Chapter 3

Biochemical characterization of DiPKS1 and delineation of MPBD biosynthetic pathway
3.1 **INTRODUCTION**

*Dictyostelium discoideum* (Dicty) is a social amoeba that initiates a complex developmental pathway on encountering unfavourable conditions. This developmental process is proposed to be regulated by small molecules. Whereas several metabolites have been characterized that mediate this differentiation and multicellularity, the presence of large number of type I polyketide synthase (PKS) genes in Dicty genome promises the inventory of these metabolites to expand even further. Two well-studied chemical molecules known to influence differentiation include Differentiation Inducing Factor (DIF-1) and 4-methyl-5-pentylbenzene-1,3-diol (MPBD) (Kay *et al*., 1999; Austin *et al*., 2006; Saito *et al*., 2006; Saito *et al*., 2008; Ghosh *et al*., 2008). PKSs are known to synthesize a host of natural products (polyketides) with diverse functions in different organisms. The first indication of involvement of DiPKSs (Dicty PKSs) in the development of Dicty came when it was established that DiPKS37 synthesizes DIF-1 (Austin *et al*., 2006). DIF-1 is a phloroglucinol compound that is known to induce pre-stalk differentiation and its chemical structure suggested that it could be a polyketide product (Kay *et al*., 1997). While DIF-1 was discovered in the 1970s (Town *et al*., 1976; Town *et al*., 1979), it was only much later in 2006 that its biosynthetic origin could be deciphered. Recently another novel compound, MPBD, was identified as a developmental regulatory factor (DRF) (Saito *et al*., 2006). Although at its time of discovery, its biosynthetic pathway was unknown (like in the case of DIF-1), it was proposed that it might also be a PKS product. Later on our group demonstrated that ACP-Type III PKS di-domain of DiPKS1 can form the des-methyl analogue of MPBD (Ghosh *et al*., 2008). Incidentally, both DiPKS1 and
DiPKS37 exhibit similar domain architecture, with a type III PKS domain fused to the C-terminus of a type I PKS domain. It was proposed that both the proteins resemble fungal type I iterative PKSs and utilize the β-keto reductive domains in a precisely programmed mechanism. However, the various steps leading to the production of the two Dicty metabolites still need experimental validation.

Owing to the tremendous versatility of PKSs, dissecting out plausible biosynthetic steps involved in polyketide production demands systematic investigation. The versatility of PKSs has been attributed to various factors which include module skipping, module stuttering, post-PKS tailoring enzymes, presence of unique auxiliary domains as part of the large PKS polypeptide, non-functional domains etc. Understanding of DiPKS metabolite biosynthesis poses a challenge due to the presence of unique domains in these multi-functional enzymes; and difficulty in expression of these large proteins in good quantity. In this chapter, the cloning, expression, purification and detailed characterization of DiPKS1 has been described that provide concrete evidence for the formation of MPBD by this type I - type III hybrid PKS. Through systematic biochemical analysis, we discuss the three crucial steps towards formation of MPBD.
3.2 MATERIALS AND METHODS

3.2.1 Materials

QuikChange site directed mutagenesis kit was obtained from Stratagene. Solvents and chemicals used for biochemical analysis were purchased from Merck and Sigma. Custom-synthesized gene was procured from DNA2.0. Other materials used in this study have been listed in chapter 2.

3.2.2 Methods

3.2.2.1 Codon-optimization of dipks1 and custom synthesis of the gene

Dipks1 gene sequence was sent to DNA2.0 for codon-optimization. Codon-optimized gene sequence was translated to give the corresponding protein sequence using Seqweb (http://sgi2.nii.res.in:8080/gcg-bin/seqweb.cgi), and this was aligned with the original sequence (using NCBI BLAST) for confirming complete identity. The gene was then custom-synthesized and cloned into pJexpress401 vector between the NdeI/XhoI restriction sites, and a C-terminus his-tag was also included in the sequence. The vector possessed kanamycin resistance cassette and the gene was placed under T5 promoter.

