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One-Pot Synthesis of Azoline-Containing Peptides in a Cell-free Translation System Integrated with a Posttranslational Cyclodehydratase

Yuki Goto,[1,2,*] Yumi Ito,[3] Yasuharu Kato,[1] Shotaro Tsunoda,[1] and Hiroaki Suga[1,4,*]
[1]Department of Chemistry, Graduate School of Science, The University of Tokyo, Tokyo 113-0033, Japan
[2]JST, PRESTO, Tokyo 113-0033, Japan
[3]Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, Tokyo 113-8656, Japan
[4]JST, CREST, Tokyo 113-0033, Japan
*Correspondence: y-goto@chem.s.u-tokyo.ac.jp (Y.G.), hsuga@chem.s.u-tokyo.ac.jp (H.S.)
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SUMMARY

Azoline moieties in the backbones of peptidic natural products are important structural motifs that contribute to diverse bioactivities. Some azoline-containing peptides (Az-peptides) are produced from ribosomally synthesized precursor peptides, in which cysteine, serine, and threonine residues are converted to their corresponding azolines by posttranslational modification through a cyclodehydratase. We have devised an in vitro biosynthesis system of Az-peptides, referred to as the FIT-PatD (*f*lexible *i*n vitro *t*ranslation) system, by the integration of a cell-free translation system with the posttranslational cyclodehydratase PatD. This system enabled the ''one-pot'' synthesis of a wide variety of Az-peptide derivatives expressed from synthetic DNA templates. The FIT-PatD system also facilitated mutagenesis studies on a wide array of precursor peptide sequences, unveiling unique in vitro substrate tolerance of PatD.

INTRODUCTION

The azolines thiazoline and oxazoline are 5-membered ring heterocycles found in the backbones of many peptidic natural products (Kalyon et al., 2011; McIntosh et al., 2009; Melby et al., 2011; Roy et al., 1999b). In some cases, the translation machinery first synthesizes precursor peptides, and Cys, Ser, and Thr (C/S/T) residues are converted to thiazoline, oxazoline, and methyloxazoline, respectively, by posttranslational cyclodehydratases. The resulting azoline-containing peptides (Az-peptides) are often further processed by other enzyme families to yield mature secondary metabolites containing azolines, azoles, or both. Recent genome-mining studies of relevant gene clusters have revealed the wide distribution of Az-peptide biosynthetic pathways in myriad prokaryotes (Donia et al., 2008; Lee et al., 2008). The enzymatic modification of C/S/T residues incorporates nonpeptidic moieties into the polyamide backbone, thereby endowing the final products with unique structural motifs and conformational strains. As a consequence, these heterocycles contribute to the diverse bioactivities of this class of natural products (Degnan et al., 1989; Drechsel and Jung, 1998; Hong and Luesch, 2012; Melby et al., 2011; Scholz et al., 2011).

For posttranslational cyclodehydration, the precursor peptides commonly require two sequence elements, a leader peptide (LP) and core regions (Arnison et al., 2013). The LP region is highly conserved among a family of precursor peptides (Leikoski et al., 2013; Oman and van der Donk, 2010). It has been proposed that the LP region acts as a recognition motif for cognate cyclodehydratase(s), as a *cis*-acting chaperone, and/or as a secretion signal (Madison et al., 1997; Oman and van der Donk, 2010; Yorgey et al., 1993). The C/S/T residues present in the LP region are unmodified by the cognate cyclodehydratase, whereas those in the core region selectively undergo cyclodehydration to yield the corresponding Az-peptide. In some precursor peptide families, in addition to a specific core region, several homologs can also be modified by a single species of cyclodehydratase (Donia et al., 2006). This property of cyclodehydratase is presumably advantageous for generating a set of secondary metabolites with minimal codon alterations in the precursor peptide genes.

Mutagenesis studies on precursor peptides have been performed to gain insight into the substrate tolerance and specificity of cyclodehydratase-associated biosynthesis (Bowers et al., 2012; Deane et al., 2013; Dunbar et al., 2012; Madison et al., 1997; Melby et al., 2012; Sinha Roy et al., 1998; Young et al., 2012). Walsh and coworkers conducted mutagenesis studies on the microcin B17 precursor (Li et al., 1996; Yorgey et al., 1994), which is modified by cyclodehydratase McbB/C, and they elegantly revealed the critical determinants of substrate recognition as follows (Madison et al., 1997; Sinha Roy et al., 1998): (1) the LP region is essential for the modification event, (2) only C and S in the core region are modified by the enzyme (i.e., T was not efficiently modified), and (3) in the modification of the $X_{-1}SCX_{+1}$ site, in which S and C are successively cyclodehydrated, the flanking X_{-1} and X_{+1} residues must be small amino acid residues, such as G and A. In another example, Mitchell and coworkers conducted a genome mining study of *Bacillus* sp. Al Hakam and discovered a new putative biosynthetic gene cluster involving BalhC/D cyclodehydratase (Dunbar et al., 2012; Melby et al., 2012). In this system, the cyclodehydratase modifies only the C/S/T residues preceded by G, and

