Characterization and Evolutionary Implications of the Triad Asp-Xxx-Glu in Group II Phosphopantetheinyl Transferases

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**Abstract**

Phosphopantetheinyl transferases (PPTases), which play an essential role in both primary and secondary metabolism, are magnesium binding enzymes. In this study, we characterized the magnesium binding residues of all known group II PPTases by biochemical and evolutionary analysis. Our results suggested that group II PPTases could be classified into two subgroups, two-magnesium-binding-residue-PPTases containing the triad Asp-Xxx-Glu and three-magnesium-binding-residue-PPTases containing the triad Asp-Glu-Glu. Mutations of two three-magnesium-binding-residue-PPTases and one two-magnesium-binding-residue-PPTase indicate that the first and the third residues in the triads are essential to activities; the second residues in the triads are non-essential. Although variations of the second residues in the triad Asp-Glu-Glu exist throughout the whole phylogenetic tree, the second residues are conserved in animals, plants, algae, and most prokaryotes, respectively. Evolutionary analysis suggests that: the animal group II PPTases may originate from one common ancestor; the plant two-magnesium-binding-residue-PPTases may originate from one common ancestor; the plant three-magnesium-binding-residue-PPTases may derive from horizontal gene transfer from prokaryotes.

**Introduction**

Phosphopantetheinyl transferases (PPTases) play an essential role in both primary and secondary metabolism [1–3]. Recently, the production of natamycin, an antifungal reagent, has been optimized through engineering of a PPTase in an industrial natamycin producer, Streptomyces chattanoogensis L10 [4]. PPTases transfer the phosphopantetheinyl group of coenzyme A (CoA) to a conserved serine residue in acyl carrier proteins (ACPs) in fatty acid synthases (FASs) and polyketide synthases (PKSs) as well as peptidyl carrier proteins (PCPs) in nonribosomal peptide synthetases (NRPSs), converting ACPs/PCPs from inactive apo-forms into active holocomplexes [5–15]. PPTases can be classified into three groups based on their structures. The group I PPTases (ACP-type PPTases) are about 120 amino acids in length, which form trimeric quaternary structures [8,11–12,16]. The group II PPTases (Sfp-type PPTases) are more than 220 amino acids in length, which form monomeric tertiary structures with 2-fold pseudosymmetry within the monomers [5,13–15]. The group III PPTases exist as domains of FASs and PKSs [17–18].

The group I PPTases are found in most organisms except animals; the group II PPTases exist in almost all organisms; the group III PPTases are only found as domains fused within FASs in fungi and some PKSs in Streptomyces [1]. In most bacteria, group I PPTases phosphopantetheinylate ACPs in FASs and group II PPTases phosphopantetheinylate ACPs/PCPs in PKSs/NRPSs [4,6,19]. Animals and few bacteria contain single group II PPTase, which phosphopantetheinylate ACPs from both primary metabolism and secondary metabolism [20–22]. The group III PPTases phosphopantetheinylate the ACPs which locate with the group III PPTases in the same peptides [17–18].

Magnesium ion is essential to PPTase activity [23–24]. X-ray crystal structure analyses of two group II PPTases, Sfp from Bacillus subtilis and AASHDPPPT from Homo sapiens, reveal that one group II PPTase binds one magnesium ion. Six ligands of magnesium ion in Sfp are two phosphates of the CoA, one water molecule, and carboxylates of Asp107, Glu109, and Ghu151 of Sfp [24]. Interestingly, although the overall structure of AASHDPPPT closely resembles that of Sfp, six ligands of the magnesium ion are two phosphates of the CoA, two water molecules, and carboxylates of Asp129 and Ghu181 of AASHDPPPT. The two magnesium binding residues of AASHDPPPT, Asp129 and Ghu181, correspond to the first and the third magnesium binding residues of Sfp (Figure 1) [25]. Sfp and AASHDPPPT represent the three-magnesium-binding-residue-PPTases and the two-magnesium-binding-residue-PPTases, respectively.
To understand the relationship between structure and activity and the evolution of PPTases will shed light on catalytic mechanisms of PPTases. Here, we carried out a systematically evolutionary analysis and a biochemical analysis of group II PPTases. Our results suggested that: (i) group II PPTases could be classified into two subgroups, two-magnesium-binding-residue-PPTases with the triad Asp-Xxx-Glu and three-magnesium-binding-residue-PPTases with the triad Asp-Glu-Glu; (ii) the first and the third residues in the triads are essential to enzyme activities; the second residues in the triads are non-essential; (iii) the animal group II PPTases may originate from one common ancestor; the plant three-magnesium-binding-residue-PPTases may derive from horizontal gene transfer from prokaryotes.

Materials and Methods

Data collection

Protein sequences of annotated PPTases of E. coli, Streptomyces, and Homo sapiens were obtained from the National Center for Biotechnology Information (NCBI) database and were used as queries for gene search using BLASTP, PSI-BLAST from NCBI protein NR databases, with e value 1e-6 as the cutoff. PPTase homologs were selected based on the following criterion: sequence identity >35%, and length coverage >70%. In order to obtain all available annotated PPTases, archaean PPTases, cyanobacterial PPTases, plant PPTases, and animal PPTases were also obtained by searching for the annotated sequences as phosphopantetheinytransferases from GenBank databases, Phytozome (http://phytozome.net), and Ensembl (http://wwwensembl.org). PPTase data from both methods were merged, and representative sequences were used for further analysis.