3.2.2.2 Expression and purification of DiPKS1 full-length protein

pDN20 was transformed in BAP-1 expression strain of E. coli for over-expression of phosphopantetheinylated DiPKS1. Protein expression was checked on small scale at different temperature conditions and IPTG concentrations in 5 mL
cultures. The following conditions were found to be optimum for DiPKS1 expression: culturing of 8 L culture (with 50 µg/mL kanamycin) at 30°C to an O.D.\textsubscript{600nm} of 0.6 and induction at 22°C for 9 hrs with 0.5 mM IPTG. After harvesting, the cells were resuspended in lysis buffer (400 mM phosphate pH: 7.0, 10% glycerol) and disrupted using french press at 1100 psi pressure. Cell debri was removed by centrifugation at 50,000 g for 40 min at 4°C. This was followed by treatment with 1.1% PEI to remove DNA contamination. 0.75 ml L\textsuperscript{-1} of Ni\textsuperscript{2+}-NTA slurry was added to the supernatant and incubated at 4°C for 2 hrs. The mixture was loaded onto a column working under gravity flow. The resin was washed with wash buffer (400 mM phosphate pH: 7.0, 10% glycerol and 5 mM imidazole) till all unbound proteins were removed. The protein was eluted using elution buffers containing increasing concentration of imidazole. Fractions containing the proteins of interest were pooled and 1 mM TCEP was added. The protein was stored at -80°C till further use.

3.2.2.3 Enzymatic assays and product characterization

\textbf{Radio-TLC} - The standard reaction mixture contained 50 µM radiolabeled M-CoA (or MM-CoA, wherever mentioned), 50 µM unlabeled M-CoA, 4 mM NADPH, 100 µM acetyl CoA, 50 µM S-adenosylmethionine, 100 µM DiPKS1 and 400 mM phosphate buffer, pH = 7.0 in a 150 µL reaction. The reaction was incubated for 6-12 hrs. The products were extracted twice in 300 µL of ethyl acetate and resolved on silica gel 60 F\textsubscript{254} TLC plates (Merck) using ethyl acetate:hexanes:acetic acid (60:40:5, v/v) solvent system. The radiolabeled product was detected and quantitated by using phosphorimager (Fuji BAS500). For kinetic analysis, the
reactions were set up at various time points and the product formed was quantitated
densitometrically using phosphorimager.

**HPLC-Mass spectrometric analysis** - Enzymatic assays were set up as described
above, with the exception of radiolabeled M-CoA being excluded. 10 such
reactions were pooled and loaded on C18 reverse phase analytical HPLC column
(250 x 4.6 mm, 5 μ, phenomenex). The products could be resolved using following
gradient: 0 to 10% B in 1 min, 40% B in 30 min, 60% B in 40 min and 100% B in
45 min till 55 min (A-water with 2% formic acid and B-acetonitrile with 2%
formic acid) at a flow rate 0.6 ml/min. The elution profile was monitored at 280
nm. The identity of peaks obtained was confirmed by TOF-MS and tandem mass
spectrometric analysis using ESI-MS (API QSTAR Pulsar i MS/MS, Applied
Biosystems).

### 3.2.2.4 Kinetic Analysis

Kinetic parameters for DiPKS1 were determined with 100 μM of the protein, and
50 – 800 μM [1-14C] acetyl CoA. Reaction was incubated for 6 hrs. which was the
time-point found in the linear range of DiPKS1 activity. Radiolabeled MPBD was
quantified using phosphorimager (BAS5001). All the experiments were carried
out in triplicates and standard deviations were estimated by using GraphPad.
3.2.2.5 Site directed mutagenesis to generate DiPKS1 mutant clone

DiPKS1 C2930A mutant was generated using QuikChange site-directed mutagenesis kit (Stratagene). Initially, a 3987 bp fragment inclusive of type III PKS domain was excised from pDN20 by *NheI/XhoI* restriction digestion. This fragment was cloned into pBS vector and used as template to carry out mutagenesis reaction in accordance with the manufacturer’s protocol. After introducing the required mutation, the mutated sequence was used to replace the original sequence from the parent clone (pDN20). The details of oligonucleotides used for generating the mutant clone are given in Table 3.1. Mutant clones were screened by restriction endonuclease analysis and confirmed by automated DNA sequencing.

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Table 3.1: Details of primers used for generating DiPKS1 C2930A mutant

3.2.2.6 Expression and purification of C2930A mutant protein

pDN21 was transformed in BAP-1 expression strain of *E. coli* for over-expression of phosphopantetheinylated C2930A. Protein expression was checked on small scale at different temperature conditions and IPTG concentrations in 5 mL cultures. Unlike the wild type protein, the mutant did not express at 22°C induction temperature. Therefore, the induction temperature was lowered to 18°C with rest of the parameters same. After harvesting, the cells were resuspended in lysis buffer (400 mM phosphate pH: 7.0, 10% glycerol) and disrupted using french
press at 1100 psi pressure. Cell debri was removed by centrifugation at 50,000 g for 40 min at 4°C. This was followed by treatment with 1.1% PEI to remove DNA contamination. 0.75 ml L⁻¹ of Ni²⁺-NTA slurry was added to the supernatant and incubated at 4°C for 2 hrs. The mixture was loaded onto a column working under gravity flow. The resin was washed with wash buffer (400 mM phosphate pH: 7.0, 10% glycerol and 5 mM imidazole) till all unbound proteins were removed. The protein was eluted using elution buffers containing increasing concentration of imidazole. Fractions containing the proteins of interest were pooled and 1 mM TCEP was added. The protein was stored at -80°C till further use.

