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Benzalacetone synthase

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Benzalacetone synthase, from the medicinal plant *Rheum palmatum* (*Rp*BAS), is a plant-specific chalcone synthase (CHS) superfamily of type III polyketide synthase (PKS). *Rp*BAS catalyzes the one-step, decarboxylative condensation of 4-coumaroyl-CoA with malonyl-CoA to produce the C_6–C_4 benzalacetone scaffold. The X-ray crystal structures of *Rp*BAS confirmed that the diketide-forming activity is attributable to the characteristic substitution of the conserved active-site "gatekeeper" Phe with Leu. Furthermore, the crystal structures suggested that *Rp*BAS employs novel catalytic machinery for the thioester bond cleavage of the enzyme-bound diketide intermediate and the final decarboxylation reaction to produce benzalacetone. Finally, by exploiting the remarkable substrate tolerance and catalytic versatility of *Rp*BAS, precursor-directed biosynthesis efficiently generated chemically and structurally divergent, unnatural novel polyketide scaffolds. These findings provided a structural basis for the functional diversity of the type III PKS enzymes.

Keywords: benzalacetone synthase, polyphenol, polyketide synthase, enzyme, biosynthesis

INTRODUCTION

The diketide benzalacetone scaffold is produced by benzalacetone synthase (BAS; EC 2.3.1.-), a member of the plant-specific chalcone synthase (CHS) superfamily of type III polyketide synthases (PKSs), by the one-step decarboxylative condensation of 4-coumaroyl-CoA with one molecule of malonyl-CoA (**Figure 1A**; Abe et al., 2001). BAS is thought to play a crucial role in the biosynthesis of the C_6–C_4 moiety of biologically active phenylbutanoids such as raspberry ketone, the characteristic aroma of raspberry fruit, and the anti-inflammatory glucoside lindleyin in rhubarb (Abe et al., 2001). In contrast, chalcone synthase (CHS; EC 2.3.1. 74), a typical type III PKS ubiquitously distributed in plants, catalyzes iterative condensations of 4-coumaroyl-CoA with three C_2 units from malonyl-CoA to produce the tetraketide chalcone (**Figure 1A**), which is the biosynthetic precursor of flavonoids (Austin and Noel, 2003; Abe and Morita, 2010). To the best of our knowledge, only five BAS-encoding cDNAs have been reported, including those from *Rheum palmatum* (*Rp*BAS; Abe et al., 2001), *Wachendorfia thyrsiflora* (*Wt*PKS1; Brand et al., 2006), *Rubus idaeus* (*Ri*PkS4; Zheng and Hrazdina, 2008), and *Polygonum cuspidatum* (*Pc*PKS1 and *Pc*PKS2; Ma et al., 2009a,b). The type III PKSs are structurally simple, homodimeric proteins, and their functional diversity is principally derived from small modifications in their active-site architectures, which generate the differences in their selection of starter molecules, numbers of malonyl-CoA condensations, and mechanisms of cyclization reactions (Austin and Noel, 2003; Abe, 2007, 2010; Abe and Morita, 2010). Recent crystallographic and site-directed mutagenesis studies have begun to reveal the intimate structural details of the enzyme-catalyzed processes (Austin and Noel, 2003; Abe, 2007, 2010; Abe and Morita, 2010). This mini-review summarizes our recent studies on the BAS from *R. palmatum* (Polygonaceae; *Rp*BAS), using an approach combining site-directed mutagenesis, X-ray crystallography, and precursor-directed biosynthesis.