Co-expression of scn ACP0-2 with SchPPT or each of SchPPT point mutant genes

All strains and plasmids used in this study are listed in Table 1. All primers used in this study are listed in Table S1. Plasmid pET44a (Novagen) was digested with NdeI/HindIII, filled in 5' overhangs to form blunt ends with DNA polymerase Klenow fragment, and then self-ligated with T4 DNA ligase, yielding plasmid pYY0040. The plasmid pYY0040 was introduced into E. coli BL21(DE3) containing pHJ0021 [4], in which snc ACP0-2 was cloned as a NdeI/HindIII fragment into pET28a (Novagen). BL21(DE3)/pHJ0021/pYY0040 was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30 °C for 4 h to overproduce scn ACP0-2. The scn ACP0-2 was purified by affinity chromatography on Ni-NTA agarose (Qiagen) and then dialyzed against 20 mM Tris-HCl (pH8.0), 25 mM NaCl, 1 mM dithiothreitol (DTT), and 10% glycerol. The scn ACP0-2 was analyzed by LC-MS as described previously [4].

SchPPT was digested with NdeI/HindIII from plasmid pHJ0024 [4], in which SchPPT was cloned as a NdeI/HindIII fragment into pET28a (Novagen), into the same sites of pET44a, yielding plasmid pYY0041. Each gene of five point mutants of SchPPT was amplified by mutagenesis PCR (QuikChange Site-Directed Mutagenesis Kit, Stratagene) from pYY0041 as template and primers HJ0117-HJ0122 and HJ0147-HJ0150, respectively. Each of the five genes was cloned into pET44a, yielding plasmids pYY0042-pYY0046, respectively. Co-expression of each plasmid pYY0041-pYY0046 with pHJ0021 in E. coli, and purification and LC-MS analysis of scn ACP0-2 are performed according to the procedures described above.

In vitro phosphopantetheinylation of scn ACP0-2 catalyzed by SchPPT, SchPPTD105A, or SchPPTE151A

SchPPTD105A and SchPPTE151A were cloned as NdeI/HindIII fragments from pET44a into pET28a, yielding plasmids pYY0062 and pYY0063, respectively. Each of the two plasmids was introduced into E. coli BL21(DE3) to overproduce proteins as N-terminally His6-tagged proteins under the induction with 0.4 mM IPTG at 30 °C for 4 h. A typical phosphopantetheinylation reaction mixture of 0.1 ml containing 100 mM Tris-HCl (pH 7.5), 1.25 mM MgCl2, 2.5 mM tris(carboxyethyl)phosphine hydrochloride (TCEP), 200 μM ACP, 20 μM PPTase, and 2 mM CoA was incubated at 25°C for 30 min. The reactions were quenched by freezing reaction mixtures with dry ice. LC-MS analysis of ACP was performed as described previously [4].

In vivo gene complement system

The SchPPT in-frame deletion mutant was constructed by using PCR targeting system as follows [26]. The cosmid pHJ0030 [4], in which SchPPT was replaced with aac(3)IV, was transferred into E. coli DH5α/β/T340 to excise the aac(3)IV gene, resulting in cosmid pHJ0034. After conjugal transfer of pHJ0034 from E. coli ET12567/pUZ8002 into S. chattanoogensis L10, exconjugants
were obtained after selection for thiostrepton. Exconjugants were then inoculated onto YMG plates for two rounds of nonselective growth before selection by replica plating for thiostrepton-sensitive colonies. The resulting strain, in which \( \text{SchPP} \) was in-frame deleted, was designated as sHJ007 and confirmed by PCR analysis using primers HJ0077/HJ0078.

A site-specific integration vector pJ8660 [27], containing \( \text{erm}^{E} \) promoter, \( \Phi 31 \) int, and \( \text{att} \), was used to construct an integration recombinant plasmid. The \( NdeI/\text{NotI} \) DNA fragments of \( \text{SchPP}_{D105A} \), \( \text{SchPP}_{E107A} \), \( \text{SchPP}_{E107D} \), and \( \text{SchPP}_{E115A} \) were cloned from pYY0042-pYY0046 into the same sites of pJ8660, resulting in the plasmids pYY0047-pYY0051. The pYY0047-pYY0051 and pHJ0033 [4] were transferred into sHJ007 via conjugal transfer from \( E. \ coli \) ET12567/pUZ8002 using standard procedures. The resulting strains were designated as sHJ008-sHJ013 and confirmed by PCR analyses using primers Pr352/WYY0014. Fermentation of \( S. \ chattanoogensis \) L10 and its recombinant strains and quantification analyses using primers Pri53/WYY0014. Fermentation of \( S. \ chattanoogensis \) L10 and its recombinant strains and quantification

