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COMMUNICATION

A type III polyketide synthase from *Rhizobium etli* condenses malonyl CoAs to a heptaketide pyrone with unusually high catalytic efficiency†

Marimuthu Jeya,[a] Tae-Su Kim,[a] Manish Kumar Tiwari,[a] Jinglin Li,[a] Huimin Zhao*[bcd] and Jung-Kul Lee*[a]

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A novel type III polyketide synthase (RePKS) from *Rhizobium etli* produced a heptaketide pyrone using acetyl-CoA and six molecules of malonyl-CoA. Its catalytic efficiency (k_{cat}/K_m = 5230 mM^{-1} min^{-1}) for malonyl CoA was found to be the highest ever reported. Molecular dynamics studies revealed the unique features of RePKS.

Type III polyketide synthases (PKSs) generate the backbones of a variety of plant secondary metabolites including chalcones, stilbenes, phloroglucinols, resorcinols, benzophenones, biphenyls, bibenzyls, chromones, acridones, pyrones, and curcuminoids.[1,2] The plant type III PKSs share 30–95% amino acid sequence identity with each other, but only 21–31% identity with those of bacterial origin. The functional diversity of the type III PKSs is attributable to differences in their selection of the starter substrate, the number of polyketide chain extensions, and the mechanisms of their cyclization reactions.

Several bacterial type III PKSs have been characterized at present; for example, RppA from *Streptomyces coelicolor* and *Streptomyces griseus*[3] and PhlD from *Pseudomonas fluorescens*.[4] This work reports the characterization of the gene product (RePKS) of AAM55027.1 from *Rhizobium etli*. The RePKS is a distinct type III PKS that catalyzes the synthesis of a heptaketide pyrone using acetyl-CoA and six molecules of malonyl-CoA. This is the first report that describes the catalytic properties of a type III PKS from *R. etli*.

Results

The amino acid alignment of RePKS with plant, bacterial, and fungal type III PKSs showed conservation of the Cys-His-Asn catalytic triad in RePKS, thus providing evidence for RePKS being a type III PKS (Fig. S1, ESI†). The RePKS encoding gene was cloned and expressed in *E. coli* BL21-CodonPlus (DE3)-RIL with an N-terminal 6×His affinity tag. The purified His_6-tagged RePKS protein gave a single protein band at ~37 kDa on SDS-PAGE. RePKS efficiently accepted malonyl-CoA as a sole substrate and yielded a single product with a parent ion peak $[M + H]^+$ at *m*/*z* 277 on LC/ESI-MS (Fig. 1). The molecular weight of the product corresponded to a heptaketide pyrone. A heptaketide pyrone was also detected in the reaction products of *Rheum palmatum* aloesone synthase and *Aloe arborescens* PKS3 which produced aloesone as a final product.[5] Hence, the product was further confirmed by the instrumental analysis as described previously.[5] In the reaction of RePKS with malonyl-CoA, a major product had a retention time of 14.7 min (Fig. 1) in HPLC that was similar to the minor heptaketide pyrone intermediate produced by *Aloe arborescens* PKS3. Instrumental analysis (HPLC, UV, MS and HRMS) of the product obtained by a large-scale enzyme reaction confirmed the product to be 6-(2-(2,4-dihydroxy-6-methylphenyl)-2-oxoethyl)-4-hydroxy-2-pyrone. It was found that acetyl-CoA resulting from decarboxylation of malonyl-CoA was a better starter substrate than malonyl-CoA for RePKS. The substrate preference was confirmed by using labeled ^{14}C malonyl-CoA

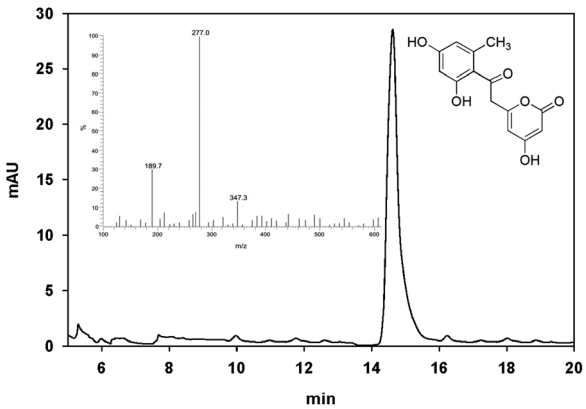

Fig. 1 HPLC analysis of the product synthesized by RePKS with malonyl CoA as a starter and extender substrate. The inset figure shows the ESI-MS chromatogram and structure of the product peak at 14.7 min which corresponds to a heptaketide pyrone (MW = 276). The reaction product of RePKS was analyzed by LC/ESI-MS in positive mode.