CLONING, EXPRESSION, AND SITE-DIRECTED MUTAGENESIS

The cDNA encoding *Rp*BAS was cloned and sequenced from young leaves of *R. palmatum* by RT-PCR amplification using degenerate primers based on the conserved sequences of the known CHSs (Abe et al., 2001). The deduced amino acid sequence of *Rp*BAS shares 60–75% identity with those of the other members of the CHS-superfamily type III PKSs of plant origin. The recombinant *Rp*BAS was heterologously expressed in *Escherichia coli*, and afforded the diketide benzalacetone as a single product from 4-coumaroyl-CoA and malonyl-CoA as substrates, within a range of pH 8.0–8.5 (**Figure 1**; Abe et al., 2001). An enzyme kinetics analysis revealed that *Rp*BAS showed a $K_M = 10.0\,\mu M$ and a $k_{cat} = 1.79\,min^{-1}$ for 4-coumaroyl-CoA, and a $K_M = 23.3\,\mu M$ and a $k_{cat} = 1.78\,min^{-1}$ for malonyl-CoA, with respect to the benzalacetone-producing activity at pH 8.0. Interestingly, there was a dramatic change in the product profile under acidic pH conditions. Instead of benzalacetone, the triketide pyrone bisnoryangonin (BNY) was obtained as almost the sole product at pH 6.0 (Abe et al., 2003).

A sequence analysis revealed that *Rp*BAS maintains an almost identical CoA binding site and the catalytic triad of Cys164, His303, and Asn336 (numbering in *Ms*CHS; Ferrer et al., 1999). In addition, the active-site residues, including Met137, Gly211, Gly216, Ile254, Gly256, Phe265, Ser338, and Pro375, are well conserved in *Rp*BAS. However, Phe215, which is conserved in all known type III PKSs, is characteristically substituted with Leu (^{214}IL instead of ^{214}LF; Abe et al., 2001). In CHS, the "gatekeeper" Phe215, located at the junction between the active-site cavity and the CoA binding tunnel, is thought to facilitate the decarboxylation of malonyl-CoA and to help orient the substrates