3.2.2.7 Enzymatic assays for C2930A

Assays were performed as described for the wild type except for a small variation. An additional step of hydrolysis was included before ethyl acetate extraction. The reaction mixture was incubated for different time points: 2 hrs, 6 hrs and 12 hrs. After the incubation period, mix was treated with 45% KOH (half the volume of reaction mixture) and incubated at 70°C for 10 min. Following this, equal volume (as that of the final volume) of 50% HCl was added and extraction was performed as before.
3.3 RESULTS

3.3.1 DiPKS1 domain organization – Bioinformatics Analysis

Domain organization of DiPKS1 was determined by using the indigenously developed web based NRPS-PKS domain search program (http://www.nii.res.in/nrps-pks.html), (Ansari et al., 2004; Ansari et al., 2008; Yadav et al., 2003), combined with Pfam analysis (http://pfam.sanger.ac.uk/search). Results of the bioinformatics analysis are shown in Fig. 3.1.

![ Significant Pfam-A Matches](image)

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Figure 3.1 Bioinformatics analysis based prediction of DiPKS1 domain organization.

3.3.2 Codon optimization of dipks1 gene and custom synthesis

Expression of *dipks1* gene was earlier attempted in our lab, however all attempts proved to be unsuccessful. Gene could not be induced to over-express either in Dicty or in the heterologous host - *E. coli*. We hypothesized that the difficulty in
protein expression in *E. coli* could be attributed to codon-bias. Analysis of the gene sequence showed GC content to be mere 28.52%, AT codon richness being a feature of Dicty genome. To overcome this, we decided to synthetically produce codon-optimized gene for protein expression in *E. coli*. After codon-optimization, GC content of the sequence was changed to 48.92%. The 9482 bp gene was then cloned into pJexpress401 vector with kanamycin as the selection marker and C-terminus his-tag (pDN20).

### 3.3.3 Expression analysis and purification of DiPKS1

The plasmid obtained from DNA2.0 was transformed into BAP-1 strain of *E. coli* to express the protein in holo-form. Preliminary investigation with small scale cultures revealed a protein band above 220 kDa to be prominently induced under the following culture conditions: growth at 30°C till 0.6 O.D., followed by induction with 0.5 mM IPTG at 22°C. The expected molecular weight of DiPKS1 is 350 kDa. On large-scale purification it was observed that induction for more than 9 hrs. led to decrease in protein concentration. In order to obtain good protein concentration for biochemical assays, the protein was purified from 8 L culture. The protein purification was so optimized that more than 90%-purity protein could be obtained by affinity chromatography with Ni²⁺-NTA agarose (Fig. 3.2).
Ion-exchange chromatography on FPLC system did not further improve the protein purity. We predicted that smaller fragments on the gel could in fact be truncated form of DiPKS1 protein and such contaminations with very large proteins are difficult to get rid of.

### 3.3.4 Confirmation of DiPKS1 by mass spectrometry

The identity of purified DiPKS1 protein was confirmed by peptide-mass fingerprinting using MASCOT search. Matching peptides mapped all over the length of DiPKS1 protein sequence and the score obtained was well above the confidence threshold (Fig. 3.3).
Mascot Search Results

Protein View

Match to: gi|160474095 Score: 297 Expect: 4.3e-015
hypothetical protein DDB0190208 [Dictyostelium discoideum]

Nominal mass (M): 354654; Calculated pI value: 6.08
NCBI BLAST search of gi|160474095 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Dictyostelium discoideum

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Deamidation (NQ), Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 21%