[a] *Department of Chemical Engineering, Konkuk University, Seoul 143-701, Korea. E-mail: jkrhee@konkuk.ac.kr; Fax: +82-2-458-3504*
[b] *Departments of Chemical and Biomolecular Engineering, Chemistry, and Bioengineering, University of Illinois, 600 South Mathews Avenue, Urbana, IL 61801, USA. E-mail: zhao5@illinois.edu; Fax: +1 217-333-5052*
[c] *Institute for Genomic Biology, University of Illinois, 600 South Mathews Avenue, Urbana, IL 61801, USA*
[d] *Center for Biophysics and Computational Biology, University of Illinois, 600 South Mathews Avenue, Urbana, IL 61801, USA*
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Table 1 Relative activity of the RePKS protein with different starter units. Results are means (n = 3) with SE values less than 15%

Starter CoA	Incorporation efficiency by RePKS (%)
Acetyl-CoA (**2a**)	100
Malonyl-CoA (**1a**)	56.7
Hexanoyl-CoA (**2b**)	5.8
Lauroyl-CoA (**2d**)	13.6
Stearoyl-CoA (**2g**)	4.7
Benzoyl-CoA (**2h**)	6.2

and acetyl-CoA. Reaction was performed by the addition of RePKS to [2-^{14}C]malonyl-CoA in the presence and absence of acetyl-CoA. Approximately a two-fold increase in the yield of the heptaketide was observed when acetyl-CoA was present in the reaction mixture. The substrate specificity of RePKS was further analyzed using CoA esters of C_2 to C_{18} straight-chain fatty acids as starter substrates, with malonyl-CoA as the extender. The incorporation efficiency of each acyl-CoA is listed in Table 1. Use of butyryl CoA as the starter substrate resulted in a heptaketide and also a small amount of a product that was identified by LC/ESI-MS to be a triketide (M_r = 154). With hexanoyl-CoA as the starter, RePKS produced a triketide (**3b**) (M_r = 182) as determined by LC/ESI-MS.

Similarly, use of octanoyl-CoA as the starter generated a triketide (M_r = 210) product. Decanoyl-CoA was also accepted by RePKS and resulted in the formation of a triketide (**3c**) (M_r = 238) and a tetraketide (**4c**) (M_r = 280). Further, RePKS showed activity with long chain acyl-CoAs including lauroyl-CoA (**2d**), myristoyl-CoA (**2e**), palmitoyl-CoA (**2f**) and stearoyl-CoA (**2g**), and aromatic substrate benzoyl-CoA (**2h**). The heptaketide pyrone was detected in all reactions with various starter-CoAs along with new products, and the molecular formulae of products were confirmed by HRMS. ESI-MS data are provided in the ESI.† The product formation by RePKS and the general structures of all the products are given in Fig. 2.

RePKS showed the highest activity towards malonyl-CoA. RppA from *S. griseus*, apart from using five molecules of malonyl-CoA as a starter to produce THN, accepted aliphatic acyl-CoAs with the carbon lengths from C_4 to C_8 as starter substrates and catalyzed sequential condensation of malonyl-CoA to yield α-pyrones and phloroglucinols.[6] PhlD from *P. fluorescens* produces phloroglucinol from three molecules of malonyl-CoA and also showed broad substrate specificity by accepting C_4–C_{12} aliphatic acyl CoAs.[4] RePKS differs from these enzymes in carrying out more condensations, *i.e.*, condensations of six molecules of malonyl-CoA with acetyl-CoA to produce a heptaketide pyrone.

Kinetic parameters were determined according to Zha *et al.*[4] Under the optimal assay conditions (30 °C, pH 8.0), the apparent k_{cat} and K_m values of purified RePKS for malonyl-CoA were 22.5 min^{-1} and 4.3 μM, respectively, giving a catalytic efficiency (k_{cat}/K_m) of 5230 mM^{-1} min^{-1}. The K_m value of RePKS is within the range of those reported for other type III PKSs.[5,7–10] However, the k_{cat}/K_m value of RePKS towards malonyl-CoA was the highest among type III PKSs reported and 2.7 fold higher than that of PhlD.