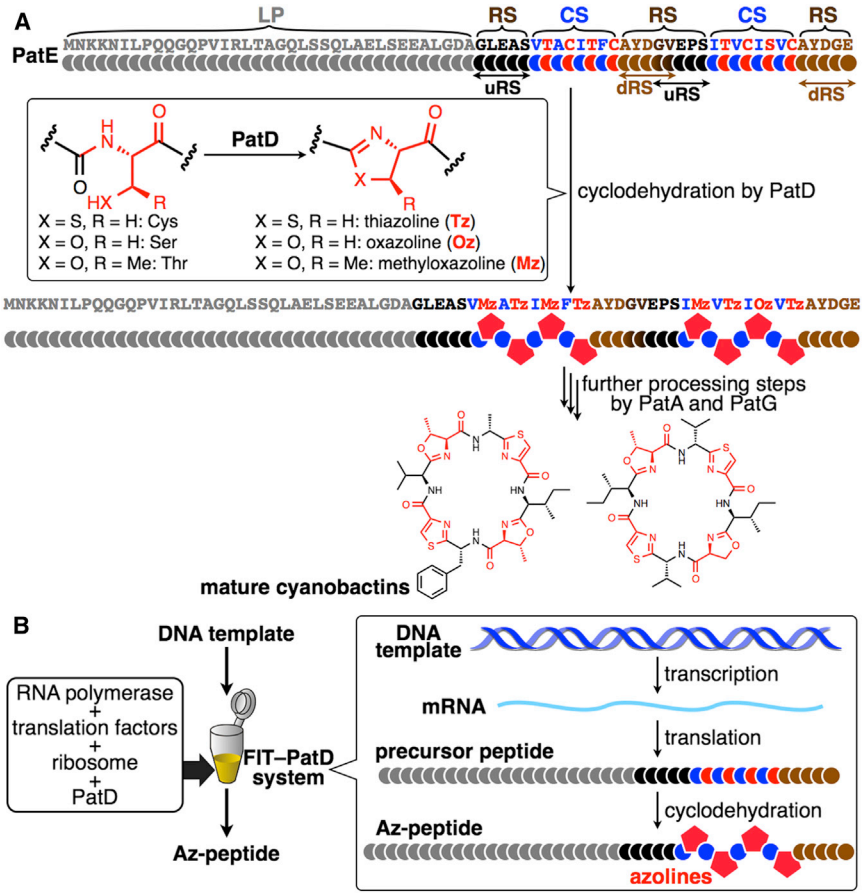

Figure 1. Present Knowledge of PatD Catalysis in Cyanobactin Biosynthesis and the Concept of the FIT-PatD System

(A) Azoline formation by PatD in the biosynthetic pathway of cyanobactins. PatD catalyzes cyclodehydration of C/S/T residues present in the CSs of PatE and installs azoline structures into the polyamide backbone. The resulting Az-peptide is further modified by other processing enzymes and eventually converted to mature cyanobactins. (Although "thiazole/oxazole-modified microcins" was recently introduced as a similar term, we use the name "Az-peptides" in this article because we feature peptides containing azolines rather than azoles, and we discuss Az-peptides including artificial ones that would not exhibit definite bioactivities, which are not included in microcins.) The N-terminal LP region, uRS, dRS, modifying C/S/T residues in the CS, and intact residues in the CS are shown in gray, black, brown, red, and blue, respectively.

(B) In vitro Az-peptide synthesis in the FIT-PatD system. A custom-made in vitro translation system in which RNA polymerase, a set of translation factors and enzymes, ribosome, and PatD are included; this FIT-PatD system allows "one-pot" synthesis of Az-peptides from the cognate DNA templates.

modification efficiency decreases with truncation of the core regions from the C terminus. These studies demonstrate that although the cyclodehydratases can accept some engineered substrates, their substrate tolerance is limited by their preference for the residues to be modified and the adjacent residues.

PatD, coded within the *pat* gene cluster of *Prochloron* spp. cyanobacteria (Schmidt et al., 2005), is a member of the cyclodehydratase family and is involved in the biosynthesis of cyanobactins (Degnan et al., 1989; Sivonen et al., 2010) that belong to a class of thiazole/oxazole-modified microcins (Melby et al., 2011). PatE, the naturally occurring substrate of PatD, consists of an N-terminal LP region, three recognition sequences (RSs), and two core sequences (CSs), aligned in the order LP-RS-CS-RS-CS-RS (Figure 1A) (Leikoski et al., 2013; Schmidt et al., 2005). The amino acid sequences of the CSs, which are the core regions in this system, are different, and both undergo cyclodehydration by PatD. The resulting azoline-incorporated CSs are further oxidized and macrocyclized by other modifying enzymes, PatG and PatA, to yield mature cyanobactins (Schmidt et al., 2005). Although the PatD cyclodehydration process shares similarities with those of McbB/C and BalhC/D, the sequence composition of PatE differs from the substrates of these enzymes, implying a unique substrate recognition mechanism and tolerance in PatD catalysis. A recent metagenomic analysis revealed the existence of several genes homologous to the PatE gene, which contains highly conserved LP and RS domains (Donia et al., 2008; Leikoski et al., 2013). Although these putative precursor peptides have various CSs, the residues flanking C/S/T in the CSs consist predominantly of hydrophobic alkyl amino acids (A, V, L, I, P, and M) or aromatic amino acids (F, Y, and H) (Figure S1 available online). Thus, knowledge regarding the requirements for LPs and RSs as well as the expandability of CSs to nonhydrophobic residues remains largely limited.

Here we have integrated PatD with a custom-made reconstituted cell-free translation system, referred to as the FIT (*f*lexible *i*n vitro *t*ranslation) system (Goto et al., 2011) (Figure 1B), and performed extensive mutagenesis and deletion analyses of PatE. This in vitro biosynthesis system, FIT-PatD, allowed us to more rapidly and readily construct a wide array of PatE variants expressed from designed DNA templates, compared with conventional methods (Roy et al., 1999a; Sinha Roy et al., 1998). We have identified the substrate recognition determinants for PatD catalysis using the FIT-PatD system, unveiling unique in vitro substrate tolerance of PatD. The results not only provide a better understanding of the inherent ability of this unique secondary-metabolite biosynthesis but also reveal its technological potential for producing a wider range of Az-peptides.