Matched peptides shown in Bold Red

1 MNRSNKIQSP NSSBWAVLGV GEKEPCHCSND PSLWNMLID GFDATITVPIK
51 ERWMTAFLRN GLIKQGCGF LKDEEWNMD PLFEGIGPKP AEFDIDPQPQRL
101 LLSLVWESLE DAYIRDELP GSNTGVFGIV SNNDTYKAGF QBNSYSIPYTT
151 MTISGSSSNLQ NRSYTFEBER GFSCITUQDAC SSLVMVSNLQ VGQSIQEMECK
201 IACCSGVNLF PDPSTSVAPS KARCQMMGSR MSNEBQASG QYRSCEACVUV
251 VLSLREQLKL DGDRIYGVRI GSSNKHGSAS NQHNSLFTP SCAQSIISINIS
301 KAMENASLSP DSIYIELEPP TCQPWCBFKP VRIALSKHPSN SNRNQINPS
351 TDGNNDIDDDQ DONTSEPPPL IGSFESNIGC LSAAGIAJLH IKCMLMNR
401 MLVPJSPICSN LMIPFPDQY NTAVIREDQ FPITIKLWNG HSNFEGGCSN
451 CHLIIQYIN MNFQMNSTC MNNNNNNDL YLIPISSSTK ELDYLILLY
501 TKNENYHCDI SEBDEYKAEQ KLMMYNLSRN MTITANDWNS FLEKSNSEFNN
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601 NNHINTLNS NVFTHQVYTM VSIILYKTPG YSSINVLRKI DHHDSHNHP
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951 ETTNEKPYQ VREDEYVWSEP LISHKNEEM QTTLLLGNHR ITDFSFPFQSV
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1251 VQFVLYTTIS KANTSRESSQ CRLPFDGDSL ILSDQQFIIK SFNKFSTKTN
1301 ETISEPDDET PDSFQMENLQ PIQPPOIQQ QPSLNSPPSF IRSKILIQDIQ
1351 FEQKSSITNN MLENEMIRKG QQQSFDINLS KHHMDQGLM ELSSISKRYL
1401 RFFTRKESSTH KVYVLIEKPN ELKLMKEIELE KYPFVQVOLL EFRVIKJGSM
1451 IPEPLFENDN MNSEMFPHQD NLLTEYFSNS NHSTFYELRD EMWYAESIRP
1501 TVREKRVFRI BSLGAGTGSN SMVULKTLLT YSLTNLSNCG SGYNNIYET
1551 FTDFSANFII GRIQETCMCN YPNVPFQKSV LDDKQIEINS SOFLMGRIDY
3.3.5 Extender unit specificity of DiPKS1

To determine the specificity of DiPKS1 AT domain, we analyzed the active site residues of the domain. It was earlier shown by Yadav et al. that a crucial Phe200 residue in the active site acts as a gate-keeper to discriminate between malonate and methyl-malonate specificity. Typically, a methyl-malonate specific AT will exhibit QQGHS[QMI]GSRHT[NS]V motif; whereas an AT domain selective for malonate will possess QQGHS[LVIFAM][GR][FP][H][ANTGESD][NHQ]V. Curiously, DiPKS1 AT active site motif (QQGHSFGR-HTNV) does not give clues to its extender unit selectivity. Therefore, we performed gel-binding assays with
both radioactive malonyl and methyl-malonyl CoA (M-CoA and MM-CoA respectively).

Protein was incubated with radioactive M-CoA and MM-CoA separately for 5 min. on ice and then loaded on 6% SDS-PAGE. Surprisingly, radiolabeling of the protein was detected with both M-CoA and MM-CoA, suggesting that DiPKS1 can take up both the extender units (Fig. 3.4).

![Image](24x0 to 589x792)

Figure 3.4 Gel-binding assay shows ability of DiPKS1 to accept both M-CoA and MM-CoA as extender units.

### 3.3.6 DiPKS1 product characterization

#### 3.3.6.1 MPBD biosynthesis by DiPKS1 - Radio-TLC based characterization

It was previously demonstrated by our lab that ACP-type III PKS domain is capable of forming des-methyl variant of MPBD (Ghosh et al., 2008). Based on this, we proposed a biosynthetic scheme for formation of MPBD by DiPKS1 as illustrated in Fig. 3.5.
According to our hypothesis, DiPKS1 takes up acetyl CoA and malonyl CoA in the first round of iteration to eventually form MPBD. To test this, we set up preliminary assays with radiolabeled malonyl CoA and acetyl CoA for tracing product formation. A typical reaction mixture also consisted of NADPH, which acts as a cofactor for the PKS reductive domains; and SAM (S-adenosylmethionine) which would be required by methyltransferase domain for methylation of MPBD. The reaction products were analyzed on TLC and different solvent systems were tried to assess the most suitable one for proper resolution of radioactive spots. Ratio of Ethyl Acetate:Hexanes:Acetic Acid were varied thus: 63:27:5, 60:40:1, 60:30:5, and 60:40:5. The latter ratio (60:40:5) was found to be
appropriate for our studies. Radio-TLC analysis revealed two prominent bands with $R_f$ of 0.62 and 0.4 respectively (Fig. 3.6). The band at $R_f$ 0.62 was seen to be co-migrating with synthetic MPBD, suggestive of DiPKS1 forming the resorcinolic product. Lane without NADPH showed absence of both the bands indicating that the activity was arising from the type I PKS domain of DiPKS1. The low intensity band at $R_f$ 0.4 corresponds to the pyrone product.