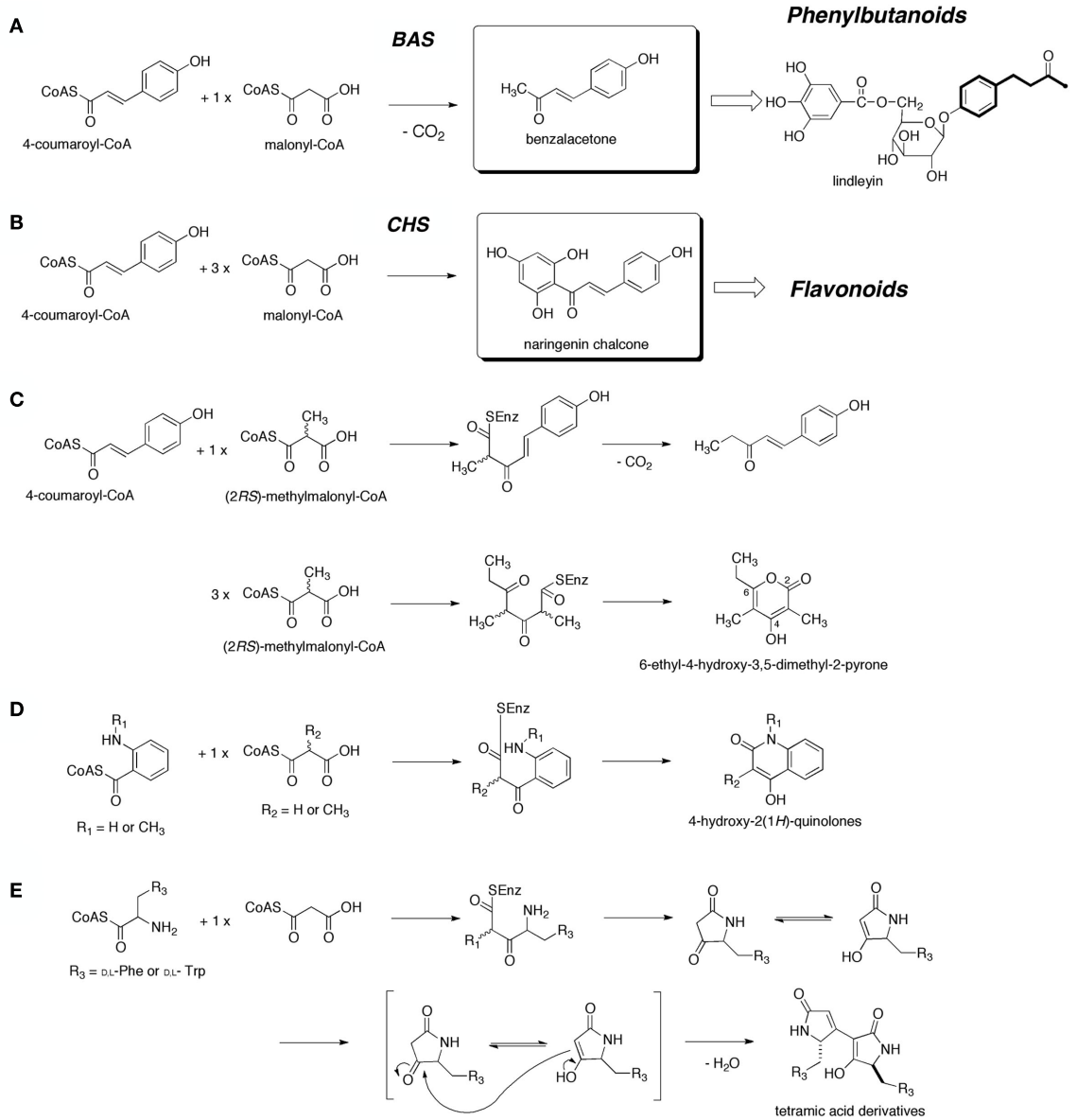

FIGURE 1 | (A) The proposed mechanisms for the formation of benzalacetone by *R. palmatum* BAS, and naringenin chalcone by CHS. The proposed mechanisms for the formation of **(B)** the C_6–C_5 diketide, **(C)** the methylated C_9 triketide pyrone, **(D)** the diketide quinolones, and **(E)** the diketide tetramic acid derivatives by *R. palmatum* BAS.

and intermediates during the condensation reactions (Ferrer et al., 1999). This led to the proposal that the unique substitution of the "gatekeeper" Phe causes the termination of chain elongation at the diketide stage, which was later supported by the observation that the *Rp*BAS I214L/L215F mutant indeed yielded naringenin chalcone by condensations with three molecules of malonyl-CoA (Abe et al., 2003). The *Rp*BAS I214L/L215F mutant showed a $K_M = 33.5\,\mu M$ and a $k_{cat} = 0.169\,min^{-1}$ for 4-coumaroyl-CoA, and a $K_M = 46.6\,\mu M$ and a $k_{cat} = 0.181\,min^{-1}$ for malonyl-CoA, with respect to the chalcone-producing activity at pH 6.5 (Abe et al., 2003). The mutant thus exhibited a 36-fold decreases in the k_{cat}/K_M for 4-coumaroyl-CoA and a 20-fold decreases in the k_{cat}/K_M for malonyl-CoA, as compared with the wild-type *Rp*BAS.

On the other hand, the conserved active-site Thr197 (numbering in *Ms*CHS), which is important for the steric modulation of the active-site cavity in a number of divergent type III PKSs (Austin and Noel, 2003; Abe and Morita, 2010), is uniquely replaced with a reactive Cys. Therefore, it was postulated that *Rp*BAS utilizes this second active-site Cys for the decarboxylation of a diketide intermediate to produce benzalacetone (Austin and Noel, 2003). To test this hypothesis, we constructed a mutant in which the Cys is substituted with Thr; however, the C197T mutant was essentially functionally identical to the wild-type *Rp*BAS, thus

excluding the possibility (Abe et al., 2007). In addition, we constructed mutants in which the active-site residues, Cys197, Gly256, and Ser338 (numbering in *Ms*CHS), were respectively replaced with Gly, Leu, and Val (Abe et al., 2007), as in the case of the *Aloe arborescens* octaketide synthase (*Aa*OKS) that produces the octaketides SEK4/SEK4b by condensations of eight molecules of malonyl-CoA (Abe et al., 2005; Morita et al., 2007). The OKS-like C197G and G256L substitutions are thought to be important for the steric modulation of the active-site cavity, thereby affecting both the substrate and product specificities of the enzyme reaction. In fact, it was previously reported that the S338V mutant of the chalcone-producing CHS yielded trace amounts of SEK4/SEK4b, by the steric modulation of a single residue lining the active-site cavity (Abe et al., 2006b). However, in *Rp*BAS, none of the mutants generated a different product pattern (Abe et al., 2007).

