 0.35, and 0.65 μM, respectively, in cells induced for enterobactin synthesis by iron starvation (Figure S2, Supporting Information). These proteins were prepared through recombinant expression according to the reported methods.[10] The half-saturation concentrations of the recombinant EntB and EntE and the maximum reaction rate of the synthetase are consistent with the previously reported values[11] (Figures S6 and S7, Supporting Information). However, the synthetase containing EntB, EntE, and EntF at their physiological concentrations in a dilute buffer produced a large amount of linear side products, which include the aminolytic release product of DHB-Ser trimer by Tris base (TT) and DHB-serine monomer (M), dimer (D), and trimer (T) (Figure 1A).[12] The correct enterobactin product was only 44% of the total turnover products. This result demonstrates that the enterobactin NRPS is not different from other NRPSs in being prone to release the precursors prematurely.[4,5] This conclusion does not contradict a previous investigation in which no linear side products were found in the initial 10 min at a low EntF concentration and high EntB/EntF ratio.[11] Actually, the linear products were formed with a mass

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(8) Nonionic small molecule detergents have been reported to enhance the macrocyclization activity of the putative terminal thioesterase domain of tyrocidine synthetase (see: Yeh, E.; Lin, H.; Clugston, S. L.; Kohli, R. M.; Walsh, C. T. *Chem. Biol.* **2004**, *11*, 1573). This detergent effect is different from the crowding effects discussed here.

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(12) The synthetase components were purified to homogeneity as shown in Figure S1 (Supporting Information). EntB and EntF were correctly pantotetheinylated as shown by MALDI-ToF spectra in Figures S3 and S4 (Supporting Information). The linear side products and enterobactin were identified by ESI-ToF mass spectrometry as shown in Figure S5 (Supporting Information).

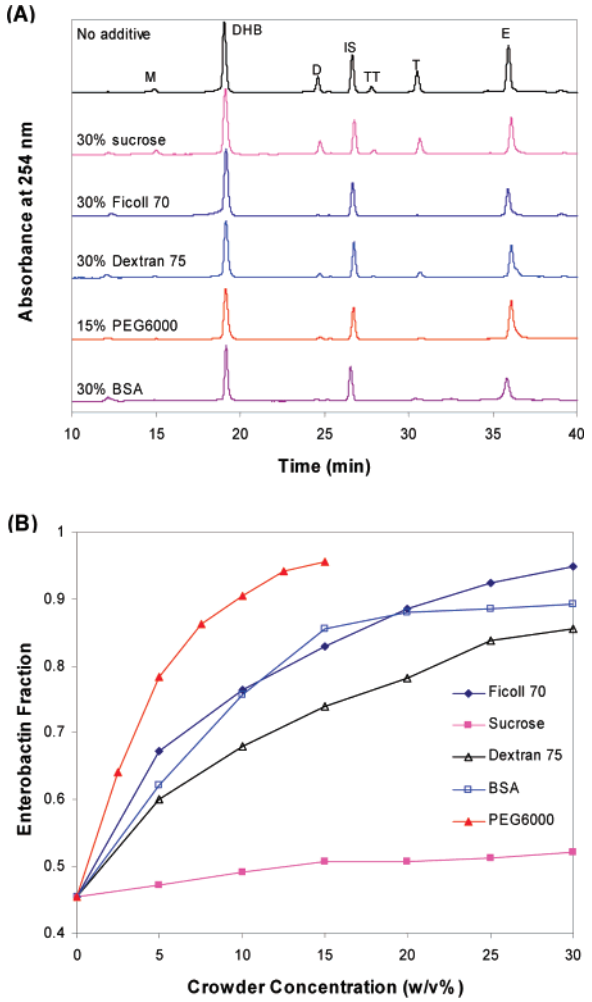

Figure 1. Effects of macromolecular crowding on the nonribosomal enterobactin synthesis. (A) HPLC analysis of the products from the reactions in the absence and presence of a crowding agent; (B) Variation of enterobactin fraction in the total turnover product with the concentration of a crowder or sucrose. See text for peak labels. IS: internal standard = ethyl 3,4-dihydroxybenzoate.

percentile yield of at least 25% when the reaction was extended beyond 10 min under those conditions.[13]

An inert polymer of sucrose recommended to mimic the intracellular crowding,[7c] Ficoll 70, was included in the reaction medium to examine its effects on the in vitro enterobactin synthesis. Interestingly, it was found that the linear side products were suppressed to negligible levels in 30% (w/v) Ficoll 70 solution (Figure 1A). In the meantime, the synthesis of enterobactin was little affected (see Table 1S (Supporting Information) for details), resulting in a percentage increase from 44% in noncrowded solution to >95% in 30% Ficoll 70 solution (Figure 1B) for enterobactin in the turnover products. In comparison, the enterobactin percentage increase was only 6% in 30% sucrose solution with a comparable polarity to 30% Ficoll 70 (Figure 1A,B), indicating that the side-product suppressing effect of Ficoll was not due to polarity change caused by the crowding agent.

