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Structural change of the enterobactin synthetase in crowded solution and its relation to crowding-enhanced product specificity in nonribosomal enterobactin biosynthesis

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ABSTRACT

Significant conformational change is detected by circular dichroism and fluorimetry for the major component of the enterobactin synthetase in crowded solutions mimicking the intracellular environment. The structural change correlates well with the extent of the crowding-induced side product suppression in nonribosomal enterobactin synthesis. In contrast, protein-stabilizing solvophobic agents such as glycerol have no effect on the formation of side products, excluding crowding-induced protein stability as a cause for the observed enhancement of the product specificity of the synthetase. These results strongly support that macromolecular crowding is an indispensable physiological factor for normal functioning of the nonribosomal enterobactin synthetase by altering the active sites to increase its product specificity.

Nonribosomal peptide products are a class of structurally diverse compounds including many important pharmaceutical products, such as the antibiotic vancomycin and immunosuppressive cyclosporin A. They are synthesized by large multifunctional enzymes called the nonribosomal peptide synthetases (NRPSs), which assemble the end product from simple amino acid monomers in a stepwise manner. There is intense interest to use these megaenzymes to produce structurally complex peptide products either in vivo in heterologous microorganisms or in vitro in an approach called 'total biosynthesis'.[1] Recent years have witnessed great progress in deciphering the chemical logic of the assembly line enzymology,[1–4] which has greatly facilitated the biotechnological utilization of the NRPSs. However, relatively little is known about the factors influencing the synthetic efficiency of the synthetases, which are generally unstable and difficult to reconstitute in vitro.[5,6] This hampers the efforts to understand the structure–activity relationship and in vitro utilization of these enzymatic systems.

Recently, we found that macromolecular crowding, which results from a high total concentration of macromolecules inside cells,[7–10] is critical to the product specificity of the *Escherichia coli* enterobactin synthetase.[11] The synthetase is a two-module NRPS comprising 2,3-dihydroxybenzoate (DHB)–adenosine 5′-monophosphate (AMP) ligase (EntE), aryl-carrier protein (ArCP) (EntB C-terminal domain), and a four-domain protein (EntF)[12] as shown in Figure 1. In dilute solution, the reconstituted enterobactin NRPS converts about a half of the starting materials to enterobactin and another half to a mixture of the linear enterobactin precursors, which include the aminolytic product of 2,3-DHB-Ser trimer by Tris base (TT) and 2,3-DHB-serine monomer (M), dimer (D), and trimer (T). The production of the side products is suppressed to a negligible level by creating a reaction medium mimicking the crowded intracellular environments using inner polymers such as Ficoll 70, Dextran, polyethylene glycol (PEG), and bovine serum albumin (BSA).[11] However, it is not known how the crowding condition enforces this side product suppression. It has been shown that the well known macromolecular crowding effect of promoting protein–protein interaction has no role in the observed suppression of the side products.[11]

Besides promoting macromolecular association, macromolecular crowding is also known to stabilize proteins.[8–10] To determine whether this protein stabilizing effect plays a role in the increased product specificity, we first investigated the possibility that the side products were generated due to instability of the enterobactin synthetase in dilute solution. The enterobactin biosynthetic proteins including EntB, EntD, EntE, and EntF were prepared as previously reported.[11] *apo*-EntB and *apo*-EntF were phosphopantetheinylated by EntD and the post-translational modifications were confirmed to be correct by an increase of mass of 340 Da, which is equivalent to addition of a phosphopantetheinyl group, in a MALDI-TOF mass spectroscopic analysis. The enterobactin synthetase was then incubated with the substrates for 1.5 h at 37 °C under non-crowding conditions as previously reported.[11] After removing the small molecules in the reaction mixture by gel filtration, the synthetase was added new substrates and its activity was reassessed. It was found that the