RESULTS

Integration of the FIT System with PatD

Cyclodehydration of engineered PatE sequences has previously been examined either by in vitro PatD reactions with PatE variants heterologously expressed and isolated from *Escherichia coli* or by coexpression of PatE variants and TruD, a PatD

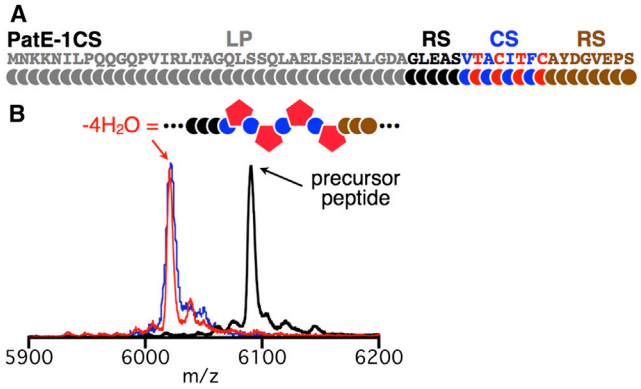

Figure 2. Synthesis of a Model Az-Peptide in the FIT-PatD System

(A) Amino acid sequence of a model substrate bearing a single CS (PatE-1CS). (B) Mass spectra of unmodified and modified PatE-1CS generated in the FIT-PatD system. The spectra in black and blue correspond to a precursor peptide synthesized in the ordinary FIT system (without PatD) and an Az-peptide generated from the FIT-expressing precursor peptide upon the treatment with PatD, respectively. The spectrum in red shows the same Az-peptide product of one-step synthesis in the FIT system containing PatD. Calculated and observed mass values are summarized in Table S2.

homolog, in *E. coli*, demonstrating that this cyclodehydratase family has some tolerance for PatE variants (Koehnke et al., 2013; McIntosh et al., 2010; McIntosh and Schmidt, 2010; Tianero et al., 2012). However, the labor-intensive preparation of substrates and isolation of Az-peptide products allows the investigation of limited numbers of subsets of their variation. To increase the throughput of mutagenesis on PatE, we used the FIT system (Goto et al., 2011), consisting of a custom-reconstituted in vitro translation system (Shimizu et al., 2005) and a flexible tRNA acylation ribozyme, so-called flexizyme (Murakami et al., 2006). The coupled transcription and translation of the FIT system can express PatE variants directly from their synthetic DNA template, thus largely facilitating the generation of substrate variants and analysis of Az-peptides.

To establish the FIT-PatD system, we first verified that recombinant PatD could modify a near-native PatE variant expressed in the FIT system. We designed a DNA template encoding a short precursor peptide consisting of LP-RS-CS-RS (PatE-1CS), in which the single CS region containing two each of C and T residues was flanked by two RS regions (Figure 2A). Upon expression of the DNA template in the FIT system, the product was analyzed by MALDI-TOF mass spectrometry (MALDI-TOF-MS), demonstrating that the expected PatE-1CS was produced without any detectable side products (Figure 2B, black line). When 6 μM of PatD was incubated with the crude PatE-1CS at 25°C for 14 hr, the original PatE-1CS peak was replaced with a peak corresponding to a 72 Da decrease in molecular mass (Figure 2B, blue line). This observation was consistent with a loss of four water molecules and indicated that the four C/T residues in the CS had been cyclodehydrated to yield the expected Az-peptide with two thiazolines and two methyloxazolines. The modified PatE-1CS peptide was digested with Glu-C endopeptidase, and the resulting C-terminal peptide fragment was subjected to tandem mass spectrometry (MALDI-TOF/TOF) (Figure S2A). The resulting fragmentation pattern further confirmed the formation of the four expected azoline moieties. We next performed "one-pot" synthesis of the Az-peptide in the FIT system in the presence of PatD. The desired peak corresponding to the Az-peptide was detected on MALDI-TOF-MS (Figure 2B, red line). Because of the facility of altering the sequences of precursor peptides expressed in the FIT-PatD system and detecting the Az-peptide products by MALDI-TOF-MS, we envisaged the utility of this system for extensive mutagenesis studies on PatE-1CS in in vitro PatD catalysis.

The Role of RSs in PatE for PatD Modification

The CSs in the native PatE are flanked by conserved G(L/V)E (A/P)S and AYDG(E/V) motifs (Donia et al., 2006; Schmidt et al., 2005). Recent biochemical and structural studies on PatA and PatG have shown that the conserved upstream RS (uRS) and downstream RS (dRS) in the native PatE (Figure 1A) play a role in defining the proteolytic sites in the macrocyclization of the CS (Agarwal et al., 2012; Koehnke et al., 2012; Lee et al., 2009). Therefore, it was postulated that the uRS and dRS are the sequence elements designating the core regions in cyanobactin biosynthesis. We here applied the FIT-PatD system to an investigation of the role of the uRS and dRS in in vitro PatD catalysis.

We designed a series of PatE-1CS mutants with altered uRS and dRS (Figure 3A). When a mutant lacking the dRS (ΔdRS) was expressed in the FIT-PatD system, all four C/T residues were cyclodehydrated, indicating that the dRS was not essential for PatD catalysis (Figure 3A, entry 2; Figure S3B). In contrast, deletion of the uRS (ΔuRS) decreased the efficiency of cyclodehydration, leaving one or two intact C/T residues (Figures 3A, entry 3, and 3B). This suggested that even though the uRS was not essential for an active substrate, it played an important role in catalytic efficiency. To identify the cyclodehydration sites, the Glu-C digested fragment of the modified ΔuRS peptide, which contains three azolines, was analyzed by using MALDI-TOF/TOF (Figure S2B). The observed fragmentations indicated that PatD was unable to modify the T residue closest to the LP region but was able to modify the other T and two C residues.

On the basis of the above results, we speculated that the uRS played a role as a spacer between the LP and CS rather than interacting with PatD as a specific sequence motif. To verify this hypothesis, we constructed precursor peptides whose uRS regions were substituted with pentaglycine (G5), pentaglutamine (Q5), pentaproline (P5), pentaglutamate (E5), or pentalysine (K5) (Figure 3A, entries 4–8; Figures S3C–S3G). These precursor peptides turned out to be fully modified after incubation with PatD, supporting the above hypothesis. We also constructed a precursor peptide with G5 inserted between the LP and uRS (G5-uRS) (Figures 3A, entry 9, and 3C). MALDI-TOF analysis of the product showed a total of five cyclodehydrations, very likely originating from the modifications of a S residue in the native uRS in addition to the four expected residues. This observation suggested that the S residue in the native uRS became a part of the CS by the insertion of the G5 spacer between the LP and uRS. Taken together, we concluded that the uRS in PatE acts as an arbitrary spacer between the LP and CS in the in vitro PatD reaction rather than as a specific RS motif for PatD.