| Table 1. Plasmids and strains used in this study. |
|--------------------------------------------------|
| **Strains**                                       | **Descriptions**                                      | **Reference** |
| S. chattanoogensis L10                           | An natamycin producing strain                        | [4,28–30]     |
| sHJ007                                           | \( \text{SchPP} \) in-frame deletion mutant of L10   | This study     |
| sHJ008                                           | Complementation of \( \text{SchPP} \) in sHJ007       | This study     |
| sHJ009                                           | Complementation of \( \text{SchPP}_{DNAQ} \) in sHJ007| This study     |
| sHJ010                                           | Complementation of \( \text{SchPP}_{E61A} \) in sHJ007| This study     |
| sHJ011                                           | Complementation of \( \text{SchPP}_{E21A} \) in sHJ007| This study     |
| sHJ012                                           | Complementation of \( \text{SchPP}_{E210A} \) in sHJ007| This study     |
| sHJ013                                           | Complementation of \( \text{SchPP}_{E210A} \) in sHJ007| This study     |
| DH5α/ET12567/pUZ8002                             | E. coli strain used for excising the DNA between two FRT site | |
| DW25113/pIJ790                                   | E. coli strain used for PCR-targeted mutagenesis      | [26]           |
| ET12567/pUZ8002                                   | Methylation-deficient \( E. \ coli \) for conjugation  | [43]           |
| Plasmids/cosmids                                 |                                                     |               |
| pHJ0021                                          | \( \text{scp ACP}0-2 \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET28a | [4]           |
| pYY0040                                          | Deletion of both His-Tag gene and Nus-Tag gene from pET44a | This study     |
| pYY0041                                          | \( \text{SchPP} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET44a | This study     |
| pYY0042                                          | \( \text{SchPP}_{DNAQ} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET44a | This study     |
| pYY0043                                          | \( \text{SchPP}_{E61A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET44a | This study     |
| pYY0044                                          | \( \text{SchPP}_{E21A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET44a | This study     |
| pYY0045                                          | \( \text{SchPP}_{E210A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET44a | This study     |
| pYY0046                                          | \( \text{SchPP}_{E210A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET44a | This study     |
| pHJ0024                                          | \( \text{SchPP} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET28a | This study     |
| pYY0062                                          | \( \text{SchPP}_{DNAQ} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET28a | This study     |
| pYY0063                                          | \( \text{SchPP}_{E61A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET28a | This study     |
| pHJ0030                                          | Derived from 3SE12, \( \text{SchPP} \) was replaced with \( \text{aac(3)IV} \) | [4]           |
| pHJ0034                                          | Derived from pHU0030, \( \text{SchPP} \) was in-frame deleted | This study     |
| pJ8660                                           | A site-specific integration vector containing \( \text{erm}^{E} \), \( \Phi 31 \) \text{int} and \( \text{att} \) | [27]           |
| pHJ0033                                          | Derived from pJ8660 with \( \text{SchPP} \) under the control of \( \text{erm}^{E} \) | [4]           |
| pYY0047                                          | \( \text{SchPP}_{DNAQ} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pJ8660 | This study     |
| pYY0048                                          | \( \text{SchPP}_{E61A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pJ8660 | This study     |
| pYY0049                                          | \( \text{SchPP}_{E21A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pJ8660 | This study     |
| pYY0050                                          | \( \text{SchPP}_{E210A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pJ8660 | This study     |
| pYY0051                                          | \( \text{SchPP}_{E210A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pJ8660 | This study     |
| pHJ0029                                          | \( \text{scp ACP} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET28a | [4]           |
| pYY0052                                          | \( \text{Hpp} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET44a | This study     |
| pYY0064                                          | \( \text{Hpp}_{E112A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET44a | This study     |
| pYY0065                                          | \( \text{Hpp}_{E114A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET44a | This study     |
| pYY0066                                          | \( \text{Hpp}_{E155A} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET44a | This study     |
| pYY0060                                          | \( \text{Sppt} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET28a | This study     |
| pYY0061                                          | \( \text{Sppt}_{E112E} \) cloned as a \( \text{NdeI-HindIII} \) fragment into pET28a | This study     |

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of natamycin production were performed in triplicate as described previously [4,28–30].

Co-expression of sch FAS ACP with Hppt or each of Hppt point mutant genes

The plasmid pYY0040 was introduced into E. coli BL21(DE3) containing pHJ0029 [4], in which sch FAS ACP was cloned as a Ndel/HindIII fragment into pET28a. BL21(DE3)/pHJ0029/ pYY0040 was induced with 0.4 mM IPTG at 30 °C for 4 h to overproduce sch FAS ACP. Sch FAS ACP was purified by affinity chromatography on Ni-NTA agarose and then dialyzed against 20 mM Tris-HCl (pH8.0), 25 mM NaCl, 1 mM diethioctetrol (DTT), and 10% glycerol. Sch FAS ACP was analyzed by HPLC as described previously [4].

Protein sequence of Hppt was obtained from NCBI database. Codons of the encoding gene were changed into the preferred codons of E. coli. The corresponding DNA sequence was chemically synthesized and cloned into the Ndel/HindIII sites of pET44a, yielding plasmid pYY0052. Each of three Hppt point mutant genes was amplified by mutagenesis PCR from pYY0052 as template and primers H112-H115(F/R), yielding plasmids pYY0064-pYY0066, respectively. Co-expression of each plasmid (pYY0052, pYY0064-pYY0066) with pHJ0029 in E. coli, purification and HPLC analysis of sch FAS ACP were performed according to the procedures described above.