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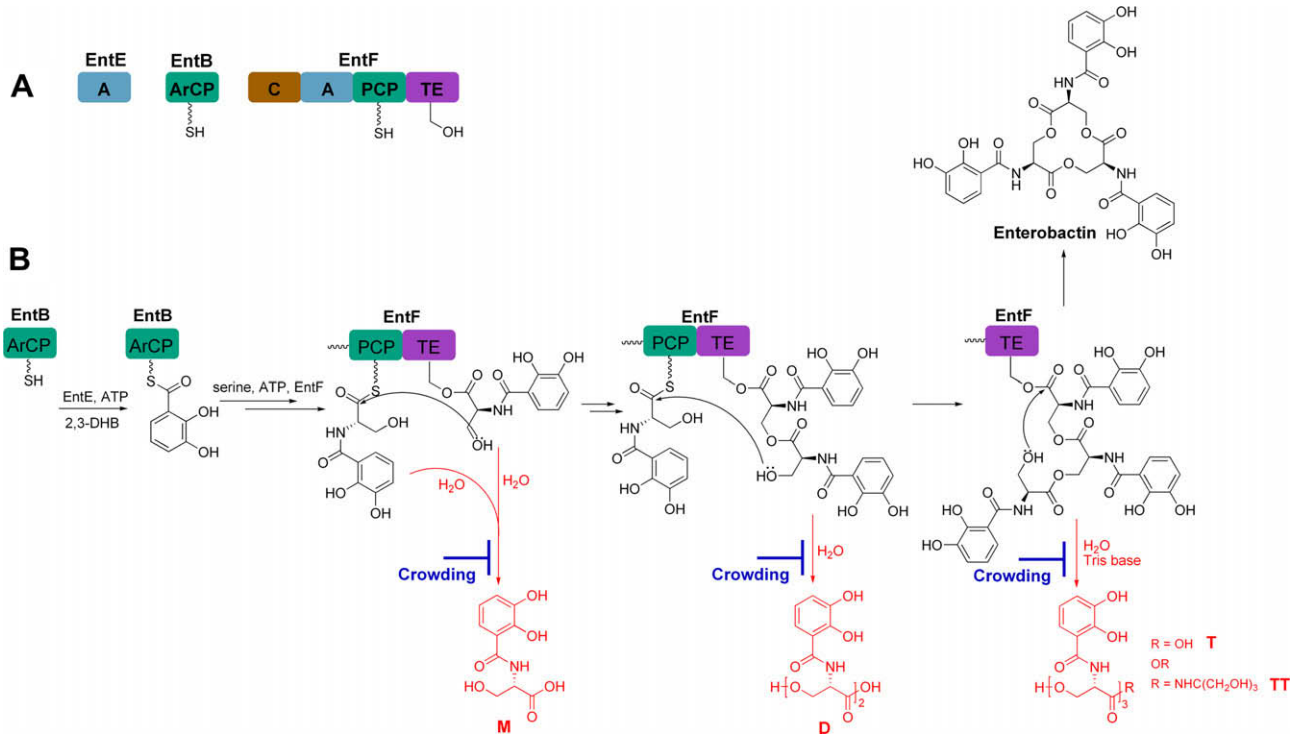

Figure 1. (A) Domain structure of the enterobactin NRPS from *Escherichia coli*. (B) The premature precursor release in enterobactin biosynthesis and its suppression by macromolecular crowding. The isochorismatase domain of EntB is omitted for simplicity. A: adenylation domain; C: condensation domain; ArCP: aryl-carrier protein; PCP: peptidyl carrier protein; TE: thioesterase domain (type I); vertical ∿∿: phosphopantetheinyl tether.

initial AMP generation rate of the spent synthetase in dilute solution was 578.4 min^{-1}, which was identical to that of the fresh synthetase (573.6 min^{-1}) within experimental errors.[13] In addition, the circular dichroism (CD) spectrum of EntF isolated from the spent synthetase through size exclusion column chromatography overlays with fresh EntF from 200 to 260 nm (data not shown). These experiments demonstrate that both the structure and the activity of the enterobactin NRPS are stable throughout the monitored reaction period and that the side products are not formed due to instability of the enterobactin synthetase under non-crowding conditions.

Next, we used glycerol to stabilize the enterobactin synthetase and observed its effect on the product specificity.[13] This small molecule is incapable of causing macromolecular crowding but is extensively used to stabilize NRPSs due to its higher solubility and stronger solvophobic effect.[14,15] At a concentration up to 30% (v/v), glycerol was found to promote premature release of the enterobactin precursors by forming an adduct with the EntF-attached 2,3-DHB-Ser dimer (gD) or 2,3-DHB-Ser trimer ester (gT) (Fig. 2), instead of suppressing the side products like crowding agents. The gD and gT adducts were confirmed by their molecular ions ($[M+Na]^+$) at 561.04 and 784.07, respectively, and were formed in addition to the previously identified hydrolytic or aminolytic products of the precursors by water or Tris base in the buffer.[11] These results again demonstrate that protein stability plays no role in the observed side product suppression by the crowding agents.