A

entry	seq. name	amino acid sequences of RS mutants	# of modified residues
1	RS-WT*	LP –GLEAS–VTACITFC–AYDGV	4
2	ΔdRS*	LP –GLEAS–VTACITFC–A(deleted)	4
3	ΔuRS	LP –(deleted) –VTACITFC–AYDGV	3, 2
4	uRS>G5*	LP –GGGGG–VTACITFC–AYDGV	4
5	uRS>Q5*	LP –QQQQQ–VTACITFC–AYDGV	4
6	uRS>P5*	LP –PPPPP–VTACITFC–AYDGV	4
7	uRS>E5*	LP –EEEEE–VTACITFC–AYDGV	4
8	uRS>K5*	LP –KKKKK–VTACITFC–AYDGV	4
9	G5-uRS	LP –GGGGG–GLEAS–VTACITFC–AYDGV	5, 4
10	uRS-G5*	LP –GLEAS–GGGGG–VTACITFC–AYDGV	4

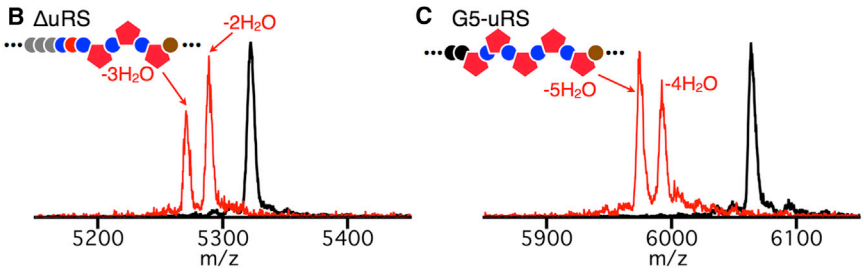

Figure 3. Studies on the uRS and dRS in PatD Catalysis

(A) RS mutants tested in this study. Dashed lines and purple residues indicate deleted and substituted or inserted residues, respectively. Red pentagons indicate the sites of azoline formation. Numbers of observed cyclodehydration are shown in the table. For mass spectra of mutants labeled with asterisks, see Figure S3.

(B and C) Mass spectra of ΔuRS (B) and G5-uRS (C). Spectra in black and red correspond to the precursor peptide and Az-peptide synthesized in the FIT and FIT-PatD systems, respectively. All variants were incubated with 6 μM PatD for 14 hr. Calculated and observed mass values are summarized in Table S2.

The Role of LP in PatE for PatD Modification

The LP region is also highly conserved among cyanobactin precursors, implying that it has an important role in biosynthesis involving PatD (Donia et al., 2006; Houssen et al., 2010; Leikoski et al., 2013). Because such LPs are commonly present in many precursor peptides in posttranslational modification systems, it has been postulated that they play a specific or multiple roles in posttranslational modification processes, such as an enzyme-recognition motif, a *cis*-acting chaperone, and/or a secretion signal (Madison et al., 1997; Neis et al., 1997; Oman and van der Donk, 2010; Yorgey et al., 1993).

To assess the importance of the LP region in PatD catalysis, we first introduced systematic deletions into the LP region of PatE-1CS and expressed the individual peptides in the FIT-PatD system. The series of LP-truncated mutants summarized in Figure 4A and Figure S4 revealed the importance of the LP C-terminal region. The substrates lacking the LP C-terminal portion (G20–A37) resulted in the Az-peptide with only a single cyclodehydrated residue (Figure 4A, entries 2 and 3). In contrast, the LP region consisting of G11–A37 (Figure 4A, entry 4) resulted in the fully (quadruply) modified Az-peptide as efficiently as with the full-length LP region. To our surprise, two LP mutants consisting of only six amino acids (Figure 4A, entries 6 and 7) were also modified by PatD to yield the fully modified Az-peptide, accompanied by partially modified Az-peptides (with two or three modifications). These results indicated that the C-terminal region of LP was important for active PatE substrates.

On the basis of the above information, we next attempted to further define critical residues within the C-terminal region of LP by means of point and multiple-site mutagenesis of PatE-1CS. To enhance the mutagenesis effect, the PatD concentration was lowered from 6 to 0.6 μM, and the reaction time was shortened from 14 to 2 hr. We first performed a point mutation in every residue within the L26–A37 region with alanine (A), asparagine (N), or glutamine (Q) (Figure 4B, entries 2–5; Figure S6, entries 2–20). In A and N point mutation studies, we found that two residues, L29 and L34, reduced the modification efficiency, while the latter mutation had more pronounced effect. To our surprise, the point mutation of acidic residues E28Q, E31Q, E32Q, and D36N had no negative effect on efficiency. Double mutations of the critical residues, L29N/L34N, yielded a singly cyclodehydrated peptide, while other double mutations, such as E28Q/E31Q and E31Q/E32Q, had a marginal negative effect (Figure 4B, entries 6–8; Figure S6, entries 21–27), indicating that L29 and L34 were far less tolerant in mutations compared with others. On the other hand, triple Q mutations of the acidic cluster in E28, E31, and E32 largely reduced modification efficiency (Figure 4B, entries 9–11; Figure S6, entries 28–32). Intriguingly, quadruple mutations of the C-terminal region of LP with P residues, which presumably broke the α-helical secondary structure, did not affect modification efficiency (Figure 4B, entry 12). These results have revealed that in LP, (1) L34 is essential; (2) L29 is important but less critical than L34; (3) the acidic cluster of E28, E31, and E32 is cooperatively important; and (4) the α-helical secondary structure is unimportant.

Although the LP C-terminal region was necessary for the full conversion of PatE to Az-peptides, we observed a single cyclodehydration even in the LP mutants lacking the C-terminal region (Figure 4A, entries 2 and 3; Figure S4, entries 2–7). Therefore, we tested a mutant lacking the entire LP region (ΔLP), that is, a precursor peptide consisting of uRS-CS-dRS. PatD modified a single residue in the ΔLP substrate along with some double modifications (Figure 4C, entry 1; Figure S5U), indicating that the LP region of the precursor peptide was not an essential determinant for an active substrate. Supplementing the PatD-ΔLP reaction with the full-length LP fragment enhanced the degree of cyclodehydration, yielding a mixture of fully (quadruply) and triply modified Az-peptides (Figure 4C, entry 2; Figure S5V). This suggested that LP is a *trans*-acting chaperone of PatD rather than an essential *cis* element of the PatE substrate for recruiting PatD.