In vitro phosphopantetheinylation of sch FAS ACP catalyzed by Sppt or SpptQ112E

Protein sequence of Sppt was obtained from NCBI database. Codons of the encoding gene were changed into the preferred codons of E. coli. The corresponding DNA sequence was chemically synthesized and cloned into the Ndel/HindIII sites of pET28a, yielding plasmid pYY0060. The SpptQ112E gene was amplified by mutagenesis PCR from pYY0060 as template and primers FK161/FK162 and cloned into the Ndel/HindIII sites of pET28a, yielding plasmid pYY0061. BL21(DE3)/pYY0060 and BL21(DE3)/pYY0061 were induced with 0.4 mM IPTG at 37°C for 4 h to overproduce Sppt and SpptQ112E, respectively. The proteins were purified by affinity chromatography on Ni-NTA agarose and then dialyzed against 20 mM Tris-HCl (pH8.0), 25 mM NaCl, 1 mM DTT, and 10% glycerol.

A typical in vitro phosphopantetheinylation reaction mixture of 0.1 ml containing 100 mM Tris-HCl (pH 7.5), 1.25 mM MgCl₂, 2.5 mM TCEP, 200 µM sch FAS ACP, 20 µM Sppt or SpptQ112E, and 2 mM CoA was incubated at 25°C for 30 min. The reactions were quenched by freezing reaction mixtures with dry ice. HPLC analysis of sch FAS ACP were performed as described previously [4].

Gene synteny analysis

We examined the chromosomal localization of PPTase homologs and neighboring genes using the Ensembl and UCSC genome browsers, with additional information obtained using the Genomics website v70.01 (http://www.dyyogen.ens.fr/genomicus-70.01/cgi-bin/search.pl) [31] or Integrated Microbial Genomes (http://img.jgi.doe.gov/cgi-bin/w/main.cgi) [32].

Phylogenetic analysis

Multiple sequence alignment (MSA) was carried out by using CLUSTALW and MUSCLE, with the default parameter setting [33–34]. The alignment was then manually improved by using BioEdit 7.1.11, and the MSA generated by CLUSTALW was used as reference for manual adjustments. The best amino acid substitution model was determined with MEGA 6 to be LG+G+F. We constructed maximum likelihood (ML) and neighbor-joining (NJ) tree using PHYML and MEGA version 6.06 [35–36]. Reliability of interior branches was assessed using bootstrap support with 1000 replicates. Tree files were viewed using MEGA, and edited by Adobe Illustrator.

Results

Variation of the magnesium binding residues of Group II PPTases

Since the magnesium binding residues of PPTases are essential for PPTase activities, we aligned the magnesium binding residues of 536 group II PPTases from databases of GenBank, Phytozome (http://phytozome.net), and Ensembl (http://wwwensembl.org). Interestingly, group II PPTases can be classified into two subgroups based on numbers of the magnesium binding residues. The three-magnesium-binding-residue-PPTases contain three magnesium binding residues, which form the triad Asp-Glu-Glu, such as Sfp. The two-magnesium-binding-residue-PPTases contain two magnesium binding residues corresponding to the first and the third magnesium binding residues of the three-magnesium-binding-residue-PPTases, forming the triad Asp-xxxx-Glu, such as AASHDPPPT. The second residues of the triad Asp-xxxx-Glu include Met, Val, Ala, Glu, Thr, Ser, Leu, and Cys. All known animal PPTases, algal PPTases, and fungal PPTases belong to two-magnesium-binding-residue-PPTases. All known animal PPTases and algal PPTases contain the triads Asp-Met-Glu and Asp-Ala-Glu, respectively. Most prokaryotic group II PPTases belong to three-magnesium-binding-residue-PPTases. All known prokaryotic two-magnesium-binding-residue-PPTases contain the triad Asp-Glu-Glu. Both three-magnesium-binding-residue-PPTases and two-magnesium-binding-residue-PPTases are found in plant. Most plant two-magnesium-binding-residue-PPTases contain the triad Asp-Val-Glu (Table 2 and Figures 1, S1, S2, S3, and S4).

Effects of magnesium binding residues of a three-magnesium-binding-residue-PPTase SchPPT

Since the second magnesium binding residues of three-magnesium-binding-residue-PPTases are missing in two-magnesium-binding-residue-PPTases, we characterized effects of three magnesium binding residues of the formers to their activities. SchPPT from S. chattanoogensis L10 was used as a model of three-magnesium-binding-residue-PPTases. SchPPT is necessary to natamycin biosynthesis since it catalyzes the phosphopantetheinylation of a three-magnesium-binding-residue-PPTases. SchPPT is not involved in the loading module of PKS, was used as a substrate of SchPPT.

