Chemistry & Biology, Vol. 11, 1573–1582, November, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.chembiol.2004.09.003

Enhanced Macrocyclizing Activity of the Thioesterase from Tyrocidine Synthetase in Presence of Nonionic Detergent

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Summary

Macrocyclization carried out by thioesterase domains of multimodular nonribosomal peptide synthetases (NRPSs) is a key step in the biosynthesis of many biologically active peptides. The thioesterase excised from tyrocidine synthetase is a versatile macrocyclization catalyst and a useful tool for chemoenzymatic synthesis of diverse cyclic peptides. However, its utility is limited by its short lifetime of catalytic activity as well as significant flux of the acyl-enzyme intermediate to hydrolysis. The addition of Brij 58, a nonionic detergent, above the critical micelle concentration, has dramatic effects on enzyme activity: catalytic activity is extended to >60 min and the rate of cyclization (but not hydrolysis) increases 6-fold, resulting in a net 150- to 300-fold increase in cyclic product yields. This enhanced activity allowed enzymatic macrocyclization of a solid phase library of tyrocidine decapeptides to identify acceptable substitutions at the Orn9 position which had previously been inaccessible for diversification.

Introduction

A variety of peptide natural products are produced nonribosomally by multimodular enzymes acting in assembly line fashion [1, 2]. Polyketide as well as hybrid nonribosomal peptide/polyketide (NRP/PK) products are biosynthesized by comparable assembly line logic. Many of these products are of therapeutic interest, such as the antitumor drugs bleomycin [3] and epothilone [4], antibiotic erythromycin [5], and immunosuppressants FK506 [6] and rapamycin [7]. Among the features that impart biological activity to these compounds are conformational constraints that lead to the biologically active conformer. NRP, PK, and NRP/PK hybrid products are constructed as linear precursors covalently attached to their dedicated synthetase. One strategy for introducing conformational rigidity, often employed in these systems, is the formation of a macrocycle from the final linear precursor with concomitant release of the acyl chain from the enzymatic assembly line. This function is carried out by C-terminal thioesterase (TE) domains [8].

In this final step of product synthesis, fully elongated product chains are transferred to the active site serine of the TE domain, the terminal domain of the NRPS or PKS assembly line, forming an acyl-O-TE intermediate (Figure 1). The fate of this intermediate varies among thioesterase domains from different synthetases. TE domains can catalyze release of these acyl chains either through hydrolysis, transferring the acyl chain to water, or through macrocyclization, directing capture of the chain by an internal nucleophile. For peptide products, the acceptor is always the carbonyl of the final residue in the peptidyl chain for both hydrolysis and macrocyclization, while the intramolecular nucleophile in macrocyclization can be either an -NH_2 or -OH group at some point within the peptidyl chain. In the case of cyclic decapeptide tyrocidine, the nucleophile is the N-terminal -NH_2 group; the resulting head-to-tail cyclization yields the macrolactam. The side chain ϵ-NH_2 of Lys7 is the competent nucleophile in the TE-catalyzed cyclization of bacitracin to generate the branched cyclic peptide. Cyclization via the side chain -OH of Ser and Thr and the phenolic -OH of Tyr residues are enabled with specific regiochemistries by NRPS assembly lines to give lariat peptidolactone structures, or depsipeptides, such as daptomycin [9], recently approved for human use as an antibiotic.

There is substantial interest in exploring the catalytic capacity of macrocyclizing TE domains excised from NRPS and PKS assembly lines. Numerous 28–35 kDa TE domains have been excised from megadalton synthetases and assessed for autonomous capacity for stereo- and regiospecific macrocyclizations. Several retain specific macrocyclization activity with head-to-tail (tyrocidine TE [10], gramicidin TE [11], epothilone TE [12]) and lariat type (surfactin TE [11], fengycin TE [13], calcium-dependent antibiotic TE [14]) cyclizations reported. Studies of the tyrocidine thioesterase domain (TycTE) have shown that it is a remarkably versatile macrocyclization catalyst. It allows the formation of head-to-tail lactones as well as lactams [15] and forms 6- to 14-membered cyclic peptides at equivalent catalytic efficiency to the natural cyclic decapeptide [11]. TycTE accepts peptides of diverse amino acid sequence as well as hybrid PK/NRP substrates for cyclization [16]. Finally, it catalyzes macrocyclization of these substrates presented on solid support, enabling library approaches to new cyclic molecules [17].

Despite these favorable characteristics, several limitations were noted in these initial studies. First, product formation by the TE domain was linear for <2 min and declined precipitously thereafter, restricting the yield of cyclic products. Second, some of the acyl-enzyme formed during the reaction was lost to hydrolysis forming the linear hydrolyzed product chain rather than the macrocyclic product, and the ratio of hydrolyzed to cyclic products tended to increase with the use of unnatural substrates. Third, a scan of the 10 amino acid residues of the decapeptide substrate indicated that D-Phe1 and Orn9 were required for cyclization, precluding the replacement of that nucleophilic side chain at residue 9 to facilitate structural modifications of the cyclic peptide scaffold [10].