To investigate the structural basis for the ability of RePKS to accept acetyl-CoA and perform condensations with six

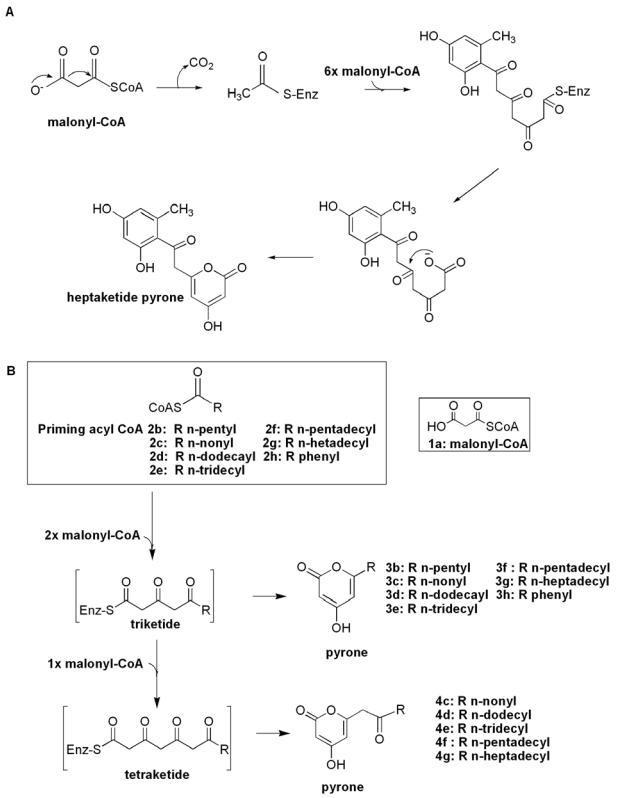

Fig. 2 Summary of RePKS reactions with various acyl-CoA starter substrates. (A) Heptaketide pyrone formation by RePKS with malonyl-CoA as a starter and extender substrate. (B) RePKS with various other acyl-CoAs as starter substrates produces corresponding tri and tetraketide pyrone products.

molecules of malonyl-CoA, a homology model of RePKS[11] was built based on the crystal structure of THNS from *S. coelicolor* (Protein Data Bank accession code 1U0M) which shares 32% sequence identity with RePKS. THNS accepts only malonyl-CoA, not acetyl-CoA. Prior biochemical and structural studies of plant and bacterial type III PKSs have established the importance of several residues near the active site in controlling substrate and product specificities. These include Thr-197, Gly-256, and Ser-338 of *M. sativa* CHS.[12] Thr-197 of CHS (or Cys-171 of THNS) is replaced by Ala-175 in RePKS (Fig. 3). The cavity volume of RePKS is larger (756 Å^3) than that of THNS (622 Å^3), which can probably explain that RePKS performs condensations of six molecules of malonyl-CoA compared to the utilization of five malonyl CoAs by THNS (Fig. S2, ESI†). The homology model of *R. palmatum* aloesone synthase (ALS) had a much larger cavity volume (1170 Å^3) than that of THNS (622 Å^3) and thus it produced aloesone after condensation with seven malonyl-CoAs.[5] Further, the size of the active site cavity physically limits the number of malonyl-CoA condensations, as implied from the X-ray crystal structures of 2-pyrone synthase and CHS.[13,14]

The RePKS enzyme showed highest catalytic efficiency towards malonyl-CoA than other reported type III PKSs (Table 2). The structures of CHS complexed with CoA, acetyl-CoA, and hexanoyl-CoA defined the overall placement of CoA in the enzyme.[13,14] Because Cys-164 is the catalytic

Table 2 Comparison of kinetic constants of malonyl CoA determined for type III PKSs

Source	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	Ref.
Heptaketide-ALS (*Rheum palmatum*)	0.027	0.31	7
PCS (*Aloe arborescens*)	0.445	6.3	15
OKS (*Aloe arborescens*)	0.094	0.98	16
BIS (*Sorbus aucuparia*)	0.430	69.35	9
BPS (*Hypericum androsaemum*)	NR	NR	8
DluHKS (*Drosophyllum lusitanicum*)	NR	NR	10
PKS3 (*Aloe arborescens*)	0.007	0.085	5
PhlD (*Pseudomonas fluorescens*)	24	1883	4
RppA (*Streptomyces griseus*)	0.770	828	6
DpgA (*Amycolatopsis orientalis*)	0.810	54	17
RePKS (*Rhizobium etli*)	22.5	5230	This study