Sucrose is another solvophobic small molecule demonstrated not to have side product suppressing effect.[11] However, there is a major difference between these two small molecule co-solvents: sucrose does not form adducts with the enterobactin biosynthetic intermediates but glycerol does. This difference strongly suggests that the active sites in the major component of the synthetase, EntF, are accessible to solvent molecules in a size-dependent manner under non-crowding conditions. Under this hypothesis, small-size molecules like water, Tris base, and glycerol are able to access

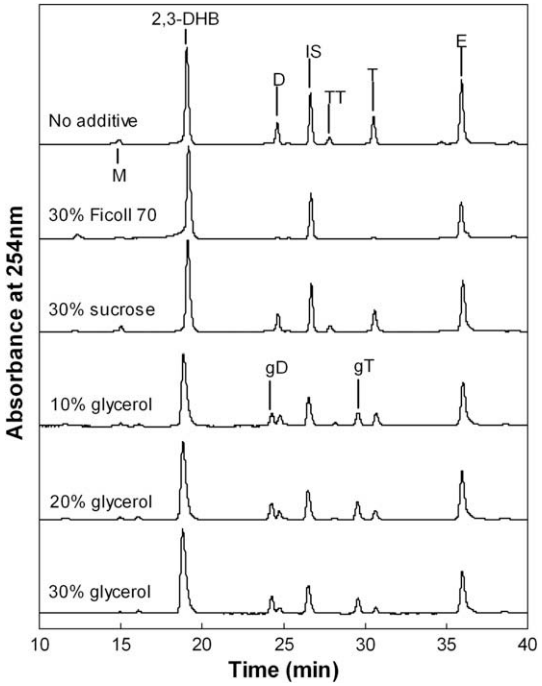

Figure 2. Effect of glycerol on nonribosomal synthesis of enterobactin. Shown are HPLC traces of the reaction products in the absence and presence of various co-solvents. IS: internal standard = ethyl 3,4-dihydroxybenzoate; E: enterobactin. See text and Figure 1 for the structure of other compounds.

the active sites to form the identified hydrolytic, aminolytic, or glycerolytic products, whereas the larger-sized sucrose is unable to penetrate the active sites to react with the biosynthetic intermediates. Under crowding conditions, this solvent accessibility of the

synthetase active sites must be blocked because the side products are suppressed to negligible levels.[11] In other words, macromolecular crowding may alter the active site structure of the synthetase to suppress the side products.

To test this possibility, the major component of the enterobactin NRPS directly associated with the formation of all the side products in the enterobactin synthesis, EntF, was examined for conformational changes under crowding conditions. A significant secondary structure change was detected by circular dichroism (CD) as indicated by change of the signal intensity at 210 nm with crowdedness of the buffer. As shown in Figure 3A, CD signal at 210 nm is progressively diminished as the level of macromolecular crowding is increased, whereas the signal at 222 nm remains essentially unchanged. In comparison, no conformational change was observed in solutions of sucrose, the Ficoll 70 monomer with comparable polarity to the crowding agent with the same weight percentile concentration. This crowding-induced structural change was further corroborated by continuous decrease of the tryptophan (Trp) fluorescence of EntF in increasingly crowded solutions of Ficoll 70 and the lack of similar fluorescence change in sucrose solutions (Fig. 3B). CD and Trp fluorescence spectrometry also found that the other two components of the enterobactin NRPS, EntB, and EntE, underwent crowding-induced conformational changes in Ficoll 70 solutions, but not in solutions of sucrose at a concentration up to 30%. However, the structural changes in EntB and EntE are not expected to contribute significantly to the side product suppression because they are not directly involved in the enzymatic steps leading to the formation of the linear side products.