An in vitro co-expression system was built up to characterize activities of point mutants of SchPPT. SchPPT is a two-magnesium-binding-residue-PPTase, we aligned the magnesium binding residues of the formers to their activities. SchPPT from S. chattanoogensis L10 was used as a model of three-magnesium-binding-residue-PPTases. SchPPT is necessary to natamycin biosynthesis since it catalyzes the phosphopantetheinylation of a three-magnesium-binding-residue-PPTases. SchPPT is not involved in the loading module of PKS, was used as a substrate of SchPPT.
2 in pET28a was co-expressed with each of the SchPPT point mutant genes in pET44a in E. coli. LC-MS data showed both SchPPTD105A and SchPPTE151A lost their activities to phosphopantetheinylate *scn* ACP0-2. However, SchPPT_E107A, SchPPT_E107V, and SchPPT_E107M were still active (Figure S5).

To exclude the possibility that abolishment of activities of SchPPT_D105A and SchPPT_E151A due to mis-folding of proteins or no expression of genes, both mutants were produced in *E. coli* as His-tagged proteins and purified to homogeneities. In vitro phosphopantetheinylolation of *scn* ACP0-2 was performed by incubation of *scn* ACP0-2 with CoA and each of the mutants by using wild type SchPPT as a positive control as described previously [4]. LC-MS data showed only wild type SchPPT but neither of the two mutants phosphopantetheinylated *scn* ACP0-2 under these conditions (Figure S6).

An in vivo gene complement system was also built up to characterize the activity of SchPPT point mutants. We in-frame deleted SchPPT in *S. chattanoogensis* L10, resulting in strain sHJ007. Fermentation of sHJ007 in YEME liquid medium in triplicate showed sHJ007 lost ability to produce natamycin, confirming the activity of SchPPT is essential to natamycin production. Then we complemented SchPPT under the control of the ermEp* promoter in the sHJ007, resulting in strain sHJ008. Fermentation of sHJ008 in YEME liquid medium in triplicate showed sHJ008 produced natamycin with the yield of 494 mg/L at 96 h, indicating SchPPT could complement sHJ007 under these conditions. Finally we complemented each of five SchPPT point mutant genes under the control of the *ermEp* promoter in the sHJ007, resulting in strain sHJ009-sHJ013. Fermentation data showed complementation of neither SchPPT_D105A nor SchPPT_E151A could produce natamycin. However, complementation of SchPPT_E107A, SchPPT_E107V, and SchPPT_E107M produced natamycin at 96 h with the yield of 434 mg/L, 482 mg/L, and 188 mg/L, respectively (Figure 2). Both in vitro and in vivo data herein reveal that the first and the third magnesium binding residues in SchPPT are essential for enzyme activity; however, the second magnesium binding residues is non-essential.

Effects of magnesium binding residues of a three-magnesium-binding-residue-PPTase Hppt

Hppt, a single PPTase in *Haemophilus influenza*, was also used as a model of three-magnesium-binding-residue-PPTases. Hppt, in

| Asp-Xxx-Glu | Glu | Met | Val | Ala | Thr | Gin | Ser | Leu | Cys | sum |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| archaea     | 4   | 0   | 0   | 1   | 1   | 20  | 35  | 0   | 0   | 384 |
| eubacteria  | 0   | 7   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 79  |
| bacteria    | 352 | 12  | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 48  |
| fungi       | 0   | 0   | 12  | 0   | 0   | 0   | 0   | 0   | 0   | 18  |
| alga        | 0   | 0   | 0   | 1   | 0   | 66  | 0   | 0   | 0   | 79  |
| plant       | 20  | 1   | 0   | 18  | 0   | 0   | 0   | 0   | 0   | 48  |
| animal      | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 18  |
| sum         | 384 | 79  | 48  | 18  | 10  | 18  | 12  | 10  | 3   | 1  |

Table 2. Distribution of group II PPTases.

![Figure 2. Natamycin production of *S. chattanoogensis* L10 and its recombinant strains.](image)

Wild type (*S. chattanoogensis* L10), ΔschPPT (sHJ007), ΔschPPT:schPPT (sHJ008), ΔschPPT:ΔschPPT (sHJ009), ΔschPPT:schPPT_E107A (sHJ010), ΔschPPT:schPPT_E107V (sHJ011), ΔschPPT:schPPT_E107M (sHJ012). doi:10.1371/journal.pone.0103031.g002
which all codons of wild type gene were changed into the preferred codons of *E. coli*, was chemically synthesized and cloned into pET44a, yielding plasmid pYY0052. The ACP of FAS in *S. chattanoogensis* L10, *sch FAS ACP*, was used as the substrate of Hppt. Plasmid pHJ0029 [4], in which *sch FAS ACP* was cloned into pET28a, was co-expressed with pYY0040 in *E. coli*. HPLC data showed *sch FAS ACP* produced from *E. coli* contained both apo-proteins and holo-proteins (Figure 3), which was consistent with the results that *E. coli* ACPS could phosphopantetheinylate *sch FAS ACP* incompletely [4]. *Sch FAS ACP* in pET28a was then co-expressed with pYY0052 in *E. coli*. HPLC data showed *sch FAS ACP* contained only holo-proteins, indicating Hppt could phosphopantetheinylate *sch FAS ACP* under these conditions. We finally constructed three point mutants of Hppt. The magnesium binding residues (D112, E114, and E155) were replaced with Ala, resulting in Hppt<sub>D112A</sub>, Hppt<sub>E114A</sub>, and Hppt<sub>E155A</sub>, respectively (Figure S1). *Sch FAS ACP* in pET28a was then co-expressed with each of the Hppt point mutant genes in pET44a in *E. coli*. Among of three Hppt point mutants, only Hppt<sub>E114A</sub> remained this activity (Figure 3).