![Figure 3.6 Radio-TLC showing DiPKS1 activity. Prominent radioactive band seen at $R_f$ 0.62 is MPBD.](image)

To ensure that the products formed were the result of DiPKS1 activity, we set up an assay with apo-form of DiPKS1 (non-phosphopantetheinylated DiPKS1 was purified from BL-21 strain of E. coli). While holo-DiPKS1 clearly showed activity, no product formation was observed with the apo-form (Fig. 3.7). Thus it was evident that DiPKS1 was indeed forming products with acetyl CoA and malonyl CoA as priming monomers.
Figure 3.7 DiPKS1 expressed in BL-21 fails to show activity.

Catalytic efficiency of the enzyme with respect to acetyl CoA was estimated by Michaelis-Menten fit (as described in materials and methods section) and \( k_{cat}/K_M \) was calculated to be 1.3 mM\(^{-1}\) min\(^{-1}\) (Fig. 3.8).

Figure 3.8 Kinetics of DiPKS1 with respect to acetyl CoA.
3.3.6.2  *HPLC-mass spectrometry based confirmation of MPBD formation*

To unambiguously confirm formation of MPBD, multiple cold assays were set up and the pooled extract was loaded on HPLC. Fig. 3.9 (a) clearly shows a peak at a retention time of 49.7 minutes which corresponds to that of synthetic MPBD. Peaks were collected and subjected to tandem MS (on negative ion mode). Molecular ion peak obtained was 193 [M - H]⁻ that agrees well with the molecular mass of 194 for MPBD. Other characteristic peaks – 151.11 [M - 42], 149.13 [M - 44] and 136.05 [M - 57], observed typically for MPBD could also be detected on MS/MS (Fig. 3.9 (b)).

![HPLC Chromatogram](image)

Figure 3.9 (a) HPLC chromatogram showing the same retention time for MPBD and the product formed by DiPKS1. (b) Mass-spectrometric analysis of the HPLC peak.
3.3.7 C-Methyltransferase mediated methylation of MPBD backbone

DiPKS1 possesses a C-methyltransferase (C-Met) domain which according to our proposed biosynthetic scheme could be responsible for mediating the transfer of a methyl group on to the MPBD backbone (Fig. 3.5). Since MM-CoA is also utilized as an extender unit by DiPKS1, it is possible that methyl group could in principle be the result of specific MM-CoA incorporation during the third round of iteration, as shown in Fig., 3.10.

![Diagram of MPBD biosynthesis by DiPKS1.](image)

Figure 3.10 Alternate mode of MPBD biosynthesis by DiPKS1.

To test this hypothesis we also performed assays by using radiolabeled MM-CoA and cold M-CoA (Fig. 3.11). As observed in lane 2 of Fig. 3.11, we did not see incorporation of radioactivity in MPBD, with radiolabeled MM-CoA. Instead, we observed a new radioactive band ‘A’ at R_f (0.51) lower than that of MPBD. However, the quantity of product formed was very low and despite our best efforts
we could not characterize this product. Lanes 3 and 4 represent reactions in which NADPH was excluded. As seen in Fig. 3.11, lane 3, MPBD synthesis was abrogated in the absence of NADPH in the assay mixture, due to lack of β-hydroxylation. In the reaction with radiolabeled M-CoA, a new product ‘B’ with an R_f value of 0.3 was observed. This product, based on our understanding of DiPKS1 biosynthetic logic could be a hemiketal formed by three rounds of M-CoA condensation with acetyl CoA in the absence of any reduction. Similar hemiketal has been earlier described for the DEBS 1/module 3+TE system of erythromycin PKS (Kao et al., 1996). The product ‘A’ observed with radiolabeled MM-CoA disappears in the absence of NADPH (Fig., 3.11, lane 4).

We compared the profile of DiPKS1 full length protein with that of the type III PKS domain (purified by Dr. Ratna Ghosh in lab). As seen in the Fig. 3.11, type III PKS domain does not show any activity when acetyl CoA is used as the starter and M-CoA as the extender unit (lane 5) or MM-CoA as the extender unit (lane 6). However, with hexanoyl CoA as the starter and M-CoA as the extender unit, it forms des-methyl form of MPBD (lane 8) which exhibits an R_f (0.59) lower than that of MPBD.
Figure 3.11 DiPKS1 activity with MM-CoA as the extender unit (along with M-CoA) and profile comparison with independent type III PKS domain of DiPKS1. Bands ‘X’ and ‘Y’ most likely represent unreacted radioactive M-CoA and MM-CoA respectively.