Finally, as described below, crystallographic analyses revealed that the characteristic substitution of the conserved Thr132 (numbering in *Ms*CHS), at the entrance of the "coumaroyl-binding pocket," with the bulky Leu causes steric contraction of the active-site of *Rp*BAS (Morita et al., 2010), which could also terminate the chain elongation at the diketide stage. Indeed, the L132T substitution also restored the chalcone-producing activity in *Rp*BAS (Shimokawa et al., 2010). The L132T mutant showed a $K_M = 4.1\ \mu M$ and a $k_{cat} = 1.60 \times 10^{-3}\ min^{-1}$ for 4-coumaroyl-CoA, with respect to the chalcone-producing activity, with a pH optimum of 6.5 (Shimokawa et al., 2010).

CRYSTAL STRUCTURE AND STRUCTURE-BASED MECHANISM

The X-ray crystal structures of both the wild-type and chalcone-producing I214L/L215F mutant of *Rp*BAS were solved at 1.8 Å resolution (Morita et al., 2010). Furthermore, the crystal structure of the wild-type *Rp*BAS, with a monoketide coumarate intermediate covalently bound to the catalytic cysteine residue, was solved at 1.6 Å resolution (Morita et al., 2010). This is the first direct evidence that type III PKS utilizes the Cys as both the nucleophile and attachment site for the growing polyketide intermediate.

The overall structures of *Rp*BAS are highly homologous to those of the previously reported plant type III PKSs, including *M. sativa* CHS (*Ms*CHS; Ferrer et al., 1999) and *Pinus sylvestris* stilbene synthase (*Ps*STS; Austin et al., 2004). On the other hand, a comparison of the active-site structure of *Rp*BAS with that of *Ms*CHS revealed that the conformational differences of Leu215 and Ser338 (numbering of *Ms*CHS) cause the loss of the CHS's "coumaroyl-binding pocket" from the active-site of *Rp*BAS. In addition, the conserved Thr197, at the entrance of the "coumaroyl-binding pocket," is characteristically substituted with the bulky Leu in *Rp*BAS. As a result, the total cavity volume (350 $Å^3$) of the active-site is much smaller than that of *M. sativa* CHS (750 $Å^3$; Morita et al., 2010).

The crystal structures revealed that, instead of the "coumaroyl-binding pocket," *Rp*BAS utilizes an alternative, novel pocket, which protrudes into the "floor" of the conventional CHS active-site, to bind the aromatic moiety of the coumarate (**Figure 2**; Morita et al., 2010). Furthermore, the F215L substitution causes not only the conformational changes at the active-site, but also a twofold increase in the surface area of the active-site entrance of *Rp*BAS, as compared to that of *Ms*CHS (Morita et al., 2010). This widening of the active-site entrance contributes to the unique substrate and product specificities of *Rp*BAS. In contrast, the crystal structure of the *Rp*BAS I214L/L215F mutant revealed that the substitutions restored the "coumaroyl-binding pocket" within the active-site of the mutant, whereas both the locations and orientations of the residues lining the active-site cavity are almost perfectly conserved with those in the wild-type *Rp*BAS (**Figure 2**; Morita et al., 2010). Thus, the I214L/L215F substitutions open a gate to the buried "coumaroyl-binding pocket," thereby increasing the chain elongation up to three condensations with malonyl-CoA, which leads to the recovery of the chalcone-producing activity.