**Construction of a three-magnesium-binding-residue-PPTase mimic based on a two-magnesium-binding-residue-PPTase Sppt**

Sppt, a single PPTase in *Synechocystis* sp. PCC6803, was used as a model of two-magnesium-binding-residue-PPTases. It has been reported that Sppt phosphopantetheinylates ACPs of type II FASs but not ACPs from secondary metabolism [20]. Sppt, in which all codons of wild type gene were changed into the preferred codons of *E. coli*, was chemically synthesized and cloned into pET28a. Sppt was produced in *E. coli* and then purified to homogeneity. *Sch FAS ACP* was also used as the substrate of Sppt. After incubation of *sch FAS ACP* with CoA in the presence of Sppt, HPLC analysis showed all apo-proteins converted into holo-proteins, indicating Sppt could phosphopantetheinylate *sch FAS ACP*. We constructed a three-magnesium-binding-residue-PPTase mimic based on Sppt. The second residue in the triad of Sppt, Q112, was replaced with Glu, resulting in a three-magnesium-binding-residue-PPTase mimic Sppt<sub>Q112E</sub> (Figure 1). After incubation of *sch FAS ACP* with CoA in the presence of Sppt<sub>Q112E</sub>, HPLC analysis showed all apo-proteins converted into holo-proteins, indicating mutation of Sppt into a three-magnesium-binding-residue-PPTase mimic remained its activity (Figure 3).

**Gene synteny and gene duplication**

To study colinearity of group II PPTases, 56 PPTases from representative species were selected for gene synteny analysis, which all codons of wild type gene were changed into the preferred codons of *E. coli*, was chemically synthesized and cloned into pET44a, yielding plasmid pYY0052. The ACP of FAS in *S. chattanoogensis* L10, *sch FAS ACP*, was used as the substrate of Hppt. Plasmid pHJ0029 [4], in which *sch FAS ACP* was cloned into pET28a, was co-expressed with pYY0040 in *E. coli*. HPLC data showed *sch FAS ACP* produced from *E. coli* contained both apo-proteins and holo-proteins (Figure 3), which was consistent with the results that *E. coli* ACPS could phosphopantetheinylate *sch FAS ACP* incompletely [4]. *Sch FAS ACP* in pET28a was then co-expressed with pYY0052 in *E. coli*. HPLC data showed *sch FAS ACP* contained only holo-proteins, indicating Hppt could phosphopantetheinylate *sch FAS ACP* under these conditions. We finally constructed three point mutants of Hppt. The magnesium binding residues (D112, E114, and E155) were replaced with Ala, resulting in Hppt<sub>D112A</sub>, Hppt<sub>E114A</sub>, and Hppt<sub>E155A</sub>, respectively (Figure S1). *Sch FAS ACP* in pET28a was then co-expressed with each of the Hppt point mutant genes in pET44a in *E. coli*. Among of three Hppt point mutants, only Hppt<sub>E114A</sub> remained this activity (Figure 3).

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**Gene synteny and gene duplication**

To study colinearity of group II PPTases, 56 PPTases from representative species were selected for gene synteny analysis,
Table 3. The 56 selected PPTases for phylogenetic analysis.