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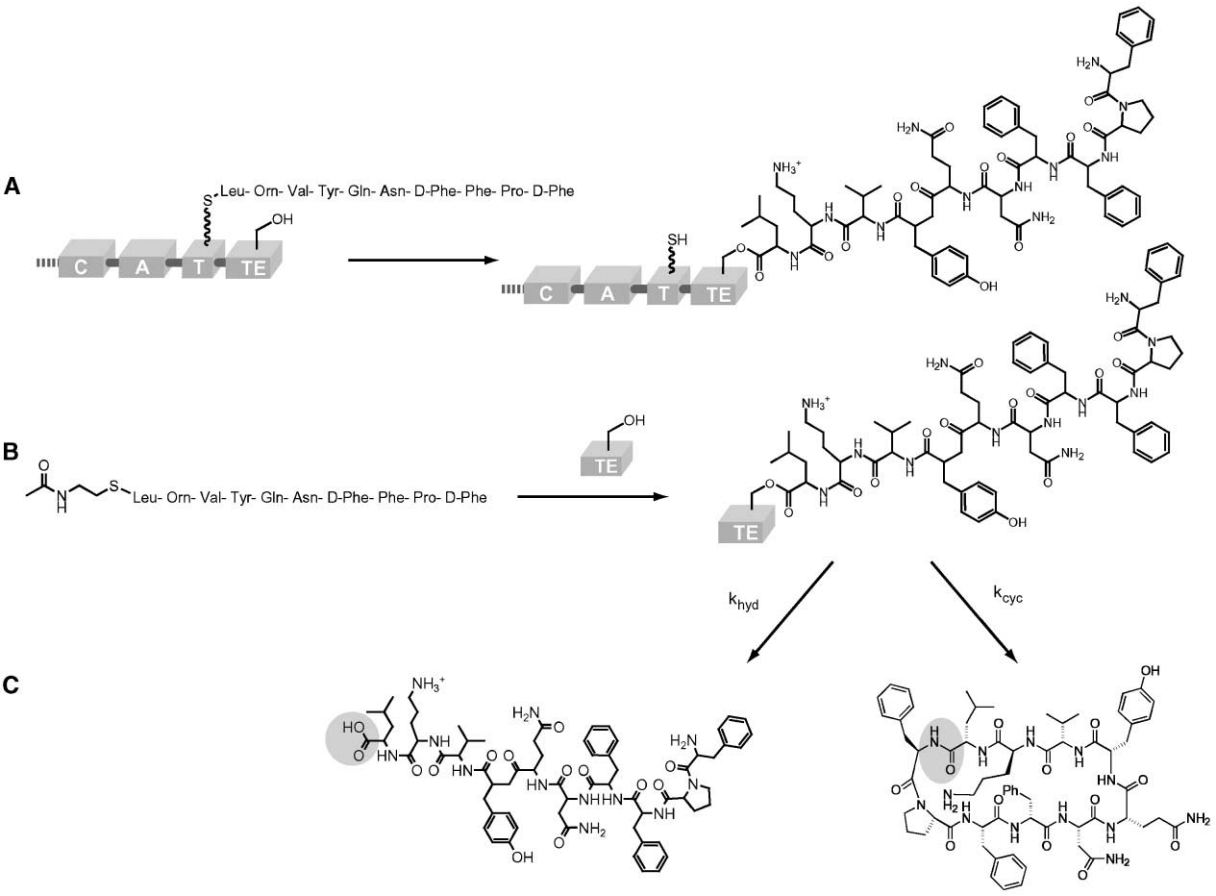


Figure 1. Partitioning of the Acyl-Enzyme Intermediate between Macrocyclization and Hydrolytic Outcomes

(A) Formation of the acyl enzyme intermediate in vivo with transfer of the peptide chain from the T domain to the TE domain within the final module.

(B) Formation of the same intermediate with peptidyl-SNAC substrate via an intermolecular transfer of the peptide chain.

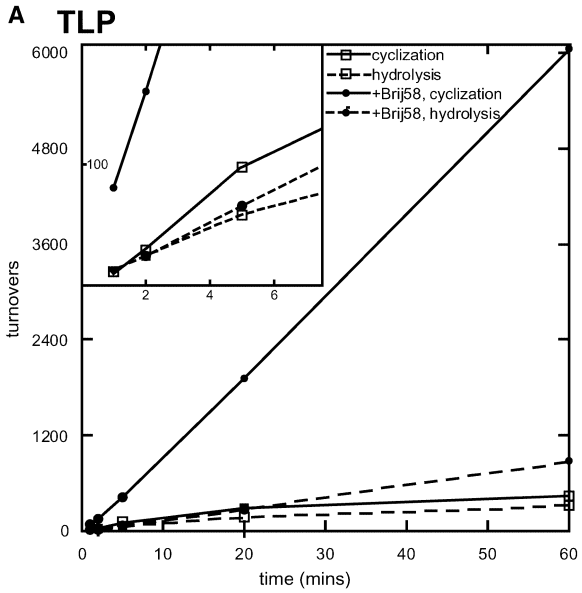
(C) Products resulting from either hydrolysis or macrocyclization of the resulting acyl-enzyme intermediate. The relevant chemical transformation for each product is highlighted in gray.

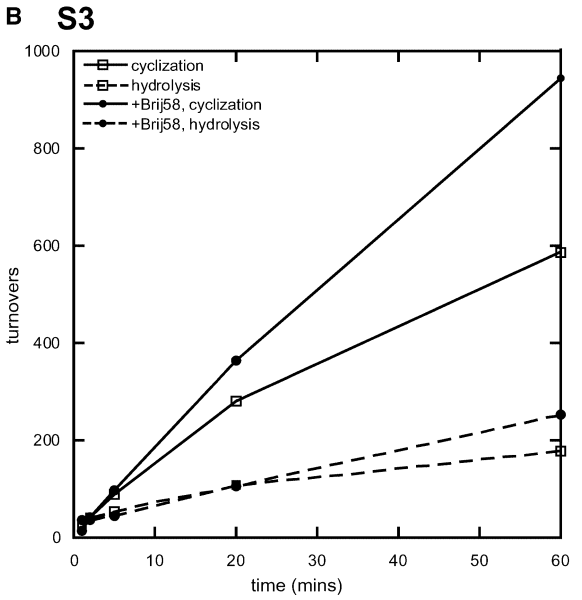
Herein, we report that the nonionic detergent Brij 58 (polyoxyethylene 20 cetyl ether, $C_{16}E_{20}$) extended the lifetime of TycTE activity, allowing linear product formation for >60 min. Addition of detergent also resulted in a preferential 6-fold enhancement in the rate of the cyclization reaction without increase in the rate of hydrolysis, markedly shifting the partitioning of the acyl-enzyme intermediate toward macrocyclization. These effects were dependent on the formation of micelles from detergent monomers. Taking advantage of these improvements in TycTE activity, we were able to explore alternate side chains at the substrate position 9 and begin to define the nature of specificity at this position. Finally, we discuss the interplay between enzyme, substrate, and detergent that may account for these intriguing effects on TycTE catalysis.