Considering the conservation of the overall folding and the catalytic triad, it is likely that *Rp*BAS and CHS utilize similar catalytic mechanisms for the initiation of the enzyme reaction and the condensation of malonyl-CoA (Ferrer et al., 1999). One of the most interesting points is the catalytic mechanism of the thioester bond cleavage of the enzyme-bound diketide intermediate, and the final decarboxylation that produces the C_6–C_4 scaffold (Morita et al., 2010). Previously, based on a crystallographic comparison of the stilbene-producing *Ps*STS with the chalcone-producing *Ms*CHS, Noel and Schröder proposed that an "aldol-switch" thioesterase-like hydrogen bond network, including a nucleophilic water molecule and Thr132, neighboring the catalytic Cys164 (numbering in *Ms*CHS), is required for the thioester bond cleavage of the tetraketide intermediate to produce stilbene in *Ps*STS (Austin et al., 2004). In contrast, the cleavage of the thioester linkage is caused by intramolecular cyclization *via* a Claisen condensation to produce chalcone in *Ms*CHS (Ferrer et al., 1999). However, a careful examination of the *Rp*BAS crystal structure negated the presence of such hydrogen bond networks, since Thr132 is replaced with the hydrophobic Leu in *Rp*BAS. Instead, the crystal structures clearly demonstrated the presence of a distinct, activated water molecule that forms hydrogen bond networks with the Cys-His-Asn catalytic triad. This finding suggested that *Rp*BAS employs unique catalytic machinery for the thioester bond cleavage of the enzyme-bound diketide intermediate and the final decarboxylation reaction, and that the β-keto acid produced by the nucleophilic attack of the water molecule, presumably activated by His303 (numbering in *Ms*CHS), subsequently undergoes decarboxylation to yield the C_6–C_4 benzalacetone (**Figure 2G**; Morita et al., 2010). Thus, the decarboxylation of the β-keto acid proceeds *via* the proton abstraction by His303, the reactivation by the Cys164 thiolate, and the formation of an enolate anion that is presumably stabilized by the His303–Asn336 oxyanion hole, as in the case of the decarboxylation of malonyl-CoA. Finally, tautomerization to the keto form produces benzalacetone and restores the Cys-His-Asn catalytic triad. This proposal is supported by the detection of the diketide β-keto acid intermediate in the reaction products of *Rp*BAS (Morita et al., 2010).

SUBSTRATE SPECIFICITY AND PRECURSOR-DIRECTED BIOSYNTHESIS

As in the cases of the other members of the type III PKSs (Abe et al., 2000; Jez et al., 2002; Austin and Noel, 2003; Abe, 2010, 2012; Abe and Morita, 2010; Morita et al., 2011), *Rp*BAS exhibits unusually broad, promiscuous substrate specificities, and readily accepts