| Protein | Organism | Accession number | Ref. |
|---------|----------|------------------|------|
| archaea Methanomethylovorans | Methanomethylovorans hollandica DSM 15978 | YP_007312575.1 |
| Methanobrevibacter | Methanobrevibacter ruminantium M1 | YP_003423257.1 | [1] |
| Thaumarchaeota Thaumarchaeota archaeon SCGC AB-539-E09 | | ZP_23951850.1 |
| Methanocella | Methanocella paludicola SANAE | YP_003335269.1 | [1] |
| cyano- bacterium Sppt | Synechocystis sp. PCC 6803 | BAA10326 | [20] |
| Pleurocapsa | Pleurocapsa sp. PCC 7327 | YP_007079598.1 | |
| Oscillatoria | Oscillatoria acuminata PCC 6304 | YP_007086947.1 | |
| PPTInv | Nodularia spumigena | AAY2632.1 | [21] |
| cyanobacterium | Streptomyces chattanoogensis L10 | AFF18625.1 | [4] |
| Sfp | Bacillus subtilis subsp. Subtilis str. 168 | CAAS4858.1 | [23–24] |
| Myxococcus | Myxococcus fulus HW-1 | YP_004668005.1 |
| Pelosinus | Pelosinus fermentans DSM 17108 | ZP_10325243.1 |
| SCO5883 (RedU) | Streptomyces coelicolor A3(2) | NP_630004.1 | [6] |
| SCO6673 | Streptomyces coelicolor A3(2) | NP_630748.1 | [6] |
| Gluconacetobacter | Gluconacetobacter diazotrophicus PAI 5 | YP_001603066.1 |
| Granulicella | Granulicella malensis MPSACTX8 | YP_005057339.1 |
| Rhodopirellula | Rhodopirellula sallentina SM41 | ZP_23722709.1 |
| Odoribacter | Odoribacter splanchicus DSM 20712 | YP_004254021.1 |
| EntD | Escherichia coli str. K-12 substr. MG1655 | NP_415115.2 | [7] |
| Sordaria | Sordaria macrospora k-hell | CCC07706.1 |
| Baudoinia | Baudoinia compniacensis UAMH 10762 | EMC91499.1 |
| Punctularia | Punctularia strigosozonata HHB-1173 SSS | EIN10680.1 |
| Cryptococcus | Cryptococcus neoformans var. grubii H99 | AFR96401.1 |
| Mixia | Mixia omsnudae IAM 14324 | GAA96409.1 |
| Wallemia | Wallemia sebi CBS 633.66 | EIM24045.1 |
| Emericella | Emericella nidulans | AAF12814.1 |
| LYS5 | Saccharomyces cerevisiae | CAA96686.1 | [40] |
| Claviceps | Claviceps purpurea 20.1 | CCE33539.1 |
| Tetrapisispora | Tetrapisispora phaffii CBS 4417 | CCE62507.1 |
| algae Coccomyxa sp. | Coccomyxa subellipsosidea C-169 | EIE27690.1 |
| Micromonas | Micromonas sp. RCC299 | XP_002501796.1 |
| Ostreococcus | Ostreococcus tauri | XP_003080746.1 |
| plant Populus trichocarpa | Potri.016G074800.1 |
| Arabidopsis thaliana | Arabidopsis thaliana | NP_974284.2 |
| Oryza sativa Japonica Group | Oryza sativa Japonica Group | NP_001061345.1 |
| Selaginella moellendorffii | Selaginella moellendorffii | XP_002969109.1 |
| Vitis vinifera (wine grape) | Vitis vinifera (wine grape) | XP_00274180.2 |
| Solanum lycopersicum | Solanum lycopersicum | XP_004246501.1 |
| Glycine max | Glycine max | XP_003518982.1 |
| Sorghum bicolor | Sorghum bicolor | XP_002448942.1 |
| Oryza sativa Japonica Group | Oryza sativa Japonica Group | NP_001065690.1 |
| animal Caenorhabditis elegans | Caenorhabditis elegans | T04G9.41 |
| Caenorhabditis elegans | Caenorhabditis elegans | T28H10.1.1 |
| Drosophila melanogaster | Drosophila melanogaster | NP_729788.1 |
| Branchiostoma floridae | Branchiostoma floridae | XP_002611588.1 |
including 4 archaeal PPTases, 4 cyanobacterial PPTases, 14 bacterial PPTases, 11 fungal PPTases, 3 algal PPTases, 10 plant PPTases, and 10 animal PPTases (Table 3 and Figures S7, S8, S9, and S10). Except six animal PPTases and three plant two-magnesium-binding-residue-PPTases, the other PPTases don’t show any gene synteny conservation (Figure 4).

Some organisms contain more than one copy of group II PPTase encoding genes. The genome of *Oryza sativa Japonica Group* contains three group II PPTase encoding genes, *oryzal*, *oryzaII*, and *oryzaIII* within the chromosome 8, the chromosome 11, and the chromosome 12, respectively. *OryzaII* and *OryzaIII* may derive from gene duplication, since both of them have the same triad Asp-Val-Glu, high DNA/protein sequence similarity/identity, and conserved gene synteny at their encoding loci. However, *OryzaI* may come from a different origin with *OryzaII* and *OryzaIII*, since *OryzaI* has a different triad Asp-Glu-Glu, low DNA/protein similarity/identity comparing with *OryzaII/QryzaIII*, and has no gene synteny with *OryzaII/QryzaIII*.

**Table 3.** Cont.

| Protein | Organism | Accession number | Ref. |
|---------|----------|------------------|-----|
| Danio   | Danio rerio | NP_001028901.1   |     |
| Xenopus | Xenopus (Silurana) tropicalis | NP_001120584.1 |     |
| Gallus  | Gallus gallus (chicken) | XP_417169.2 |     |
| Lizard  | Anolis carolinensis | ENSACAG0000001121 |     |
| Mus     | Mus musculus (house mouse) | AAH30043.1 |     |
| AASHDPPT| Homo sapiens (human) | Q9NRN7.2 | [25] |

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Phylogenetic relationships between the PPTases