A

entry	seq. name	amino acid sequences of LP mutants	# of modified residues
1	PatE-1CS	MNKKNILPQQGQPVIRLTAGQLSSQLAELSEEALGDA– (uRS CS dRS)	4
2	LP1–10	MNKKNILPQQ----------–	1
3	LP11–19	M----------GQPVIRLTA----------–	1, 0
4	LP11–37	M----------GQPVIRLTAGQLSSQLAELSEEALGDA–	4
5	LP26–37	M----------LAELSEEALGDA–	4, 3
6	LP26–31	M----------LAELSE----------–	4, 3, 2
7	LP32–37	M----------EALGDA–	4, 3, 2

B

entry	seq. name	amino acid sequences of LP mutants	# of modified residues
1	PatE-1CS	MNKKNILPQQGQPVIRLTAGQLSSQLAELSEEALGDA– (uRS CS dRS)	4
2	L29A	MNKKNILPQQGQPVIRLTAGQLSSQLAE**A**SEEALGDA–	4, 3
3	L29N	MNKKNILPQQGQPVIRLTAGQLSSQLAE**N**SEEALGDA–	4, 3, 2, 1
4	L34A	MNKKNILPQQGQPVIRLTAGQLSSQLAELSEE**A**GDA–	4, 3, 2, 1
5	L34N	MNKKNILPQQGQPVIRLTAGQLSSQLAELSEE**N**GDA–	2, 1
6	L29N/L34N	MNKKNILPQQGQPVIRLTAGQLSSQLAE**N**SEE**N**GDA–	1, 0
7	E28Q/E31Q	MNKKNILPQQGQPVIRLTAGQLSSQLA**Q**LS**Q**ALGDA–	4, 3, 2
8	E31Q/E32Q	MNKKNILPQQGQPVIRLTAGQLSSQLAELS**QQ**ALGDA–	4, 3, 2, 1
9	E28Q/E31Q/D36N	MNKKNILPQQGQPVIRLTAGQLSSQLA**Q**LS**Q**EALG**N**A–	4, 3
10	E31Q/E32Q/D36N	MNKKNILPQQGQPVIRLTAGQLSSQLAELS**QQ**ALG**N**A–	4, 3, 2, 1
11	E28Q/E31Q/E32Q	MNKKNILPQQGQPVIRLTAGQLSSQLA**Q**LS**QQ**ALGDA–	2, 1
12	LP4P	MNKKNILPQQGQPVIR**P**TAGQ**P**SSQL**P**ELSEE**P**LGDA–	4

C

entry	seq. name	amino acid sequences of LP mutants	# of modified residues
1	ΔLP	M----------–	2, 1
2	LP + ΔLP	MNKKNILPQQGQPVIRLTAGQLSSQLAELSEEALGD +	4, 3

Figure 4. Studies on the LP Region in PatD Catalysis

(A) Selected LP deletion mutants. The PatE variants were incubated with 6 μM PatD for 14 hr. Dashed lines denote deleted residues in LP, and the "# of modified residues" column indicates the numbers of modified residues observed in MALDI-TOF spectra (Figure S5) of the Az-peptide(s). A full set of LP deletion mutants is shown in Figure S4.

(B) Selected point and multiple-site mutants. The PatE variants were incubated with 0.6 μM PatD for 2 hr. Mutated residues are shown in black bold characters. The C-terminal region of LP (residues 26–37), to which extensive mutations were introduced, is underlined in entry 1. Hydrophobic and acidic residues present in this region are shown in green and purple, respectively, and the essential five residues identified by this analysis are underlined with red lines. A full set of point and multiple-site mutants is shown in Figure S6. For mass spectra of these mutants, see Figures S7 and S8.

(C) PatD modification of a precursor peptide lacking the entire LP region with and without *trans*-presenting LP. The PatE variants were incubated with 6 μM PatD for 14 hr. The corresponding mass spectra are shown in Figure S5.

An Extensive Collection of CSs for PatD Modification

Recent genome mining of *Prochloron* spp. has revealed a series of different CS components in PatE homologs (Donia et al., 2006; Leikoski et al., 2013). Although this suggests tolerance of PatD toward such CS variants, the sequences are biased toward the following trends (Figure S1): (1) CS lengths are limited to six to eight residues, (2) CSs consist primarily of hydrophobic and/or aromatic residues, and (3) successive C/S/T residues to be modified are not found, in contrast to the cyclodehydratase McbB/C system, in which successive azolines are formed (Li et al., 1996). Although it has been reported that TruD can modify engineered CSs containing nonnatural phenylalanine analogs (Tianero et al., 2012), their sequence composition also followed these trends. Therefore, tolerance of PatD toward CS motifs beyond the naturally observed trends was unknown.

To gain insight into the in vitro substrate tolerance of PatD, we designed precursor peptides having a wide variety of noncanonical CSs. These CS mutants were expressed in the FIT-PatD system, and their degrees of modifications were analyzed (Table 1; Table S1). This analysis revealed the following extraordinary properties of PatD: (1) PatD accepted diverse CS lengths as short as two residues with a single modifiable site and as long as 36 residues with 18 modifiable sites (Table 1, entries 2–7; Table S1, entries 1–9); (2) PatD was capable of modifying any of the C/S/T residues, but C was the most efficiently modified (Table 1, entries 8–10; Table S1, entries 26–35); (3) PatD modified residues independently of the sequence context (i.e., even those with adjacent nonhydrophobic resides could be modified) (Table 1, entries 11–17; Table S1, entries 10–25); and (4) PatD modified consecutive residues to yield successive azolines (Table 1, entries 19–21). Taken together, these results indicate that PatD has remarkable substrate tolerance toward a wide array of CSs.