Results

Effect of Detergent on the Time Course of Product Formation

The activity of excised TycTE has been demonstrated on a linear peptidyl N-acetylcysteamine (SNAC) substrate constructed from the tyrocidine peptide sequence [10]. In the reaction of this decapeptidyl-SNAC (**TLP**) with purified TycTE, enzyme turnover was constant for several minutes, then rapidly declined for both hydrolysis and cyclization reactions (Figure 2A, inset). When enzyme turnover was plotted as a function of time, the resulting curve was consistent with that of enzyme undergoing rapid exponential decay with a half-life of about 1.5 min (data not shown). The cause of this rapid decay of catalytic activity is unknown but may be due to the destabilization of enzyme structure by substrate and/or product molecules, as no loss of initial activity was observed when enzyme was preincubated in reaction buffer at 25°C (assay temperature) for 20 min before addition of substrate (data not shown). Neither was there any proteolysis of the enzyme observed during the course of the reaction. The addition of 1 mM Brij 58 in the reaction stabilized enzyme activity and produced linear kinetics of product formation for up to 60 min (Figure 2A). A comparison of the initial rates for the cyclization reaction also showed a 6-fold acceleration in the formation of cyclic product when detergent was present. In contrast, initial rates for substrate hydrolysis





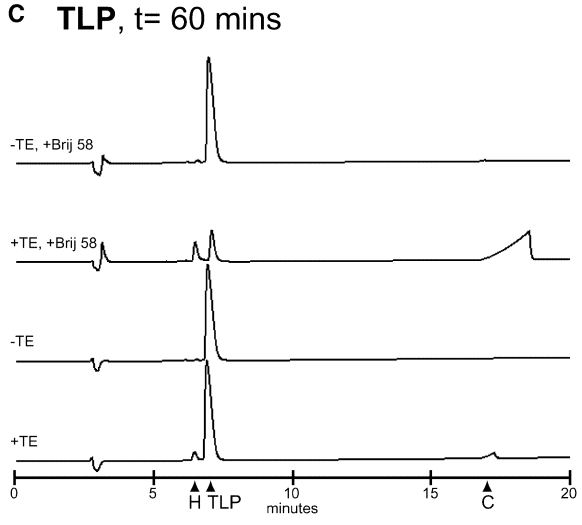


Table 1. Effect of Detergent on Kinetic Parameters for TycTE Cyclization and Hydrolysis

Detergent	Product	k_{cat} (min^{-1})	K_m (μM)	Cyc:Hyd
+0.1% (w/v) Brij 58	cyclization	78.1	2.4	
				6.9
	hydrolysis	11.3	11.3	
None	cyclization	23.9	3.0	
				1.2
	hydrolysis	19.3	3.6	

remained unchanged. Nonenzyme catalyzed cyclization, which was not observed in the absence of detergent, occurred at slow rates when Brij 58 was added (Figure 2C). Thus, detergent alone generated a substrate conformer capable of macrocyclization even in absence of the enzyme. Under all conditions, background hydrolysis and cyclization rates were less than 1% of enzyme-catalyzed rates.

Effect of Detergent on k_{cat} and Product Ratio

To investigate the changes in rates of cyclization versus hydrolysis and the resulting shift in the cyclic to hydrolyzed product ratio, we determined the kinetic parameters for TycTE activity in the presence and absence of Brij 58 (Table 1). Enzyme turnover of cyclic product increased from 23.9 min^{-1} to 78.1 min^{-1} in presence of detergent. The k_{cat} for hydrolysis product decreased from 19.3 min^{-1} to 11.3 min^{-1}. As a result, the partition ratio between cyclization to hydrolysis increased from 1.2 in absence of detergent to 6.9 in its presence, a 6-fold enhancement. Brij 58 induced a dramatic change in product composition, shifting the flux of the acyl-enzyme intermediate in favor of macrocyclization. K_m was 3 μM for cyclization reactions both in the presence and absence of Brij 58. A previous report had determined k_{cat} for cyclization of 59 per min with cyclization to hydrolysis ratio of 6:1 in absence of detergent [10], greater than observed in the present study. Variability in these parameters has been noted with different preparations of enzyme. In the current study, 3 separate purifications yielded enzyme with cyclization activity 15–30 turnovers per min and cyclic to hydrolyzed product ratio 1:1–3:1. The detergent enhancement was consistent, and the K_m as well as the specificity for alternate substrates was similar to previously described results. Therefore, the variability could be another limitation of the decreased stability of TycTE.

Similar improvements in the product ratio were observed for TycTE on alternative substrates: gramicidin linear peptide SNAC (**GLP**) [11] and **TLP** containing a Pro2 → Ala mutation and one containing D-Phe4 →

Figure 2. Brij58 Increases the Catalytic Lifetime of TycTE

(A) Reaction of 100 μM **TLP** ± 1.0 mM (0.1% w/v) Brij 58 with 35 nM TE in which both a linear time course and accelerated cyclization rates are observed. Inset, magnification of time course from t = 0–7.5 min showing loss in enzyme activity when detergent is absent.

(B) Reaction with **S3** in which linearity can be seen but rate enhancement for cyclization does not occur.

(C) HPLC traces of reaction products after 60 min incubation with **TLP**, monitoring at 220 nm (H = hydrolyzed and C = cyclic product).

Table 2. Screen of Different Classes of Detergents for Effect on TycTE Activity

Detergent	Type	CMC (mM)	Amount in Assay (mM)	Cyclization Yield (μM)	Hydrolysis Yield (μM)	Product Ratio, R
None	—	—		4.1	1.5	2.7
Cholate	A	14.0	28.0	5.1	1.8	2.8
Deoxycholate	A	5.0	10.0	0.5	0.1	6.4
CTAB	C	1.0	2.0	*0*	*0.3*	*0*
BRIJ-58	N	0.007–0.077	0.15	**25.1**	2.8	**8.9**
CYMAL-6	N	0.56	1.1	**40.6**	4.0	**10.1**
Octylglucoside	N	24.5	49.0	**29.1**	2.6	**11.1**
Reduced Triton X-100	N	0.25	0.5	**21.8**	2.5	**8.6**
CHAPS	Z	8.0	16.0	4.5	0.8	5.4
Zwitt3-12	Z	4.0	8.0	*0*	*0*	*0*

Seventy-five micromolar **TLP** was incubated with twenty-five nanomolar TycTE and detergent. Reactions were quenched after 15 mins. Bold values indicate those in which the cyclization yield was enhanced >5-fold; italics, those in which cyclic product formation was abolished. For detergent types, A = anionic; C = cationic; N = nonionic; and Z = zwitterionic.