Additionally, we performed experiments in the absence of S-adenosylmethionine (SAM) in the reaction mixture. Radioactivity profile under these conditions showed very different pattern (Fig. 3.12). The most prominent band was obtained at Rf 0.4, which corresponds to triketide pyrone and a very faint band at Rf 0.62. Our results thus suggest that methylation on MPBD is a result of C-Met mediated transfer of methyl group via SAM.
3.3.8 Understanding functional cross-talk between type I and type III

**PKS in the DiPKS1 hybrid protein**

DiPKS1 is a unique hybrid PKS protein in which type I PKS is fused with a type III PKS. The starter units in type I PKSs are loaded at the KS domain, while in type III PKSs, same active site accepts both the starter and extender units. It is interesting to note that both the KS domain and type III PKS possess the same catalytic ‘Cys’ at the active site residue, where starter units are covalently acylated. Our studies with DiPKS1 suggested that type I PKS synthesizes the hexanoyl group by using acetyl CoA and two rounds of M-CoA condensation and all the three associated reductions. Further, this chain, through another round of M-CoA condensation and by using the C-Met domain forms a branched octanoyl chain (without any reduction). This chain is then further transferred to type III PKS domain to perform two more rounds of condensation and cyclization to form
MPBD. We wanted to understand whether altered starter unit provided at the KS domain could change the product profile.

Surprisingly, the usage of butyryl CoA and hexanoyl CoA as the starter units did not alter the product profile of DiPKS1; instead MPBD was formed in both the cases (lanes 2 in Fig. 3.13 (a) and 3.13 (b) respectively). Such an activity of DiPKS1 can be argued based on reduced number of iterations. For example, addition of butyryl CoA would reduce one iteration involving M-CoA and associated reductive steps; and addition of hexanoyl CoA would decrease two rounds of iterations.

![Figure 3.13](image)

Figure 3.13 (a) MPBD formation with acetyl CoA or butyryl CoA as the starter units. No product formation without NADPH (lanes 3 and 4). (b) MPBD formation with hexanoyl CoA; reduced activity in the absence of NADPH.
Interestingly, no such activity was observed for octanoyl CoA. It is important to note that eventually octanoyl chain that gets transferred to type III domain must have a methyl modification and should not be completely reduced. Instead we observed the formation of a des-methyl resorcinolic product, whose $R_f$ is higher than des-methyl MPBD (lane 1, Fig. 3.14 (a)). Further, exclusion of SAM from the reaction mixture did not have any detrimental effect on product formation (Fig. 3.14 (c)). Decanoyl CoA gives a faint product with an $R_f$ value slightly higher than the product for octanoyl CoA (lane 2, Fig. 3.14 (a)). However, no activity was observed with lauroyl- and palmitoyl CoA (lanes 3 and 4, Fig. 3.14 (a)). This product profile can also be achieved by directly using a type III PKS domain (lanes 5 and 6, Fig. 3.14 (a)). The removal of NADPH in the assays of octanoyl- and decanoyl CoA had no effect on this product formation, again indicating that type I PKS of DiPKS1 is not involved this catalytic function (lanes 2 and 4, Fig. 3.14 (b)). In contrast, the acetyl-, butyryl- and hexanoyl CoA assays in the absence of NADPH resulted in almost negligible activity (although, activity was better in the case of hexanoyl CoA); confirming that these products are formed by combination of the type I and type III PKS domains (lanes 3 and 4, Fig. 3.13 (a); lane 3, Fig. 3.13 (b)). Our studies thus emphasize that type I PKS has a stringent specificity to produce a branched octanoyl chain and it is only after the synthesis of this chain, that ACP transfers the acyl group to type III PKS domain. This suggests a tight-coordination of specificity between type I and type III PKS domains to synthesize the MPBD product.
Figure 3.14 (a) Activity profile of full-length DiPKS1 and type III PKS domain with C₈-C₁₆ starter units. Product Rₚ of C₈ and C₁₀ starters is correspondingly higher with respect to MPBD. No activity seen with lauroyl CoA or palmitoyl CoA. (b) DiPKS1 activity with octanoyl CoA and decanoyl CoA is unaffected by absence of NADPH. In all reactions, radioactive M-CoA was used as the extender unit. (c) DiPKS1 activity with octanoyl CoA in the presence and absence of SAM.
3.3.9 Inactivation of type III PKS domain of DiPKS1

3.3.9.1 Site directed mutagenesis to generate C2930A mutant of type III PKS domain

To understand the interaction between type I PKS domain and the type III domain, we decided to inactivate the latter. All known CHS related enzymes contain four conserved residues in their active sites (Cys164, Phe215, His303 and Asn336 of *Medicago sativa*). In mycobacterial type III PKS - PKS18, Cys175, His313 and Asn346 residues form the catalytic triad. Sequence alignment of PKS18 and type III PKS domain of DiPKS1 was carried out to identify the corresponding residues in the Dicty PKS (Fig. 3.15).