PatD Modification of Short Precursor Peptides Consisting of Engineered LP, Linker, CSs, and Terminator

On the basis of our knowledge of the minimal PatD substrate composition, we designed two engineered precursor peptides for transformation to Az-peptides by PatD catalysis. The construct sLS-G5-CS4-A was composed of a short (13-mer) LP region and a G5 linker (sLP-linker), followed by a tetramer CS with two C residues, and terminating with a single A. The construct sLS-G5-CS16-A consisted of a 16-mer CS with eight C residues flanked by the sLP-linker and terminator. In both cases, the FIT-PatD system yielded the expected fully modified Az-peptides (Figures 5A and 5B). These results demonstrated that precursor peptides consisting of the sLP-linker and noncanonical CSs were compatible with PatD catalysis.

DISCUSSION

Our mutagenesis studies using the FIT-PatD system have shown that the conserved sequence motifs in the native PatE are not necessarily essential for the cyclodehydration reaction step catalyzed by PatD in vitro. Our experiments provide the following insights (Figure 6): (1) LP can act not only as a *cis* but also as a *trans* element, enabling us to postulate that it plays a role in PatD activation; (2) the residues in the C-terminal region of LP,

Table 1. Studies on the CS Region in PatD Catalysis

Entry	Sequence Name	Cassette Sequence	Possible Maximum Modifications	No. of Modified Residues
1	PatE-1CS (WT)	VTA*C*I*T*F*C*	4	4
2	CS-2	V*C*	1	1
3	CS-4	V*C*A*C*	2	2
4	CS-14	V*C*A*C*I*C*F*C*V*C*A*C*V*C*	7	7
5	CS-16	V*C*A*C*I*C*F*C*V*C*A*C*V*C*I*C*	8	8
6	CS-22	V*C*A*C*I*C*F*C*V*C*A*C*V*C*I*C*Y*C*F*C*I*C*	11	11
7	CS-36	V*C*A*C*I*C*F*C*V*C*A*C*V*C*I*C*Y*C*F*C*I*C*F*C*A*C*V*C*I*C*Y*C*F*C*I*C*	18	18
8	CS-allC	V*C*A*C*I*C*F*C*	4	4
9	CS-allT	V*T*A*T*I*T*F*T*	4	4, 3, 2
10	CS-allS	V*S*A*S*I*S*F*S*	4	4, 3, 2
11	CS-I5D	V*T*A*C*D*T*F*C*	4	4
12	CS-I5N	V*T*A*C*N*T*F*C*	4	4
13	CS-I5K	V*T*A*C*K*T*F*C*	4	4
14	CS-14-1R3R	RCRCICFCVCACVC	7	7, 6
15	CS-14-7R9R	VCACICRCRCACVC	7	7, 6
16	CS-14-11R13R	VCACICFCVCRCRC	7	7
17	CS-R2D2	RCDCDCRC	4	3
18	CS-C	VFALIMF*C*	1	1
19	CS-CC	VFALIM*C*C*	2	2
20	CS-CCC	VFALI*C*C*C*	3	3
21	CS-CCCC	VFAL*C*C*C*C*	4	4

C/S/T residues in CS are shown in italics. All variants were incubated with 6 μM PatD for 14 hr. The "No. of Modified Residues" column indicates the numbers of modified residues observed in MALDI-TOF spectra (Figure S9) of the Az-peptide(s). Additional CS mutants are also shown in Table S1.

especially E28, L29, E31, E32, and L34, are important for the function of LP; (3) the uRS acts as a spacer sequence whose primary role is to accommodate the downstream CS in the PatD active site; (4) the dRS is largely dispensable; and (5) a wide variety of functional CSs can be designed that expand on the naturally observed CS trends.

Our data, generated by extensive deletion and mutagenesis studies (Figures 4A and 4B), revealed that L29/L34 and acidic clusters in the C-terminal region are critical to retain the substrate activity. Indeed, the residues at the 28th to 34th positions are highly conserved in the cyanobactin precursor peptide family (Leikoski et al., 2013). Intriguingly, we observed that partial LP deletion mutants retaining the N-terminal region (Figure S4, entries 2–7) exhibited lower modification efficiencies than the complete LP deletion mutant, ΔLP (Figure 4C, entry 1). This means that the N-terminal LP region containing hydrophilic and basic residues could weakly inhibit PatD, which also indirectly suggests that the above sequence in the C-terminal region is the specific activator of PatD. Note that the observed full retention of the modification efficiency in an α-helix-broken mutant by proline substitutions (Figure 4B, entry 12) has contradicted previously reported data in which LP alone has an α-helical conformation in 50% aqueous trifluoroethanol (Houssen et al., 2010). Because our data reflect the functional importance of LP, we conclude that the α-helical secondary structure of LP is not critical for the activation of PatD when the critical L29/L34 and the acidic cluster are present in the C-terminal region.

With regard to the hypothesis that LP is an activator of PatD, we propose that without LP, PatD remains in a passive state (Figure 6). This passive state may be important for preventing indiscriminate modification of the proteome. Binding of LP activates PatD's catalytic ability, leading to the selective modification of precursor peptides. It should be noted that our hypothesis is partly contradictory to a model recently proposed for TruD (a homolog of PatD), in which the LP is not involved in the recognition of the CS's C-terminal Cys residue but is necessary for modification of the interior Cys residues (Koehnke et al., 2013). Our observation for the *trans*-activity of LP in the PatD catalysis suggests that the LP simply acts as an activator of PatD. We cannot yet rule out a possibility that this observation is limited to the in vitro activity of PatD or possibly related enzymes. However, similar observations of such *trans*-acting LPs have been also reported for lantibiotic and thiazole/oxazole-modified microcin biosynthetic systems (Dunbar and Mitchell, 2013; Khusainov and Kuipers, 2012; Oman et al., 2012; Patton et al., 2008). Thus, such an activation mechanism may be generic for the biosynthesis of ribosomally synthesized and posttranslationally modified peptides.