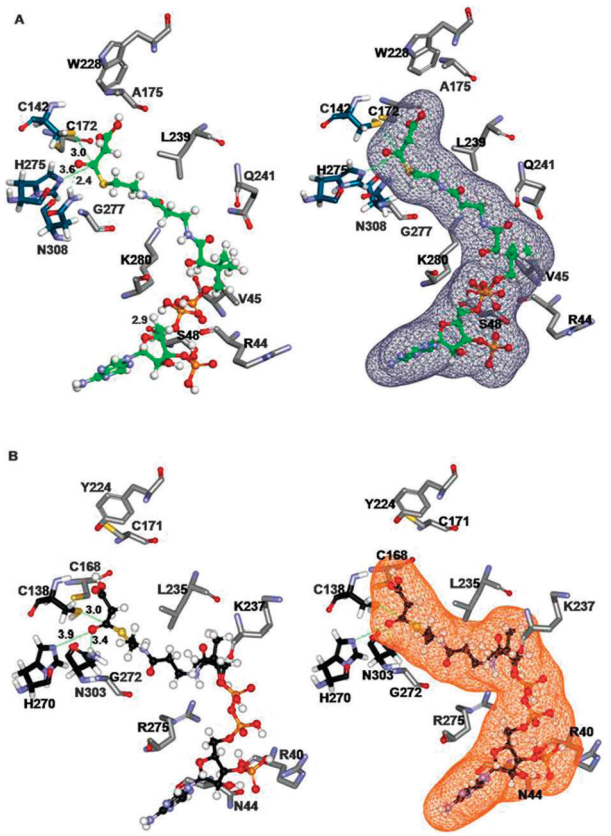

Fig. 3 Substrate docking of RePKS and THNS with malonyl CoA in the CoA binding tunnel. (A) Malonyl-CoA docking into the CoA binding tunnel of RePKS. Malonyl-CoA was bound through hydrogen bonds (green dotted lines) with Asn-308 (2.4 Å). Right figure represents the CoA binding tunnel of the modeled RePKS. Amino acid residues are shown in a stick model, residues in the CoA binding tunnel are colored with elemental carbon while catalytic amino acid residues are shown with blue color carbon. Malonyl-CoA is represented in a ball and stick model with green color carbon. (B) Malonyl-CoA docking into the CoA binding tunnel of THNS. Malonyl-CoA showed interacting distances of 3.4 Å, 3.9 Å and 3.0 Å with Asn-303, His-270, and Cys-138, respectively. Right figure represents the CoA binding tunnel of THNS. Amino acid residues are shown in a stick model, residues in the CoA binding tunnel are colored with elemental carbon while catalytic amino acid residues are shown with black color carbon. Malonyl-CoA is represented in a ball and stick model with black color carbon.

nucleophile required for tethering of the starter group and the growing polyketide chain, the CoA-thioester carbonyl carbon must be accessible to the cysteine S_γ. Interaction between the thioester carbonyl oxygen and the side-chains of Asn-336 and His-303 of CHS facilitates substrate binding and stabilizes the transition state during nucleophilic attack of Cys-164 on the thioester carbonyl.[13,14] With these constraints, we modeled the binding of malonyl-CoA to both the RePKS modeled structure and THNS structure (Fig. 3). When malonyl-CoA was docked into the CoA binding site of RePKS, malonyl-CoA was bound through hydrogen bonds (green dotted lines) with Asn-308 (2.4 Å). An interacting distance of 3.6 Å has been observed between the thioester carbonyl oxygen and nitrogen (Nε) atom of His-275. While a distance of 3.0 Å was observed between the sulphur of the catalytic cysteine and the thioester carbonyl group of malonyl-CoA. Other potential hydrogen bond interactions were also observed with Ser-48 (2.9 Å) (Fig. 3A). However, when malonyl-CoA was docked into the CoA binding site of THNS, there were no hydrogen bonding interactions between malonyl CoA and enzyme. Malonyl-CoA showed interacting distances of 3.4 Å, 3.9 Å and 3.0 Å with Asn-303, His-270, and Cys-138, respectively (Fig. 3B). Moreover, superimposition of the RePKS–malonyl–CoA complex and the THNS–malonyl–CoA complex (Fig. S3, ESI†) clearly defined the differences in amino acids (Trp228/Tyr224 and Ala175/Cys171) in the active site pocket and amino acid side chains (Gln-241/Lys-237; Lys-280/Arg-275; Ser-48/Asn-44) in the CoA binding site between these two enzymes.

To investigate the role of the residues (Gln-241, Lys-280, and Ser-48) in catalytic efficiency, they were each mutated to Ala. The recombinant enzymes carrying K280A, Q241A, and S48A mutations were expressed and purified. Their activity with malonyl-CoA was measured and compared with that of the wild-type RePKS. The k_{cat}/K_m value of the mutants was decreased from 50 to 72-fold compared to that of the wild type RePKS (Table S1, ESI†), suggesting that all of these residues play a significant role in the turnover of malonyl-CoA. The role of these residues in catalysis will be further investigated by site-directed mutagenesis.

Conclusions

RePKS characterized in the present study produced a heptaketide pyrone product from acetyl-CoA and malonyl-CoA. Although RePKS displayed broad substrate specificity, it exhibited the highest catalytic efficiency towards malonyl-CoA among type III PKSs reported. Overexpression of RePKS in engineered *E. coli* strain (with the improved cellular malonyl-CoA level)

will be useful for biosynthesis of polyketide products in higher yields. Crystal structure analyses combined with mutational studies are now in progress to probe the structural basis for the starter substrate specificity and the catalytic properties of RePKS.

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