Besides Ficoll 70, three other commonly used crowding agents, namely dextran 75, bovine serum albumin (BSA), and poly(ethylene glycol) (PEG6000), were also found to suppress the linear side products and increase the enterobactin percentage in the turnover products to a similar extent (Figure 1A,B). Noticeably, like Ficoll 70, all of these crowding agents seems to only reduce the production of the linear side products; production of the normal enterobactin product of the synthetase is minimally affected (Figure 1A). This side-product suppressing effect from all of the structurally distinct crowders can only be caused by the common interaction of all of the crowding agents with the synthetase, which is the nonspecific repulsion interaction—the only influence exerted by macromolecular crowding.[7] This effect, therefore, is a true macromolecular crowding effect. As shown in Figure 1B, PEG6000 is able to achieve a high enterobactin-enhancing effect at a much lower concentration in comparison to other crowding agents, probably due to its additional interaction with the proteins as demonstrated in a previous investigation.[14]

All the crowding agents were also found to decrease the reaction rate of the in vitro enterobactin synthesis (Figure 2), while the reaction rate was less affected in 30% sucrose solution. This rate reduction is consistent with the HPLC observation (Figure 1A) that normal production of enterobactin is little affected while production of the linear side products is suppressed by the crowding agents. In contrast, this rate reduction is unlikely due to the diffusion-impeding effect of crowding[7] on components of the synthetase because the reaction rate is far from the diffusion limit.

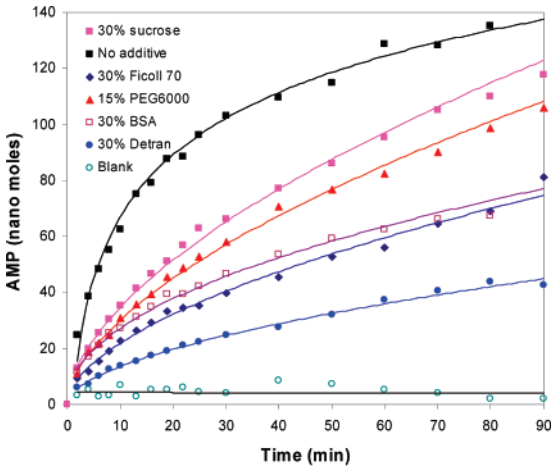

Figure 2. Crowding agents decrease the rate of AMP generation in the nonribosomal enterobactin synthesis.

(13) The EntB saturation reported in ref 11 is able to reduce the side products from >80% to 25% at a low EntF concentration, but unable to completely suppress their formation. It actually increases the side product formation at the physiological EntF concentration. See Figure S8 in the Supporting Information for more details. The premature release of linear side products is universally suppressed by 30% Ficoll 70 to < 5% irrespective of the composition of the synthetase.

(14) Minton, A. P. *Mol. Cell. Biochem.* **1983**, *55*, 119.

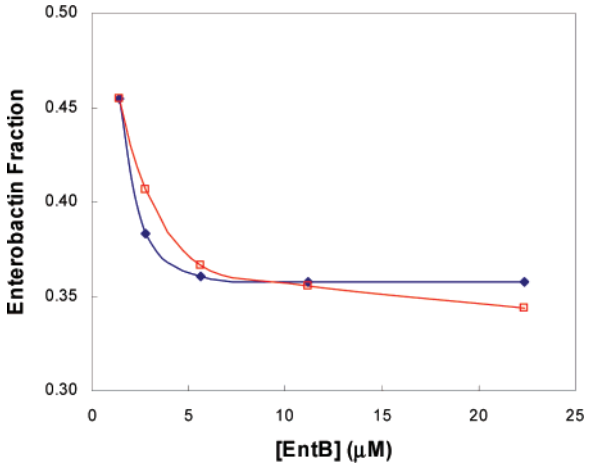

Figure 3. Decrease of enterobactin fraction in the total turnover product with increasingly saturating EntB at the physiological concentration of EntF and a saturating concentration of EntE. The reaction was carried out in 75 mM Tris·HCl buffer (pH 7.5) containing 0.65 μM EntF, 1.5 mM DHB, 1.5 mM serine, 10 mM ATP, 10 mM $MgCl_2$, 5 mM DTT, varied concentrations of EntB, and 0.35 μM EntE (♦) or 2 μM EntE (□) at 37 °C for 2 h.

Macromolecular crowding might affect the enterobactin synthesis through influencing the specific enzyme−substrate interaction between EntB and EntF, based on its well-known effect of promoting protein−protein interaction.[7] This EntB-EntF interaction was indeed slightly strengthened as indicated by the decrease of EntB half-saturation concentration from 0.50 μM in noncrowded buffer to 0.36 μM in 30% Ficoll 70 (Figure S7, Supporting Information). To examine how the product specificity was affected by this affinity increase, the EntB−EntF interaction was alternatively strengthened by increasing the EntB concentration under non-crowding conditions. Surprisingly, the premature release of the precursors in the enterobactin synthesis was actually increased (Figure 3). This result clearly showed that the strengthened EntB−EntF interaction is not a cause of the observed side-product suppressing effect of macromolecular crowding. Future investigation of the cause of this crowding effect should focus on potential crowding-induced structural changes in protein components of the synthetase, which have been suggested to alter the functional activities of several monofunctional enzymes.[15]

In summary, we have shown that macromolecular crowding suppresses premature precursor release from the enterobactin synthetase. Since the synthetase shares the same modular domain structure and thiotemplate catalytic mechanism with other NRPSs, this crowding effect is unlikely limited to the enzymatic synthesis of enterobactin but is more likely general to nonribosomal synthesis of other peptide natural products. Results presented here suggest that macromolecular crowding is not only critical to the correct understanding of the functions of NRPSs, but also an indispensable factor in their biotechnological utilizations.

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Supporting Information Available: Experimental procedures, Table S1, and Figures S1−S8. This material is available free of charge via the Internet at http://pubs.acs.org.

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