We have also observed that PatD accepts not only the naturally conserved G(L/V)E(A/P)S motif of the uRS but also other sequence motifs, such as G5, Q5, P5, E5, and K5 (Figure 3A, entries 4–8). Moreover, the dRS is completely dispensable (Figure 3A, entry 2). These observations imply that PatD does not rely on specific sequences in the CS flanking regions but rather can adapt to the RS requirements of other enzymes (Agarwal et al., 2012; Koehnke et al., 2012; Lee et al., 2009) in the biosynthesis of cyanobactins.

Naturally occurring PatE derivatives have a smaller set of sequence variations compared with the possible PatD

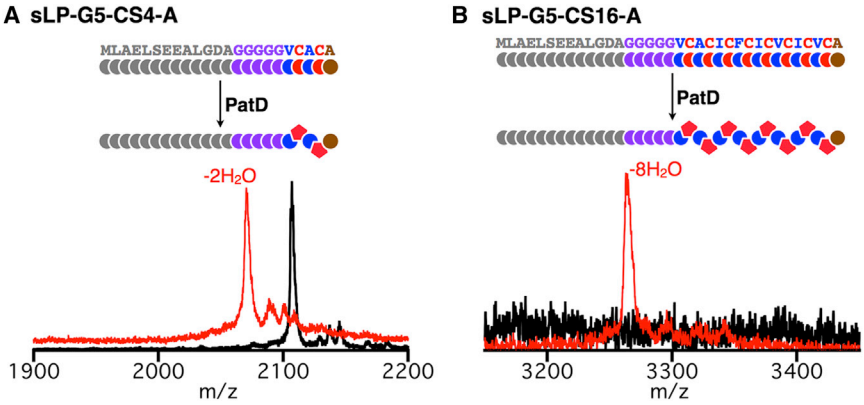

Figure 5. PatD Modification of Short Precursor Peptides Consisting of Engineered LP, Linker, CSs, and Terminator

(A and B) The engineered short precursor peptides, (A) sLS-G5-CS4-A and (B) sLS-G5-CS16-A, with their corresponding Az-peptides. Spectra in black and red correspond to the designed substrates without and with PatD treatment, respectively. The peptides were incubated with 6 μM PatD for 14 hr. The mass peak of the precursor sLS-G5-CS16-A peptide was undetectable, because cysteine-rich peptides are often difficult to recover in SPE desalting or to ionize on MALDI-TOF, resulting in very poor signal detection. Calculated and observed mass values are summarized in Table S2.

substrates demonstrated in this study. The selection and evolution of precursor peptides that could produce secondary metabolites with favorable bioactivities may have resulted in the biased sequence variation in nature. Alternatively, the subsequent enzyme reaction catalyzed by PatG may have less tolerance to the sequence variation. We also cannot rule out another possibility: that because the experiments in this study were performed in vitro under conditions in which a stoichiometric or an excess amount of PatD was used in the FIT-PatD system, the high substrate tolerance to LP, RS, and CS mutants observed in this study may not be necessarily applicable to in vivo reactions. Nevertheless, the present data show the remarkable catalytic potential toward a variety of PatE derivatives, at least in vitro. Moreover, the FIT-PatD system is applicable to the synthesis of diverse kinds of Az-peptides containing not only proteinogenic amino acids but also nonproteinogenic amino acids incorporated by the genetic code reprogramming methodology. In particular, a potential use of the FIT-PatD system is an integration with mRNA display (Hayashi et al., 2012; Hipolito and Suga, 2012; Kawakami et al., 2013; Kawakami and Murakami, 2012; Kodan et al., 2014; Morimoto et al., 2012; Nemoto et al., 1997; Passioura et al., 2014; Roberts and Szostak, 1997; Tanaka et al., 2013; Yamagishi et al., 2011), which will provide an opportunity to select bioactive Az-peptides against therapeutic targets from their libraries with extraordinarily high complexity. This is our clear direction of the future development of the FIT-PatD system.

In summary, we have established an in vitro FIT-PatD system that facilitates not only investigation of the sequence requirements of the substrate peptides but also generation of diverse Az-peptides. In principle, the FIT system can be coupled with other posttranslational modification enzymes, and such in vitro systems allow us to accelerate studies on their biosynthesis and expanding the chemical diversity of natural products as "pseudonatural products."

SIGNIFICANCE

The FIT-PatD system, an in vitro biosynthetic system for Az-peptides, was established by integrating a posttranslational cyclodehydratase PatD with a FIT system. The FIT-PatD system allows us to express diverse substrate variants and test their modification efficiencies in a one-pot fashion. Extensive mutagenesis and deletion analyses of PatD substrate identified the substrate recognition determinants for PatD catalysis. The high substrate tolerance of PatD implies that the FIT-PatD system could be a powerful tool to produce a wider array of Az-peptides.

EXPERIMENTAL PROCEDURES

Preparation of DNA Templates Coding PatE Mutants

Double-strand DNA templates coding PatE mutants, which are used for Az-peptide synthesis in the FIT-PatD system, were prepared using multistep PCR (method A) or primer extension followed by multistep PCR (method B). Primers and PCR templates used for the construction of DNA templates are summarized in Table S3. Sequences of all primers used in this study are listed in Table S4. The PCR mixture used in this study contained 10 mM Tris·HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM deoxyribonucleotide triphosphates, 0.1% (v/v) Triton X-100, and *Taq* DNA polymerase.

In method A, from the first to the penultimate PCR, an appropriate template DNA (see Table S3) was amplified using the designated forward and reverse primers (1 μM each). PCR was conducted with a 100 μl scale by 5 cycles of denaturing (95°C for 40 s), annealing (50°C for 40 s), and extending (72°C for 40 s). When the plasmid was used as a template DNA, the PCR cycle was increased to 20 cycles. The resulting PCR mixture was 200-fold diluted and used as a template DNA for the following PCR step. The final PCR was conducted with a 200 μl scale by 12 to 25 cycles of denaturing (95°C for 40 s), annealing (50°C for 40 s), and extending (72°C for 40 s) and final extension at 72°C for 3 min.