D-Glu (data not shown; see Supplemental Data [available online at http://www.chembiol.com/cgi/content/full/11/11/1573/DC1/] for structures of substrates used in this study). Curiously, two peptide substrates, **IT2** [18] and **S3** [15], showed no changes in the product ratio although their time courses still exhibited improved linearity. The time course for **S3**, in which the 3 amino acids Gln6-Val8 of the tyrocidine sequence were replaced with the spacer 8-amino-3,6-dioxaoctanoic acid, is shown in Figure 2B. The factors that govern these differences are unknown. However, the results demonstrate that there must be more than one mechanism for the observed enhancement of TycTE activity by detergent, one of which involves interaction of detergent molecules with the peptide substrate.

The specificity of these effects for particular detergents was also explored. Detergents from different classes were screened for their ability to increase cyclization rates and enhance the cyclic to hydrolyzed product ratio. As for Brij 58, these effects were found for all nonionic detergents tested, including glucopyranoside, CYMAL-6, and reduced Triton X-100 (Table 2). Detergents with charged or zwitterionic head groups either had no effect or were inhibitory. The cationic detergent CTAB and zwitterionic Zwit3-12, both strongly denaturing detergents, were found to abolish cyclic product formation. The associated reduction in hydrolyzed product with these ionic detergents indicated a global effect on enzyme activity, likely through destabilization of enzyme structure. The interaction of substrate and/or enzyme with detergent molecules is specific for the class of nonionic detergents due to either structural or chemical properties of this class.

Dependency on Monomer to Micelle Transition

The effects of detergent on enzyme activity could be several, including preorganization of substrate or enzyme in an active conformation for cyclization, stabilization of enzyme structure, or removal of cyclic amphipathic product. These could depend on interaction with a detergent micelle. We assayed TycTE cyclization over a range of Brij 58 and CYMAL-6 concentrations encompassing their literature-reported critical micelle concentration (CMC) value. In a narrow concentration range around the CMC, detergent monomers undergo a cooperative transition to form micelle structures. We confirmed this transition from detergent monomer units to larger aggregate structures by dynamic light-scattering analyses of detergent solutions at concentration equal to 0.5× CMC and 2× CMC and observed a larger molecular weight species formed at 2× CMC which was not observed in the detergent solution at 0.5× CMC (data not shown). The observed effect of these detergents, Brij 58 and CYMAL-6, to increase cyclization activity was also dependent on this transition with maximal effects at concentrations above their CMC (Figure 3). In contrast, the accumulation of hydrolyzed product was largely unchanged across the same concentration range. Representative time courses were taken at detergent concentrations equal to 0.5× CMC and 2× CMC. Both linearity of the time course and rate enhancement of the cyclization reaction were seen only at detergent concentration above CMC (data not shown).

After the increases in cyclic product yield and product ratio at concentrations near CMC, further increases in detergent concentration did not alter product formation. The activity of a catalyst with a substrate accessed from a micelle (for example, as occurs when an enzyme such as phospholipase interacts with its substrate phospholipid, a membrane component) has been quantified by surface dilution kinetics [19, 20]. This model of substrate, micelle, and enzyme interaction predicts a dependency of enzyme kinetics on surface concentrations of substrate, expressed by its mole fraction in combination with detergent monomers. Incubations of different mole fraction ratios of substrate TLP-SNAC to Brij58 monomers showed no significant differences in k_{cat} or K_m (data not shown), arguing against presentation of the substrate in the micellar phase as the mechanism of detergent on the TycTE domain. For example, if the hydrophobic face of the amphipathic peptidyl substrate were buried in the micelle to facilitate an active conformation, it may have to be accessed from the micellar phase. However, failure to observe surface dilution kinetics does not rule out the possibility that enzyme or substrate molecules associate with detergent micelles, such as in the "bound" water layer that is believed to surround the polar heads of surfactant molecules.