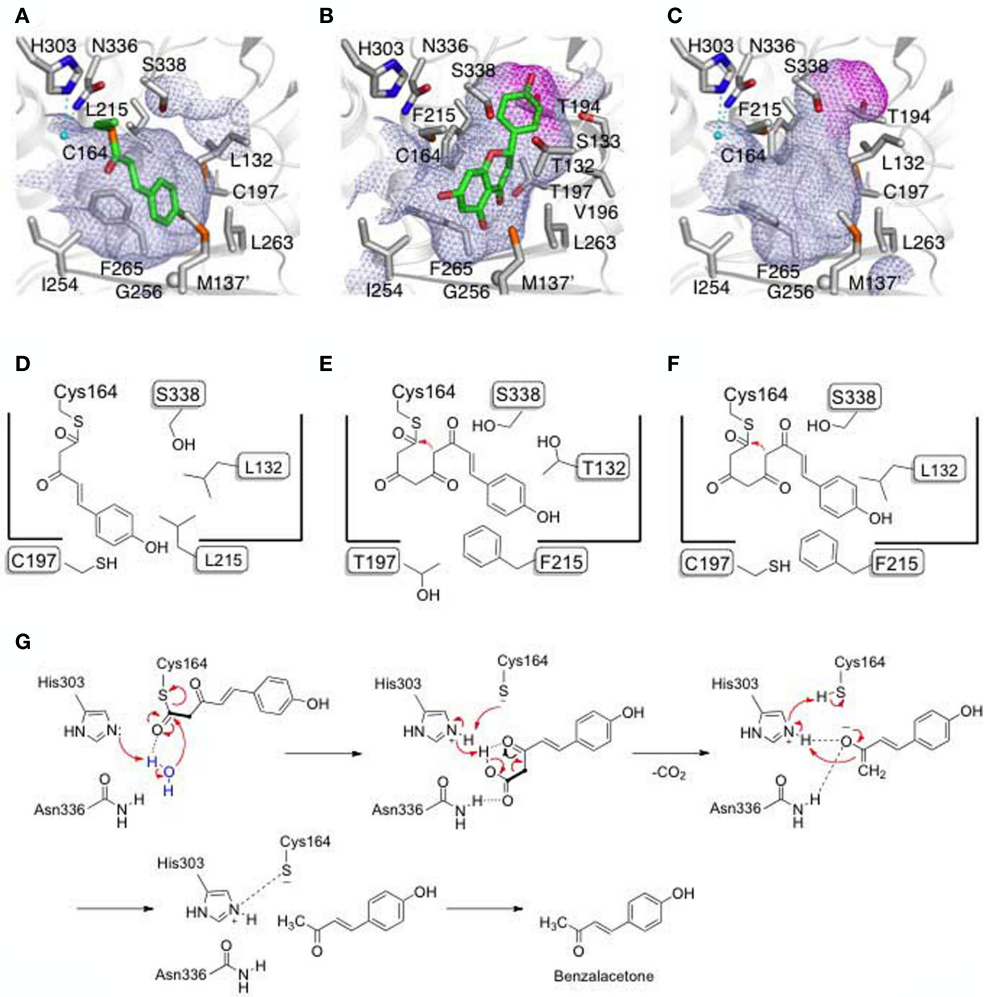

FIGURE 2 | Surface and schematic representations of the active-site cavities of (A,D) wild-type *R. palmatum* BAS, (B,E) *M. sativa* CHS, and (C,F) the *R. palmatum* BAS I207L/L208F mutant (numbering in *Rp*BAS). The bottoms of the "coumaroyl-binding pocket" are highlighted as purple surfaces. The covalently bound coumarate, the water molecules and the hydrogen bonds are highlighted. Proposed mechanism for the BAS enzyme reaction **(G)**.

diverse non-physiological substrates to produce a variety of chemically and structurally distinct unnatural polyketides. Furthermore, as described, the crystal structures of *Rp*BAS revealed that the characteristic substitution of the "gatekeeper" Phe residue causes not only conformational changes at the active-site, but also a twofold increase in the surface area of the active-site entrance of *Rp*BAS, as compared to that of *Ms*CHS (Morita et al., 2010). This widening of the active-site entrance contributes to the unique substrate specificities of *Rp*BAS, allowing it to accept bulky non-physiological substrates.

For example, *Rp*BAS can accept (2*RS*)-methylmalonyl-CoA as an extender substrate, and a 4-coumaroyl-CoA starter, to produce an unnatural novel diketide, 1-(4-hydroxyphenyl)pent-1-en-3-one (**Figure 1B**; Abe et al., 2002). The C_6–C_5 skeleton was thus produced by the one-step decarboxylative condensation of 4-coumaroyl-CoA with the C_2 unit of methylmalonyl-CoA. Notably, since a diketide carboxylic acid was not detected in the reaction mixture, the decarboxylation of the enzyme-bound intermediate should be tightly controlled by the enzyme (Abe et al., 2002). Interestingly, *Rp*BAS also accepts (2*RS*)-methylmalonyl-CoA as the only substrate to produce a methylated C_9 triketide, 6-ethyl-4-hydroxy-3,5-dimethyl-2-pyrone, as a single product from three molecules of (2*RS*)-methylmalonyl-CoA (**Figure 1C**; Abe et al., 2006c). It is remarkable that the type III PKS accepts (2*RS*)-methylmalonyl-CoA as both a *starter* and an *extender* substrate, and catalyzes the formation of the C_9 triketide pyrone.