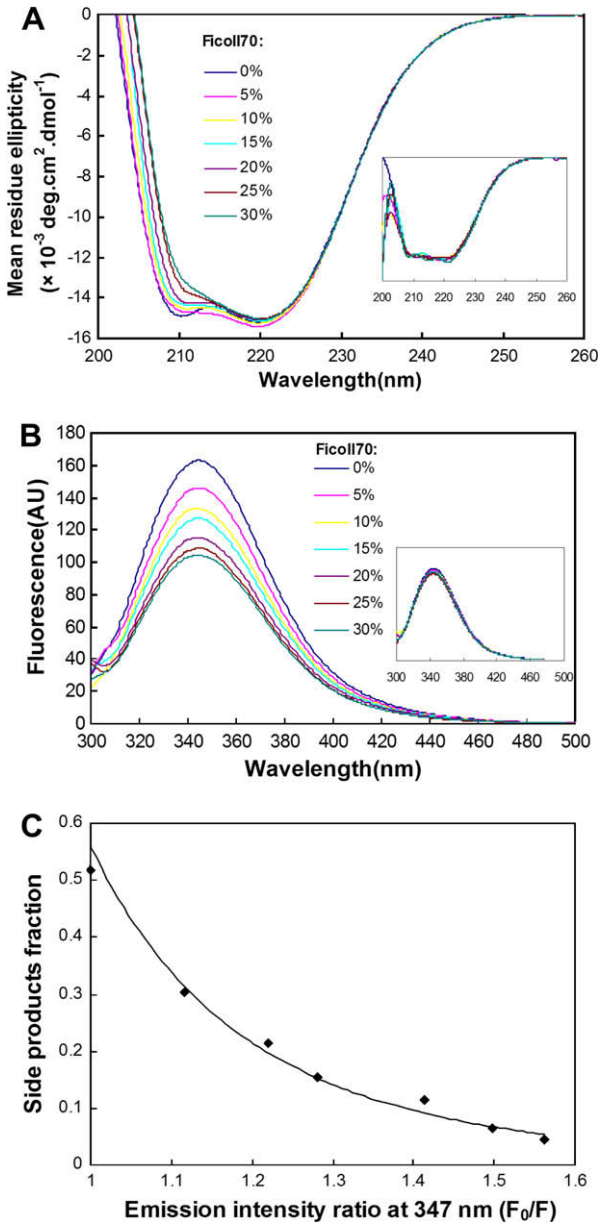

Figure 3. (A) Far-UV circular dichroism (CD) spectra of EntF in Ficoll 70 and sucrose (inset) solutions. (B) Tryptophan fluorescence spectra of EntF in Ficoll 70 and sucrose (inset) solutions. Color code for sucrose concentration in the inset is the same as that for Ficoll concentration. (C) The weight fraction of the side products in the total turnover products is exponentially decreased with the crowding-induced structural change as indicated by tryptophan fluorescence decrease at 347 nm. F_0 and F represent tryptophan fluorescence of EntF at 347 nm in buffers containing no crowding agent and a varied amount of Ficoll 70, respectively.

The fraction of the side products in the total turnover products generated in the nonribosomal enterobactin synthesis was found to decrease exponentially with increasing change in EntF structure, which was indicated by the crowding-induced EntF tryptophan fluorescence decrease at 347 nm in Ficoll 70 solution (Fig. 3C). This direct correlation lends strong support to the hypothesis that macromolecular crowding causes structural change in EntF to reduce the accessibility of the solvent molecules to its active sites, thus leading to the observed suppression of the side products.[11]

Macromolecular crowding has been shown to cause repacking of several monofunctional enzymes and alters their active sites to affect their catalytic activities.[16–18] The secondary structural change indicated by CD and fluorimetry suggests that domains in the multifunctional EntF also undergo repacking. Such structural change likely makes EntF active sites more compact and less accessible to small solvent molecules, thereby stop or reduce release of the biosynthetic intermediates by water, Tris base, or glycerol from the bulk solution. Additionally, macromolecular crowding may also affect domain dynamics to better coordinate inter-domain transfer of the activated precursors in EntF to shorten their exposure to solvent molecules and prevent them from premature release. This probable dynamic effect is consistent with the finding that crowding is able to alter flap dynamics of HIV-1 protease[19] and a recent structural study showing that the dynamics of the PCP and thioesterase (TE) domains of EntF are crucial to the activity of the NRPS synthetase.[20]

In summary, we have shown that the structure of EntF of the enterobactin synthetase is significantly changed in crowded solutions mimicking the intracellular environment. This structural change correlates well with the extent of the observed side product suppression in the nonribosomal enterobactin synthesis by macromolecular crowding. In contrast, protein-stabilizing solvophobic agents such as glycerol have no effect on the formation of side products, excluding crowding-induced protein stability as a cause for the observed enhancement of the product specificity of the synthetase. These results strongly support that macromolecular crowding is an indispensable physiological factor for normal functioning of the nonribosomal peptide synthetase by altering its active sites to increase its product specificity.