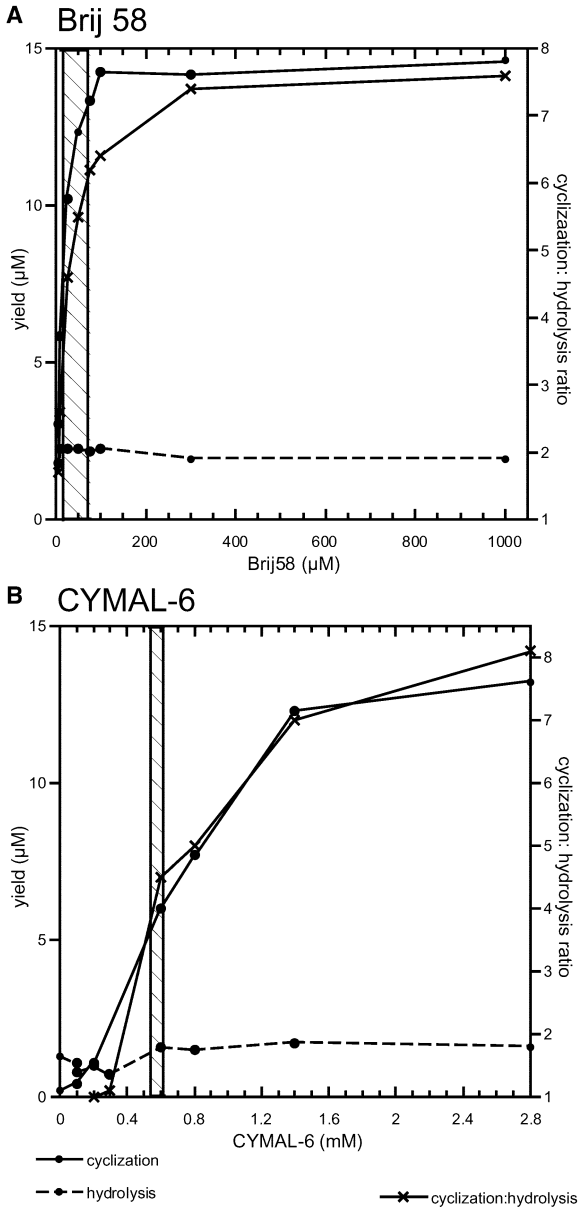


Figure 3. Rate Acceleration Is Dependent on Detergent Micelle Formation

Reactions containing 50 μM **TLP**, 10 nM TycTE, and detergent were incubated for 15 min. Under these conditions, no background cyclization was observed without TE. Background hydrolysis was subtracted to calculate yields. Gray box indicates range for literature-reported CMC values for Brij 58 (0.007–0.077 mM) and CYMAL-6 (0.56 mM).

Substitution of Ornithine at Position 9 and Evidence for Saturation Kinetics on Solid Phase Substrates

With the improvements in TycTE cyclization activity and lifetime afforded by addition of nonionic detergent, we sought to explore the ability of TycTE to catalyze the head-to-tail cyclization of peptidyl substrates lacking a cationic or nucleophilic side chain at position 9, using the previously described solid phase method employing peptides conjugated to oxoester beads [17]. Previously, a scan of the 10 residues of **TLP** had indicated Glu could not replace ornithine at position 9 [10]. Substitution of Orn9 in solution phase assays had also been limited by the decreased solubility of the peptide upon replacement of this charged residue. Taking advantage of the diversity of substitutions that could be explored on solid phase, a library of 86 different peptides containing natural and unnatural amino acid substitutions at position 9 was synthesized on solid phase and tested for activity with TycTE. Table 3 shows 21 residues that, when substituted for Orn9, yielded cyclic macrolactam product released from solid phase beads by the action of TycTE. The relative yields varied over 75-fold with the native peptide containing ornithine at position 9 at highest abundance followed by lysine, arginine, and derivatives of these amino acids. Four *N*-alkyl nonproteinogenic amino acids, isopropyl-, dimethyl-, and trimethyl- lysine as well as dimethylarginine, were cyclized by Tyc TE at 2- to 20-fold lower yields than native peptide. Fourteen of twenty-one residues, including nine with the highest yields, were positively charged. Loss of the positive charge resulted in a minimum of 30-fold decrease in yields. All substitutions accepted for cyclization were L-amino acids. Screening for accepted substrates was aided by addition of detergent to increase yields, such that product of less robust substrates could only be detected in presence of detergent. Product ratios with detergent as compared to without were typically 2- to 5-fold greater in favor of cyclic product formation for synthesis on solid phase. Eight of these macrocyclic products were prepared by large scale enzymatic cyclization of the peptide-SNAC and tested for antibiotic activity against *B. subtilis*. Of the eight tested, 6 variants demonstrated a minimal inhibitory concentration (MIC) of 1.5 μM, comparable to the native TycA peptide (Supplemental Table S1, available online at http://www.chembiol.com/cgi/content/full/11/11/1573/DC1/). Nonspecific hemolysis of human red blood cells was also observed for these variants with similar therapeutic indices (MHC/MIC) as TycA.

It was not clear if TycTE recognition of peptidyl oxoesters attached to the surface of solid phase beads by a pantetheinyl mimetic linker followed catalytic efficiency patterns for substrates presented in solution. Previously, the poor kinetic properties of TycTE in absence of detergent had prohibited kinetic profiles for candidates that were not robust substrates for TycTE. Therefore, 10 of the substituted peptides were synthesized as SNAC thioesters and assayed in solution in the presence of Brij 58 for kinetic characterization (peptides substituted with Gln and Cit did not yield cyclic product when assayed in solution; results are shown in Supplemental Table S2). Seven of the eight alternatives substrates were cyclized with k_{cat} values ranging from 4.6 to 83 min^{-1} (the peptide containing His9 had background hydrolysis rate of 0.07 min^{-1} that prevented measurement of low level enzymatic turnover). The 3-pyridylalanine substitution at position 9 had 25-fold decrease in turnover compared to the native tyrocidine decapeptide, but otherwise substitutions at this position clustered around 2- to 5-fold drops in k_{cat}. K_m values were also

Table 3. Substitutions Accepted by TycTE for Ornithine at Position 9

Amino Acid Substitution	Cyclization Yield (μM)	Hydrolysis Yield (μM)	Product Ratio, R	$R_{+Brij58}/R_{-dtg}$
Ornithine	75.0	4.3	17	2.4
Lysine	58.5	5.4	11	2.8
N-Dimethyl-arginine	37.0	4.5	8	2.0
Arginine	27.2	1.9	14	2.3
N-Dimethyl-lysine	14.1	2.1	7	3.5
Homoarginine	10.0	0.8	12	4.4
N-Isopropyl-lysine	8.2	2.4	3	3.0
Diaminobutyric acid	3.5	2.4	1.5	+
N-Trimethyl-lysine	3.5	0.6	5	+
N-Nitro-arginine	2.6	1.3	2	5.0
N-Formyl-lysine	2.2	0.6	3	18.8
Homocitrulline	1.7	0.0	—	+
Citrulline	1.6	0.4	4	+
Glutamine	1.4	0.4	3	+
Histidine	1.2	7.1	0.2	+
p-Amino-phenylalanine	1.2	0.7	2	+
N-Nicotinyl-lysine	1.0	0.5	2	+
3-Pyridylalanine	1.0	0.6	2	+
Diaminopropionic acid	1.0	12.6	0.07	3.5
4-Pyridylalanine	0.8	0.6	1	3.6
N-Acetyl-lysine	0.8	0.2	4	+
Methioninesulfoxide	0.7	0.3	3	+
Homoserine	0.5	0.4	1	+

Accepted substitutions in D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr-Val-X-Leu peptides are listed in order of most to least abundant yields.
+ indicates cyclic product was formed only in presence of detergent.

largely unaffected with 1- to 3-fold increase for 5 of the 7 peptides. Except for diaminobutanoic acid and 3-pyridylalanine substitutions with 28- and 13-fold increase in K_m, respectively, yields of cyclic product from solid phase substrates correlated strongly with observed k_{cat} values for soluble substrates, indicating saturating conditions on solid phase for these peptides. The rates of TycTE acting on peptidyl oxoesters arrayed on solid phase beads is mimicked by soluble peptidyl thioester substrate concentration of about 20 μM.