Anthranilic acid has been postulated to be a key intermediate in the biosynthesis of quinolone and acridone alkaloids, which occur in their greatest abundance in plants from the family of Rutaceae. Interestingly, *Rp*BAS efficiently catalyzes the condensation of *N*-methylanthraniloyl-CoA with one molecule of either malonyl-CoA or (2*RS*)-methylmalonyl-CoA, to produce the corresponding diketide, 4-hydroxy-2(1*H*)-quinolones (**Figure 1D**; Abe et al., 2006a). The enzyme reaction with the anthraniloyl

starter thus proceeds without the decarboxylation step, and amide formation immediately follows the condensation reactions with malonyl-CoA. Notably, the best yield was obtained with the combination of the non-physiological *N*-methylanthraniloyl-CoA and the (2*RS*)-methylmalonyl-CoA. Steady-state enzyme kinetics for the formation of 4-hydroxy-1,3-dimethyl-2(1*H*)-quinolone revealed a $K_M = 23.7\,\mu M$ and a $k_{cat} = 1.48\,min^{-1}$ for *N*-methylanthraniloyl-CoA, which were comparable to those for the formation of benzalacetone. This result suggests the existence of an as yet unidentified novel type III PKS that produces quinolones from the CoA thioester of anthranilic acid (Abe et al., 2006a).

Finally, *Rp*BAS also accepts a series of aminoacyl-CoA thioesters as starter substrates, and catalyzes their condensation with one molecule of malonyl-CoA to produce the tetramic acid (2,4-pyrrolidinedione) derivatives (**Figure 1E**; Wakimoto et al., 2011). For example, the enzyme afforded a 1:10 mixture of two products from L-phenylalanoyl-CoA and malonyl-CoA. The minor product was a tetramic acid monomer, which is produced by the condensation of L-phenylalanoyl-CoA with malonyl-CoA, followed by intramolecular lactamization from the enzyme-bound intermediate. On the other hand, the major product was a tetramic acid dimer. Since the active-site of *Rp*BAS is apparently large enough for the monomer, but not the dimer, the dimer is likely to be produced by the non-enzymatic, spontaneous dimerization between each tautomer. Interestingly, *Rp*BAS efficiently accepted both L- and D-phenylalanoyl-CoAs as substrates. Steady-state kinetics analyses revealed a $K_M = 11.7\,\mu M$ and a $k_{cat} = 27.8\,min^{-1}$ for L-phenylalanoyl-CoA, and a $K_M = 3.3\,\mu M$ and a $k_{cat} = 8.1\,min^{-1}$ for D-phenylalanoyl-CoA. Furthermore, *Rp*BAS also efficiently accepted both L- and D-tryptophanoyl-CoAs as substrates, to produce the corresponding monomeric and dimeric tetramic acid derivatives in nearly equal yields (Wakimoto et al., 2011). The structurally simple *Rp*BAS thus initially accepts the aminoacyl-CoA as a starter, and then recruits malonyl-CoA for a Claisen condensation to generate the γ-amino-β-ketothioester. The free γ-amino group of the enzyme-bound intermediate could cleave the thioester bond, with concomitant intramolecular lactamization. The results suggests the possibility of the further preparation of tetramic acids from various amino acids.

SUMMARY

Crystallographic and site-directed mutagenesis studies on the functionally diverse type III PKSs have begun to reveal the intimate structural details of the enzyme-catalyzed processes. It is remarkable that the catalytic plasticity of the structurally and mechanistically conventional type III PKSs evolved from simple modifications of the substrate structures and the active-sites of the enzymes. By exploiting the remarkable substrate tolerance and catalytic versatility of the enzymes, the precursor-directed biosynthesis will contribute to the further production of chemically and structurally divergent, unnatural novel polyketides.

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