![Sequence alignment](image)

Figure 3.15 Sequence alignment of type III PKS domain of DiPKS1 (subject) with mycobacterial PKS18 (query).

The catalytic Cys residue in DiPKS1 was determined to be Cys2930 and this was chosen for mutation to *Ala* for abolishing the activity of the type III PKS domain.
dipks1 gene region from 5491 bp to 9482 bp (which includes the entire type III PKS domain) was excised from pDN20 by digesting the plasmid with NheI/XhoI. The 3987 bp fragment was then cloned into pBS vector (pDN21) and used as template for site-directed mutagenesis (SDM) for conversion of Cys2930 to Ala2930 (TGC → GCC). Primers with the appropriate base changes were used for the SDM PCR. Introduction of the desired mutation was confirmed by DNA sequencing. The mutated fragment was excised from the mutant pBS clone (pDN22) and re-inserted into the parent clone to yield pDN23. Clones were confirmed by restriction digestion and automated DNA sequencing (Fig. 3.16 and Table 3.2).

![Image](image-url)  
**Figure 3.16** Restriction fragmentation analysis of dipks1 C2930A mutant. Expected fragment sizes in bp are indicated in parentheses.  
1. 1kb ladder  
2. pDN21 NheI, XhoI (3987, 2958)  
3. 1kb ladder  
4. pDN22 NheI, XhoI (3987, 2958)  
5. pDN23 NheI, XhoI (9411, 3987)  
6. 1kb ladder

<table>
<thead>
<tr>
<th>Clone description</th>
<th>Name of the clone</th>
<th>Restriction enzymes – Size of fragments generated (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type III PKS domain in pBS</td>
<td>pDN21</td>
<td>NheI, XhoI 3987, 2958</td>
</tr>
<tr>
<td>Type III PKS domain C2930A in pBS</td>
<td>pDN22</td>
<td>NheI, XhoI 3987, 2958</td>
</tr>
<tr>
<td>Type III PKS domain C2930A in pDN20</td>
<td>pDN23</td>
<td>NheI, XhoI 9411, 3987</td>
</tr>
</tbody>
</table>

**Table 3.2** Restriction digestion pattern of dipks1 C2930A mutant clone. Fragments sizes are in bp.
3.3.9.2  **Purification of C2930A mutant of type III PKS domain**

The mutant clone – pDN23 was transformed into BAP-1 strain of *E. coli*. Same culturing conditions as for the wild type protein were used initially. However, the mutant protein was seen to be going into pellet. Induction temperature was therefore reduced from 22°C to 18°C. With the changed condition, induction of C2930A could be clearly seen in the supernatant. Batch cultures of 8 L were set up and the mutant protein was purified by Ni²⁺-NTA chromatography (Fig. 3.17).

![SDS-PAGE analysis of Ni²⁺-NTA purified C2930A](image)

Figure 3.17 SDS-PAGE analysis of Ni²⁺-NTA purified C2930A. Protein band at ~350 kDa represents C2930A.

1. Protein marker  
2. Elution with 50 mM imidazole

3.3.9.3  **Biochemical characterization of C2930A mutant of type III PKS domain**

We first checked for the extender unit specificity of C2930A. Analogous to the wild type protein, mutant also showed the ability to take up both M-CoA and MM-CoA (Fig. 3.18). Combined with the studies discussed in the previous section, it is apparent that both type I PKS and type III PKS domains of DiPKS1 can accept MM-CoA as the extender unit to form products distinct from MPBD.
Figure 3.18 Extender unit specificity of C2930A checked by gel-binding assay. Mutant protein also accepts both M-CoA and MM-CoA.

Activity assays for C2930A were set up as for the wild type protein. No product formation was observed, which could be attributed to non-transfer of the intermediate chain from type I PKS domain to the type III domain. With the biosynthesis stalled mid-way and in the absence of a chain-release mechanism (due to inactivation of type III domain), we decided to check if any bound intermediate could be observed by hydrolysis. Therefore, after incubating the reaction mix overnight, KOH hydrolysis was carried out and this was followed by ethyl acetate extraction of released products. We presumed that the intermediate formed after the final round of iteration by type I PKS domain would undergo decarboxylation as a result of hydrolysis (instead of being transferred to type III PKS domain) as has been represented in Fig. 3.19.
However, no radiolabeled product could be observed on TLC (Fig. 3.20). Since the proposed product is unstable in nature, we reduced the reaction incubation time and tried two different time points – 2 hrs. and 6 hrs. But we still failed to detect any released intermediates.
Next we checked for the activity of the mutant protein when incubated along with the independent type III PKS domain. Notably, co-incubation of the two proteins could lead to detectable levels of product formation (Fig. 3.21). Increased product formation was also observed on increasing the amount of the mutant protein. It is therefore apparent that type I PKS domain of the mutant protein is able to trans-load the intermediate to the independent type III PKS domain, although with lower efficiency as compared to cis-transfer.