Discussion

Several reports have documented significant increases in the enzymatic activity of a variety of biological catalysts with addition of surfactants [21]. For example, cytoplasmic glycerol-3-phosphate dehydrogenase is stimulated 1.5-fold by Brij 35, alcohol dehydrogenase exhibits 4- to 7-fold activation in presence of deoxycholate, and nonionic detergent increases firefly luciferase activity up to 7-fold. The rate enhancement reported in these systems are comparable to that observed in this study on TycTE upon addition of Brij 58 and other nonionic detergents. The term "enzyme superactivity" has been applied to these cases in which a water/surfactant and/or solvent system generates improved catalytic activity compared with that in aqueous buffer alone [22]. Various mechanisms can account for such observed "superactivity," all involving the productive interaction of detergent monomers or micelles with enzyme, substrate, and/or product to facilitate catalysis. Due to complexity of these two-phase systems, deciphering these different interactions and their effects on catalysis is often not straightforward [22].

Recently, rate acceleration of chymotrypsin activity by the cationic detergent CTAB was found to depend on significant conformational changes in the enzyme induced by the detergent [23]. Studies of firefly luciferase suggest the combined interaction of enzyme and substrate luciferin with Triton micelles is critical in the 5-fold stimulation of light output in that system [24]. The motivation for studying such mixed surfactant/water systems is based in the view that aggregate systems such as these more fully recapitulate the environment of the cell, wherein enzymatic reactions take place under conditions of concentrated solutes and macromolecules, within macromolecular complexes, and at or near aqueous medium/membrane interfaces [21, 25]. These circumstances may indeed play a role in understanding the effects of detergent in the current study. In the following discussion, we offer insight into the phenomenon of enzyme superactivity in the present study and the complexity of the interactions that underpin these effects.

Two effects of nonionic detergent were observed on TycTE activity: an increased lifetime of enzyme activity and a catalytic rate acceleration which shifted the product ratio in favor of cyclization. The first effect reflects the stability of the catalytic TE domain under assay conditions, specifically destabilization of the enzyme upon addition of substrate and/or formation of product as neither preincubation at assay temperature nor enzyme dilution in assay buffer without substrate caused decrease in subsequent activity. The 28 kDa TycTE removed from its normal position at the C terminus of the 720 kDa TycC subunit is reconstructed as an excised domain lacking the context of its normal protein interactions with the rest of the synthetase. As such, the excised domain may be more susceptible to unfolding. In addition, it has been suggested that the NRPS assembly localizes at the cytoplasmic membrane of the producing

Bacillus [26, 27]. This subcellular localization may be required for the released cyclic tyrocidine to be shipped out by a dedicated ABC transporter, keeping the intracellular concentration low in the producing organism as part of its self defense [28]. As the final domain of the synthetase responsible for product release, the TE may be an important mediator of the interaction with this cellular structure. Interaction of the excised domain with detergent micelles may serve to mimic the normal contacts of the TE domain with the larger synthetase and/or the cell membrane.

Alternatively, the stabilizing effects of nonionic detergent may be indirect through sequestration of substrate and/or product molecules. The mechanism of tyrocidine A as an antibiotic is to insert into bacterial membranes [29–31]. TycA is an amphipathic molecule with a tendency to aggregate. The accumulation of product molecules, even in low quantities, at its point of generation could cause it to associate with and destabilize the enzymes responsible for its synthesis (though no protein aggregation was detected by dynamic light-scattering analysis of a concentrated enzyme sample upon addition of small quantities of the TycA product [data not shown]). Removal of these products into detergent micelles could mimic the normal mode of removal through efflux pumps or cellular transport.

Detergent stabilization of the TycTE protein structure could also account for the second effect observed in these studies, the change in the product ratio of TycTE induced by detergent. TE domain catalysis entails the transfer of the product chain synthesized by the NRPS to the -OH of an active site serine in the TE domain, generating an acyl-enzyme intermediate. The features that govern hydrolysis or macrocyclization of this intermediate are not well understood but must encompass the productive orientation of molecular water versus an intramolecular nucleophile in the active site of the acyl-enzyme. In order to shift the product ratio, nonionic detergents must affect this partitioning of the common acyl-enzyme intermediate between cyclization and hydrolysis. Embedding of the excised domain in detergent micelles may provide, in addition to structural stability, a hydrophobic environment that prevents capture of the acyl-enzyme intermediate by a water molecule. This could involve repositioning of the mobile lid, seen in the X-ray structure of the Srf TE [32], over the active site to exclude bulk water.

However, evidence from this study indicates that the product ratio change is not a result of detergent-enzyme interactions but rather is mediated through detergent interactions with the peptide chain itself either as the substrate peptidyl-SNAC and/or in the acyl-enzyme. First, incubation of substrate with detergent resulted in an acceleration of nonenzyme catalyzed cyclization. Second, acceleration of cyclic product formation is abolished for certain substrates, such as **S3**, although detergent still exerts its stabilizing effects on the enzyme consistent with two separable effects, one dependent on interactions with the enzyme and another dependent on interactions with the peptide chain. The peptide-detergent interaction may mimic the functional interaction of these amphipathic peptides with the cell membrane which is believed to be the mechanism of their antibiotic effect. If this interaction were to occur between detergent micelles and substrate peptidyl-SNACs, micelles could provide an optimal surface whereby the amphipathic substrate is presented to the enzyme, prefolded for subsequent cyclization with hydrophobic side chains buried in the micelle and polar residues exposed on the surface. However, this mode of interaction requires part of the substrate to be buried in micelles and inaccessible to the enzyme from the aqueous phase and thus is less likely according to surface dilution kinetics since cyclization rates were independent of the mole fraction of the substrate [19, 20]. Moreover, the K_m for cyclization remained unchanged at 3 μM in the presence and absence of detergent, suggesting no effect on the K_d for substrate recognition. Instead, we suggest that substrate is still accessible to the enzyme from the aqueous phase (rather than in the micellar phase), either as free substrate in bulk water or as substrate associated with the micelle in the "bound" water layer [22].