To study evolutionary relationship among group II PPTases, the above selected 56 PPTases were also analyzed by different phylogenetic methods. The maximum like tree can be separated into three-magnesium-binding-residue-PPTases part and two-magnesium-binding-residue-PPTases part (Figure 5). The two-magnesium-binding-residue-PPTases part included animal PPTases, algal PPTases, fungal PPTases, and plant two-magnesium-binding-residue-PPTases. The three-magnesium-binding-residue-PPTases part included plant three-magnesium-binding-residue-PPTases and prokaryotic three-magnesium-binding-residue-PPTases. All of these 10 animal PPTases form one clade. These animal PPTases may originate from one common ancestor since (i) they have the same amino acids (Met) at the second position of the triad Asp-Glu-Glu; (ii) they are closely related homologs in the phylogenetic tree; and (iii) the vertebrate PPTases even have gene synteny. Notably, the phylogenetic tree of the animal PPTases is consistent with animal species evolution (Figures 5 and S10). Interestingly, the 10 plant PPTases are distinctly separated into two clades, the clade of plant two-magnesium-binding-residue-PPTases and the clade of plant three-magnesium-binding-residue-PPTases. The plant two-magnesium-binding-residue-PPTases may originate from one common ancestor since (i) they have the same amino acids (Val) at the second position of the triad Asp-Glu-Glu; (ii) they are clustered in the phylogenetic tree; (iii) and they have gene synteny. The three-magnesium-binding-residue-PPTases part included plant three-magnesium-binding-residue-PPTases and prokaryotic three-magnesium-binding-residue-PPTases. All of these 10 animal PPTases form one clade.

**Table 4.** The DNA/protein sequence similarity/identity of PPTases.

| Protein sequence similarity/identity | DNA sequence identity (including introns) |
|--------------------------------------|------------------------------------------|
| Oryzal/OryzalI                       | 96%/94%                                   | 92% |
| Oryzal/Oryzal                        | 43%/24%                                   | No identity |
| Oryzal/OryzalII                      | 42%/24%                                   | No identity |
| SCO5883/SC06673                      | 43%/21%                                   | No identity |
| EntD/AcpT                            | 43%/28%                                   | No identity |
| C.elegansV/C.elegansX                | 52%/36%                                   | No identity |

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Discussion

To date, all known group II PPTases contain two or three magnesium binding residues. Three-magnesium-binding-residue-PPTases contain three conserved magnesium binding residues forming the triad Asp-Glu-Glu, including most prokaryotic group II PPTases and some plant group II PPTases. Two-magnesium-binding-residue-PPTases with the triad Asp-Xxx-Glu contain two conserved magnesium binding residues, which correspond to the first and the third magnesium binding residues of the three-magnesium-binding-residue-PPTases, including most eukaryotic group II PPTases.

Here characterization of the point mutants of two three-magnesium-binding-residue-PPTases (SchPPT and Hppt) showed mutations of the first residues and the third residues in the triad abolished their activities. Our data are consistent with the results that mutations of the first residues and the third residues in the triads of Sfp, Lys5, and AASHDPPT abolished the activities or decreased the activities with more than 20-fold [23,25,39–40]. Our results here showed mutations of SchPPT and Hppt into two-magnesium-binding-residue-PPTase mimics and mutation of Sppt (a two-magnesium-binding-residue-PPTase) into a three-magnesium-binding-residue-PPTase mimic remained their activities. However, it is unknown if replacement of triad Asp-Xxx-Glu in a two-magnesium-binding-residue-PPTase with triad Asp-Glu-Glu result in a bona-fide three-magnesium-binding-residue-PPTase with lack of the structural information.

Conservations of the first and the third residues in the triads of all known PPTases and our biochemical results suggested that the first and the third residues in the triads of group II PPTases are essential to the activities. The variations of the second residues in the triads and our biochemical results suggested that the second residues in the triads are non-essential to the activities. However, although the second residues in the triads are not critical to their functions, they are conserved in animals (Met), algae (Ala), plants (Val and Glu), and most prokaryotes (Glu). Therefore, the variation in this site is not random and can be used for species classification. The fixation of the second residues in the triads in different taxa may be due to selective sweep or other evolutionary forces. Most likely, the mutations of the second Mg residue may be due to random genetic drift, and the fixation of this residue in separate clades is largely independent of fitness, which could be explained by random fixation of very slightly deleterious mutations, as suggested by neutral evolution theory. A better understand of the evolution of PPTases gene family will shed new insights into the mechanism of this important enzyme in systems level [41–42].
Figure S3 Protein alignment of plant and algal group II PPTases. The red words represent the proteins selected for phylogenetic analysis. (TIF)

Figure S4 Protein alignment of animal group II PPTases. The red words represent the proteins selected for phylogenetic analysis. (TIF)

Figure S5 Co-expression of scn ACP0-2 with schPPT and the point mutants of SchPPT. (A) HPLC analyses. (B) MS analyses. (TIF)

Figure S6 In vitro phosphopantetheinylation of scn ACP0-2 catalyzed by SchPPT and the point mutants of SchPPT. (A) HPLC analyses. (B) MS analyses. (TIF)

Figure S7 Cladograms of bacteria and cyanobacteria (http://www.tolweb.org). The selected group II PPTases for gene synteny analysis and phylogenetic analysis are in red. (TIF)

Figure S8 Cladograms of fungi (http://www.jgi.doe.gov). The selected group II PPTases for gene synteny analysis and phylogenetic analysis are in red. (TIF)

Figure S9 Cladograms of plants and algae (http://phytozone.net). The selected group II PPTases for gene synteny analysis and phylogenetic analysis are in red. (TIF)

Table S1 Primers used in this study. (DOC)

Author Contributions
Conceived and designed the experiments: HJ YQL. Performed the experiments: YYW YDL XXR YYG. Analyzed the data: JBL NNR XC. Wrote the paper: HJ YYW.

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The Triad Asp-Xxx-Glu in Group II PPTases

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