In method B, for primer extension, appropriate forward and reverse primers (1 μM each; see Table S3 for further information) were mixed in the PCR mixture. The primer extension reaction was conducted with a 100 μl scale by denaturing (95°C for 1 min), followed by 5 cycles of annealing (50°C for 1 min) and extending (72°C for 1 min). The resulting reaction mixture was 200-fold diluted and used as a template DNA for the following PCR step. From the first to the penultimate PCR, the template DNA (the product of the preceding extension reaction or PCR) was amplified using the designated forward and reverse primers (1 μM each; see Table S3 for further information). PCR was conducted with a 100 μl scale by 5 cycles of denaturing (95°C for 40 s), annealing (50°C for 40 s), and extending (72°C for 40 s). The resulting PCR mixture was 200-fold diluted and used as a template DNA for the following PCR step. The final PCR was conducted with a 200 μl scale by 12 to 25 cycles of denaturing (95°C for 40 s), annealing (50°C for 40 s), and extending (72°C for 40 s) and final extension at 72°C for 3 min.

Amplification of the final PCR product was checked by agarose gel electrophoresis. The resulting DNA was purified by phenol/chloroform extraction and ethanol precipitation and then dissolved in 20 μl of water (giving a 0.1–0.6 μM solution) and directly used without adjustment of concentration for in vitro translation reaction using the FIT-PatD system.

FIT-PatD System

Translation factors, enzymes, and ribosome were prepared and mixed as previously described to reconstitute an in vitro transcription-translation coupled system (Goto et al., 2011). The reaction mixture contained a final concentration of 50 mM HEPES·K (pH 7.6), 100 mM KOAc, 2 mM guanosine triphosphate,

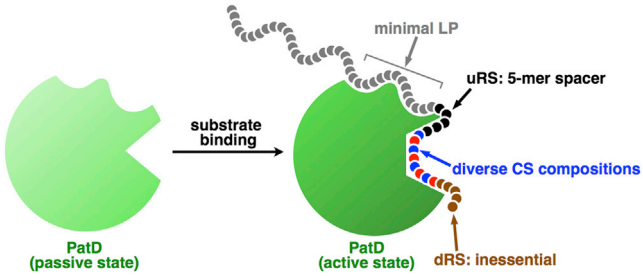

Figure 6. A Model for the Interaction of PatD with the Substrate
The LP region, uRS, and dRS are shown in gray, black, and brown, respectively. In the CS, residues modified by PatD are shown in red, while unmodified residues are shown in blue. The C-terminal region of LP (minimal LP) activates PatD upon its binding, and the uRS acts as a spacer accommodating the downstream CS to the PatD active site. Diverse CS compositions can be cyclodehydrated by PatD.

2 mM ATP, 1 mM cytidine triphosphate, 1 mM uridine triphosphate, 20 mM creatine phosphate, 12 mM $Mg(OAc)_2$, 2 mM spermidine, 2 mM dithiothreitol (DTT), 1.5 mg/ml *E. coli* total tRNA (Roche), 1.2 μM ribosome, 0.6 μM methionyl-tRNA formyltransferase, 2.7 μM IF1, 0.4 μM IF2, 1.5 μM IF3, 30 μM EF-Tu, 30 μM EF-Ts, 0.26 μM EF-G, 0.25 μM RF2, 0.17 μM RF3, 0.5 μM RRF, 4 μg/ml creatine kinase, 3 μg/ml myokinase, 0.1 μM pyrophosphatase, 0.1 μM nucleotide-diphosphatase kinase, 0.1 μM T7 RNA polymerase, 0.73 μM AlaRS, 0.03 μM ArgRS, 0.38 μM AsnRS, 0.13 μM AspRS, 0.02 μM CysRS, 0.06 μM GlnRS, 0.23 μM GluRS, 0.09 μM GlyRS, 0.02 μM HisRS, 0.4 μM IleRS, 0.04 μM LeuRS, 0.11 μM LysRS, 0.03 μM MetRS, 0.68 μM PheRS, 0.16 μM ProRS, 0.04 μM SerRS, 0.09 μM ThrRS, 0.03 μM TrpRS, 0.02 μM TyrRS, 0.02 μM ValRS, 200 μM each proteinogenic amino acids, and 100 μM 10-HCO-H_4folate.

In the two-step method, the translation reaction was performed at 37°C for 30 min at a 2.5 μl scale that contained 0.25 μl of DNA template. To the resulting translation mixture (2.5 μl) was added 2.5 μl of PatD mixture I (12 μM PatD, 90 mM HEPES·K [pH 8.4], 16 mM DTT, and 1 mM ATP), and the mixture was further incubated at 25°C for 14 hr. For the point and multiple-site mutagenesis analysis of LP, the translated PatE variants were incubated with 0.6 μM PatD at 25°C for 2 hr.

In the one-pot method, modified reaction mixture was used; 6 μM PatD was added, and the concentrations of HEPES buffer, DTT, and ATP were changed to 95 mM (pH 8.0), 10 mM, and 2.5 mM, respectively. DNA template (0.25 μl) was incubated in the reaction mixture (2.5 μl) at 37°C for 30 min, followed by at 25°C for 14 hr.

For precursor peptide synthesis without PatD treatment, the translation reaction was performed at 37°C for 30 min at a 2.5 μl scale that contained 0.25 μl of DNA template. To the resulting translation mixture (2.5 μl) was added 2.5 μl of a buffer (90 mM HEPES·K [pH 8.4], 16 mM DTT, and 1 mM ATP), and the mixture stood at 25°C for 14 hr as a control before analyzing the peptide using MALDI-TOF-MS.

Mass Spectrometry of Precursor Peptides and Az-Peptides Synthesized in the FIT-PatD System

The translation mixture or FIT-PatD reaction mixture was desalted with a solid phase extraction (SPE) column (C-Tip C18; Nikkyo Technos) and eluted with matrix solution (80% acetonitrile, 0.5% acetic acid, and half-saturated matrix). As the matrix, sinapinic acid and α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) was used for products above and below 4,500 Da, respectively. MALDI-TOF-MS was carried out on an autoflex II TOF/TOF (Bruker Daltonics) or an ultrafle Xtreme (Bruker Daltonics) externally calibrated with protein calibration standard I (Bruker Daltonics) or peptide calibration standard II (Bruker Daltonics).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, ten figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.04.008.

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