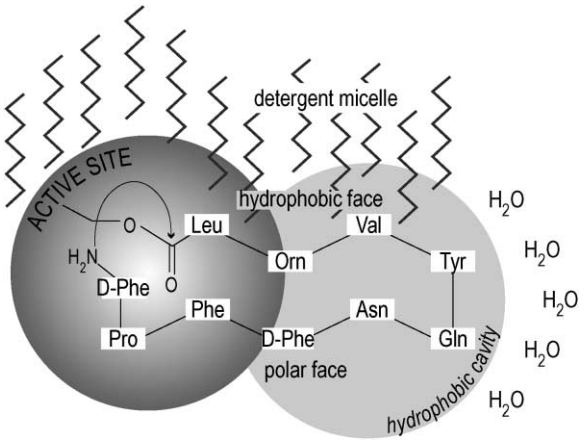


Figure 4. Model for the Interaction of Detergent Micelle with Acyl-Enzyme Intermediate

Interaction of the enzyme (gray) with the detergent micelle ensures proper folding of both the active site and hydrophobic binding cavity. The well-folded active site increases the lifetime of activity of TycTE. In the protected environment of the binding cavity, water molecules are effectively excluded and cannot hydrolyze the peptide chain. In addition, detergent interaction with the hydrophobic face of the peptide helps orient the substrate in a conformation leading to cyclization, setting up the amine nucleophile for attack of the acyl-enzyme ester bond.

As such, it seems likely that the effect of detergent on TE mechanism occurs at the level of the acyl-enzyme (Figure 4). A covalent peptidyl-O-TE intermediate is anticipated in the action of every chain terminating NRPS TE domain. The increase in k_{cat} with flux directed to macrocyclization reflects a specific lowering of the energy barrier for acyl-O-TE breakdown via intramolecular capture. Detergent interactions with the peptidyl chain in the active site of the TE could facilitate the folding of the chain into an active conformation leading to cyclization. If detergent does indeed facilitate this deacylation/macrocyclization step in TE catalysis, it would suggest that deacylation is rate-limiting in the TE mechanism, as the lowering of the energy barrier for acyl-O-TE breakdown results in improved overall product turnover rates. It may be worthwhile to explore detergent effects in

other TE systems that macrocyclize amphipathic cyclic peptides.

Finally, the practical consequence of Brij 58 inclusion in TycTE assays is a much longer catalyst life time coupled to dramatic redirection of flux toward macrocyclization. The combined effects can result in up to 300 times more yield of cyclic products. For example, detergent inclusion was essential in the solid phase enzymatic synthesis of tyrocidine variants substituted at position 9 to accumulate enough products for quantitation and also in solution phase to obtain kinetic parameters for these variants. In order to be accepted by TycTE, the positive charge at this position had to be maintained while the potential nucleophile or H-bond donor could be removed without penalty. Previously, the Orn9 residue was thought to be involved in an intramolecular H-bond within the peptidyl chain to help orient the terminal amine for nucleophilic attack [15]. But the acceptance by TycTE of positively charged residues that cannot H bond (e.g., trimethyl-Lys and dimethyl-Arg) at position 9 make this proposed interaction less likely. Instead, there may be a charge interaction of the position 9 residue with a negatively charged residue in the enzyme. Eight of the variant products, in which Orn9 was replaced with nonnucleophilic cations, retained antibiotic activity, as indicated by nearly unchanged MIC values. Other modifications in addition to substitution of Orn9 can now be enabled and may be required to improve the therapeutic index (MHC/MIC), as the undesirable hemolytic effects of the position 9 substituted peptides were comparable to native TycA. The removal of the side chain amine nucleophile at position 9 is an important step toward further structural diversification of this cyclic decapeptide antibiotic, setting up the system for other reaction manifolds—for example, chemical or chemoenzymatic glycosylation [33–35] and fatty acid acylation—to generate lipoglycopeptide scaffolds.

Significance

Thioesterases found as the final domain of nonribosomal peptide synthetases catalyze a critical macrocyclization step in the biosynthesis of many biologically active cyclic peptides. Previous studies have demonstrated the utility of the thioesterase excised from tyrocidine synthetase (TycTE) as a chemoenzymatic tool in generating diverse cyclic peptides for screening. However, its use was limited by its short lifetime of catalytic activity and flux of the acyl-enzyme intermediate to nonproductive hydrolysis which lowered product yields. The requirement for a nucleophilic ornithine residue at substrate peptide position 9 also hindered structural diversification of the cyclic product. Herein we report that the addition of nonionic detergent greatly enhances the activity of this macrocyclization catalyst, increasing yields of the cyclic product up to 300-fold. The addition of nonionic detergent both extends the catalytic lifetime of the enzyme and induces a significant shift in the product ratio of TycTE in favor of macrocyclization (over the nonproductive hydrolysis reaction). This latter effect may be due to the action of detergent in facilitating the folding of the amphipathic peptidyl chain within the active site of TycTE and may be applicable in other systems which biosynthesize amphipathic products. Furthermore, the observed rate acceleration (comparable to that reported for other biological catalysts upon addition of surfactants) may provide important insight into the cellular context of catalysis by terminal TE domains, either at or near the cell membrane or as part of the macromolecular complex of the NRPS. Finally, the improved yields allowed an exploration of substitutions for the nucleophilic ornithine at position 9 of the tyrocidine decapeptide that could be accepted for enzymatic cyclization. Removal of the side chain nucleophile at Orn9 opens possibilities for further structural diversification of the cyclic peptide products generated by this macrocyclization catalyst.