Figure 3.21 Co-incubation of mutant DiPKS1 with the wild type type III PKS domain, shows trans interaction between type I and type III PKS domain. Increased product formation seen on increasing the concentration of independent type III PKS domain in the assay (right panel).

Thus, our studies unambiguously prove that DiPKS1 is responsible for MPBD formation and the biosynthesis involves three key intermediate steps. Methylation is necessary for the transfer of ketide intermediates from type I PKS domain to the type III domain and the latter is indispensable in product formation.
3.4 DISCUSSION

Expression of high molecular weight proteins like PKSs (approximately 300 kDa) in Dicty has been a daunting task. This poses a major bottleneck in understanding their biosynthetic functions. Previously, attempts at purifying these proteins (even in small fragments) from E. coli strains optimized for rare-codon expression have not led to much success (Austin et al. 2006). Recent studies on fungal iterative PKSs have unraveled new modes to decipher their functions. For example, it has been indicated that PKS gene clusters could be silenced by histone - methylation mediated chromatin regulation (Bok et al., 2009). This silencing can be therefore reversed by removing genes responsible for repressive chromatin configuration. In another study, a cryptic gene cluster in A. nidulans was activated by replacing the promoter of its transcriptional activator with an inducible alcA promoter (Chiang et al., 2009). In our studies we were able to successfully express the complete DiPKS1 protein by systematically changing the codon-bias. Previously in the lab, type III PKS domain and ACP-type III PKS di-domain were expressed. Together, all these proteins provided means to dissect out mechanistic understanding of this unique type I-type III fusion protein.

Biochemical analyses of DiPKS1 clearly established the biosynthetic scheme for MPBD. Three main steps during the biosynthesis can be readily delineated based on our studies: 1) acetyl CoA and malonyl CoA condensation occurs on ACP, followed by KR, DH and ER catalyzed reduction steps. Subsequent to this, another round of M-CoA condensation and reductive steps occur, giving rise to C6.
intermediate. 2) Third round of iteration involves condensation with another molecule of M-CoA and C-Met mediated methylation. Our results clearly demonstrate that this methylation is essential for transfer of the intermediate to type III PKS domain for further iterations. This observation further highlights the precisely regulated interaction between the type I and the type III domains. 3) Eventually, the methylated intermediate is transferred by ACP to the type III PKS domain for further two rounds of M-CoA condensation and the tetraketide intermediate thus formed undergoes aldol condensation to give the final MPBD structure. Acetyl CoA could be replaced by the longer chain acyl CoAs (C₄ and C₆) to produce MPBD. Our studies thus show that longer chains restrict the number of iterations, thereby continuing to produce identical products, as with acetyl CoA.

Another interesting aspect of our study is the formation of a new metabolite when MM-CoA is used as the extender unit. It is yet to be ascertained whether this metabolite can be formed under in vivo conditions. Analysis of Dicty metabolic pathways on KEGG database indicates the presence of MM-CoA biosynthetic pathway. Yet no studies have actually investigated MM-CoA formation in Dicty. It can be speculated that the same enzyme is capable of forming two different products depending on the choice of extender unit during the course of biosynthesis. It would be interesting if this choice is dictated by specific environmental conditions leading to altered metabolic flux, and thus producing a modified morphogen. Further work needs to be done to validate this hypothesis.
Our results with mutant DiPKS1 (with inactivated type III PKS domain) suggested that the type I domain can interact with the type III domain in trans to form the final MPBD product. The trans-association however, does not seem to be very efficient, as gauged by the low quantity of product formed. A similar observation was reported for the interaction of TE domain with DEBS erythromycin polyketide synthase (Gokhale et al., 1999). TE domain in trans produced 100-fold less product as compared to the fused construct. Broadly, it can be speculated that type III protein places itself similar to chain-releasing domains in this multi-functional complex. The trans-interaction exhibited by the type III PKS domain gives further hints on the versatile functioning of PKSs and raises possibilities of engineering novel metabolites by domain “mix-and-match” in Dicty PKSs.