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References and notes

1. Sattely, E. S.; Fischbach, M. A.; Walsh, C. T. *Nat. Prod. Rep.* **2008**, *25*, 757.
2. Finking, R.; Marahiel, M. A. *Annu. Rev. Microbiol.* **2004**, *58*, 453.
3. Sieber, S. A.; Marahiel, M. A. *Chem. Rev.* **2005**, *105*, 715.

4. Fischbach, M. A.; Walsh, C. T. *Chem. Rev.* **2006**, *106*, 3468.
5. von Döhren, H.; Keller, U.; Vater, J.; Zocher, R. *Chem. Rev.* **1997**, *97*, 2675.
6. Kleinkauf, H.; von Döhren, H. *Prog. Drug Res.* **1997**, *48*, 27.
7. Fulton, A. B. *Cell* **1982**, *30*, 345.
8. Zimmerman, S. B.; Minton, A. P. *Annu. Rev. Biophys. Biomol. Struct.* **1993**, *22*, 27.
9. Ellis, R. J. *TIBS* **2001**, *26*, 597.
10. Zhou, H. X.; Rivas, G.; Minton, A. P. *Annu. Rev. Biophys.* **2008**, *37*, 375.
11. Guo, Z.-F.; Jiang, M.; Zheng, S.; Guo, Z. *Org. Lett.* **2008**, *10*, 649.
12. Gehring, A. M.; Mori, I.; Walsh, C. T. *Biochemistry* **1998**, *37*, 2648.
13. The reaction rate and the turnover products of the enterobactin NRPS were assayed with reported high pressure liquid chromatography (HPLC)-based methods.[11] A typical reaction contained 1 μM EntE, 15 μM *holo*-EntB, 50 nM *holo*-EntF, 1.5 mM 2,3-DHB, 10 mM adenosine 5′-triphosphate (ATP), 1.5 mM L-serine in 75 mM Tris–HCl (pH 7.5) buffer supplemented with 10 mM $MgCl_2$ and 5 mM DTT. The pH value of the mixture was re-adjusted after addition of the additives. Aliquots (100 μL each) were taken from a reaction mixture at an appropriate time interval and quenched immediately by addition of 15 μL of 1 N HCl. In investigation of the glycerol effect, enzyme concentrations were at their physiological concentrations (EntE 340 nM; *holo*-EntB 1.4 μM and *holo*-EntF 650 nM) and the reaction mixture was incubated at 37 °C for 1.5 h before quenching with HCl.
14. Billich, A.; Zocher, R. *J. Biol. Chem.* **1987**, *262*, 17258.
15. Banko, G.; Demain, A. L.; Wolfe, S. *J. Am. Chem. Soc.* **1987**, *109*, 2858.
16. Casati, D. F. G.; Aon, M. A.; Iglesias, A. A. *Biochem. J.* **2000**, *350*, 139.
17. Jiang, M.; Guo, Z. *J. Am. Chem. Soc.* **2007**, *129*, 730.
18. Morán-Zorzano, M. T.; Viale, A. M.; Muñoz, F. J.; Alonso-Casajús, N.; Eydallín, G. G.; Zugasti, B.; Baroja-Fernández, E.; Pozueta-Romero, J. *FEBS Lett.* **2007**, *581*, 1035.
19. Minh, D. D. L.; Chang, C.-E.; Trylska, J.; Tozzini, V.; McCammon, J. A. *J. Am. Chem. Soc.* **2006**, *128*, 6006.
20. Frueh, D. P.; Arthanari, H.; Koglin, A.; Vosburg, D. A.; Bennett, A. E.; Walsh, C. T.; Wagner, G. *Nature* **2008**, *454*, 903.