Experimental Procedures

Synthesis of Peptide-SNAC

Automated SPPS was performed on a Symphony multiple synthesizer. Fmoc-protected amino acids were incorporated on 2-chlorotrityl resin derivatized with L-leucine (coupling reagent HBTU/HOBt). The final N-terminal amino acid contained Boc protecting group. The peptide was cleaved off the resin in 1:1:3 acetic acid: trifluoracetic acid (TFA): dichloromethane for 2 hr. Acetic acid was removed as an azeotrope with *n*-hexanes. Following rotary evaporation, the protected peptide (1 eq) was dissolved in tetrahydrofuran. DCC (2 eq), HOBt (2 eq), *N*-acetylcysteamine (10 eq), and diisopropylethylamine (10 eq) were added. The reaction was stirred for 3 hr, then filtered. The filtrate was then concentrated, deprotected in 95:2.5:2.5 TFA: triisopropylsilane: water, and precipitated in cold ether. Reversed phase HPLC purification on C18 column (20%–50% acetonitrile in 0.1% TFA/water over 30 min) yielded the purified peptide-SNAC in >95% purity. The product was verified on Shimadzu QP8000 ESI LC/MS.

Enzyme Assays

Recombinant TycTE was purified as previously described [10]. Enzyme activity was assayed in 50 mM MOPS (pH 7.0) buffer ± detergent with varying substrate concentrations. Reactions were performed at 25°C and quenched by addition of TFA to 0.1% and flash frozen in N_2 (l). Samples were thawed, and acetonitrile was added to final 20% before loading onto Vydac peptide/protein C18 column. Product was eluted on 20%–100% acetonitrile gradient over 35 min, monitoring at 220 nm.

Cyclization Reactions of Substituted Peptide Library

The peptide library was synthesized as described [17]. The first amino acid (corresponding to Leu10) was coupled by 1-(mesitylene-2-sulphonyl)-3-nitro-1H-1, 2, 4- triazole (MSNT, 5 eq) and 1-methylimidazole (3.75) in DMF. The mono-substituted resin was distributed in a 96-well filter plate. A library of 86 amino acids plus 10 L-ornithine controls was coupled by standard FMOC chemistry. In a final coupling step, the dipeptide resin was extended by a protected octapeptide (corresponding to positions 1–8 of the tyrocidine sequence) which had been synthesized by automated SPPS. Resin-bound peptides were deprotected in 95: 2.5: 2.5 TFA: triisopropylsilane: water. Excised TycTE was expressed and purified (dialyzed into 20 mM NaCl, 50 mM MOPS [pH 7.0]) [10]. Enzyme-catalyzed cyclization was carried out by incubation of resin-bound peptides with 10 μM TycTE for 2 hr in 50 mM MOPS (pH 7.0), 20 mM NaCl, and 0.1% Brij 58 in 800 μl volume. Product was eluted by centrifugation, and resin washed with 200 μl acetonitrile (combined with product filtrate). Product peaks were identified on Shimadzu QP8000 electrospray ionization LC/MS and quantified by analytical HPLC using the determined extinction coefficient for the tyrocidine peptide at 220 nm.

Preparation of Cyclized TycA Variants Substituted at Position 9

Linear peptide *N*-acetylcysteamine thioesters (SNAC) were synthesized as described above. For the preparation of cyclic peptides, 500 μM peptide SNAC was incubated for 3 hr with 1 μM Tyc TE in 25 mM MOPS (pH 7) and 0.1% Brij 58. The cyclic peptides were purified by preparative HPLC. Lypholized cyclic peptides were dissolved in methanol. The purities were checked by analytical HPLC on a 20%–100% gradient of buffer B (B = acetonitrile/0.1% TFA; A = water/0.1% TFA), and the concentrations were determined by comparing the area of absorption at 220 nm with that of a known concentration of **TLP**. Masses as determined by LCMS and HPLC retention times for the **TLP** peptides substituted at position 9 were as follows: ornithine, HPLC retention time (RT) = 22.5 min, MS m/z 1270; lysine, RT = 22.2 min, MS m/z 1284; arginine, RT = 22.7 min, MS m/z 1312; isopropyllysine, RT = 22.9 min, MS m/z 1326; dimethyllysine, RT = 22.4 min, MS m/z 1312; trimethyllysine, RT = 22.3 min, MS m/z 1327; 3-pyridylalanine, RT = 22.6 min, MS m/z 1304; histidine, RT = 22.4 min, MS m/z 1293; and diaminobutyric acid, RT = 22.1 min, MS m/z 1256.

MIC and MHC Determination for Variant Cyclic Peptides

Cyclic peptides were serially diluted in methanol and dried under vacuum. For minimal inhibitory concentration (MIC) testing, an overnight *B. subtilis* PY79 culture was diluted (1/10,000) with LB media, and 80 μl of the diluted culture was added to each well. After overnight incubation at 30°C, the concentrations required for complete inhibition of bacterial cell growth were determined by visual inspection. Minimal hemolytic concentration (MHC) values were determined by addition to dried peptides of 80 μl of human red blood cells (Research Blood Components, Boston, MA) diluted (1/100) with PBS buffer (pH 7.4). RBCs were incubated at room temperature overnight, and concentrations required for complete lysis were determined visually.

Acknowledgments

This work was supported in part by grants from the Medical Scientist Training Program (E.Y. and R.M.K.), Jane Coffins Child Foundation (H.L.), NSERC (Canada, S.L.C.), and the National Institutes of Health (Grant GM 20011, C.T.W.). We thank Professor Michael Gelb for his helpful discussion concerning surface-dilution kinetics. We also thank Oleg Tsodikov and Xiaohui Lu for providing detergent reagents used in these studies.

Received: July 7, 2004
Revised: August 18, 2004
Accepted: September 7, 2004
Published: November 29, 2004

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