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Insights into the structure-function relations of amorpha-4,11-diene synthase

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Terpenoids are considered the biggest and most varied class of natural products. They are involved with a wide range of applications from food to cosmetics to medicine. All terpenoids originate from the same C5 isoprene building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), but have a diverse range of complex chemical structures with multiple chiral centers and/or chemical modifications. This variation in terpenoidal chemical structures can be accredited to the enzymes responsible for their production. Terpene synthases are an enzyme family in control of catalyzing the rearrangement and/or cyclization of the isoprene precursors to produce different terpenoids. Terpene synthases perform complex chemical rearrangements leading to the production of terpenoids. The structural diversity of terpenoids is centered on the positioning of their substrate in the active site of the terpene synthase so it can undergo the required cyclizations and/or modifications to give the desired structure of the final product (1,2). Terpene synthases generally, and sesquiterpene synthases precisely, perform intricate catalytic mechanisms to produce their final product. Sesquiterpene synthases bind the substrate in a large active site where a tri-nuclear metal cluster liganded by DDXXD and (N,D)DXX(S,T)XXXE, the conserved metal ion binding motifs, prompts the removal of the diphosphate group from the substrate producing a highly reactive carbocation. Then, cyclization and/or rearrangement of the carbocation leads

to the formation of one specific sesquiterpene (15 carbons) major product (3).

Tailoring enzymes to meet the specific demands of the research and industrial communities gained massive attention in the last few decades. The ideal goal of enzyme engineering is to optimize the target enzyme by either enhancing the catalytic activity or maintaining the activity of the wild type enzyme while altering certain properties that make the enzyme more useful. A level of knowledge about the three-dimensional structure of the enzyme and its catalytic mechanism is the corner stone in rational engineering of any enzyme (4,5). In line with such research, engineering terpenes synthases with increased catalytic activity garnered attention as an important factor for upscaling production of terpenoids using synthetic biology.

Amid the most famed sesquiterpene synthases is amorphadiene synthase (ADS) which catalyzes the first rate limiting step in the biosynthesis of the important antimalarial drug artemisinin in the plant *Artemisia annua*. Currently, artemisinin combination therapy is the most effective treatment against malaria especially multi-drug resistant strains resulting in the very high demand for artemisinin (6,7). Hence, there is an urgency to understand the steps involved in the biosynthesis of artemisinin in order to improve its production and increase its availability at cheap price. Since ADS controls the first committed step in the biosynthesis of artemisinin, in-depth research of its

structure-function relationships is necessary to improve its role. There has been no report of the X-ray crystal structure of ADS. However, several reports attempted to examine the mechanism and structure-function relations of ADS by sequence alignment with other sesquiterpene synthases in addition to computational modeling (8-10). Researchers generally agree that ADS mechanism begins with an isomerization step at the C2-C3 double bond of the substrate farnesyl pyrophosphate (FPP) to produce nerolidyl diphosphate (NPP). Then, NPP is ionized to yield a 2,3-cis-farnesyl cation that will primarily undergo 1,6-cyclization to produce bisabolyl cation trailed by 1,10-ring closure and deprotonation to create amorphadiene as the major product (11,12).

In a recent article, Fang *et al.* attempted to identify the functional residues involved in the catalytic mechanism of ADS (13). They generated a homology model of ADS using 5-epi-aristolochene synthase (TEAS) as a template. Then the substrate FPP was modeled into the active site based on its position in the crystal structure of TEAS and the different carbocation intermediates were docked in the active site. However, it is worth to mention that TEAS is a transoid sesquiterpene synthase while ADS is cisoid and they only have around 38% sequence similarity. In contrast, bisabolol synthase (BOS) is another cisoid sesquiterpene synthase with 89% sequence similarity with ADS and it has a reported crystal structure. Hence, using BOS as a template to generate a model of ADS should be more reliable (8,14). Fang and colleagues used sequence alignment of six sesquiterpene synthases from *A. annua* along with site-directed mutagenesis to identify the functional residues. They first attempted to pinpoint the ADS residues involved with ring closures. They designed a mutagenesis strategy based on switching active site residues of ADS with the different residues in BOS and germacrene A synthase (GAS). BOS and GAS were chosen because they catalyze a single ring closure, 1,6- or 1,10-, respectively, while ADS perform both 1,6- and 1,10-ring closures. Swapping ADS residues with GAS which cannot perform 1,6-ring closure identified a T296V mutant that lost cyclization activity. The same results were also observed in BOS in which 1,6-ring closure is necessary similar to ADS. This suggests that residue T296 is essential for 1,6-cyclization. They proposed that the role of T296 is dependent on the proximity of the hydroxyl group to the first isoprene unit. However, the involvement of the hydroxyl group should be ruled out since it has been reported that the T296A mutant retained 1,6-cyclization activity producing amorphadiene (15). It

would be a better assumption that the size of the side chain of the residue at 296 has a direct effect on its role in 1,6-ring closure. In a similar manner, ADS residues were swapped for the different residues in the BOS active site to determine the residues involved in 1,10-ring closure in ADS. None the less, this procedure was not sufficient to identify the functional residues involved in the 1,10-cyclization. Thus, the authors researched further by comparing the identical residues of ADS and BOS to GAS, twelve of these residues were different in GAS. Replacing the respective twelve ADS residues with those of GAS resulted in mutants F370L, L374Y, G400S, G401A, and G439S with disrupted 1,10-cyclization leading to accumulation of bisabolyl derived products. Also, the authors docked the bisabolyl cation in the active site of the ADS model and discovered that residues L374, L404, L405 and G439 are in close proximity to the bisabolyl cation. In addition, the tetra-substitution mutant of these residues produced 80% bisabolyl derived product and only 10% amorphadiene, confirming the involvement of these four residues in 1,10-ring closure.

After that, Fang and co-workers aimed at determining the residues responsible for deprotonation of the formed bicyclic ring skeleton to produce the major product amorphadiene. It has been previously reported that T399 is responsible for regioselective deprotonation where T399L (corresponding to leucine at the same position in BOS) produced less of the main product amorphadiene and more of amorphadiene-7 compared to the wild type (14). Similarly, T447G (corresponding to glycine at the same position in BOS) showed the same pattern of products indicating that both T399 and T447 are involved in the deprotonation process. In addition, substituting T399 and T447 with different amino acids showed that only the serine mutants acted like the wild type which confirms the importance of the hydroxyl group in the regioselective deprotonation. Since the hydroxyl group cannot directly remove a proton from the carbocation intermediate and the side chains of basic residues in the model are pointing away from the active site, they suggested that enzyme-bound water can perform the deprotonation and act as the active site base. However, further investigation of all residues in the active site for their possible role in the deprotonation should be performed before deciding on the active site base. The generated ADS model was used to look for the positioning of all these residues in relation to the conserved metal ion binding motifs in the active site. They found that T296 is located on the same helix as the DDXXD motif while G439 along with the DSE/NTE motif that contains

Table 1 Steady-state kinetic parameters of ADS reported in literature

Enzyme	K_{cat} (s^{-1})	K_m (μM)	K_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	Method	Reference
ADS	0.186	2.03	0.92×10^5	GC-MS	(13)
ADS	1.370	3.56	3.85×10^5	GC-MS	(14)
ADS	0.016	15.30	0.01×10^5	Malachite Green	(15)
ADS	0.030	8.90	0.03×10^5	Malachite Green	(16)
ADS	0.006	2.00	0.03×10^5	Radioactive	(16)

ADS, amorphadiene synthase.

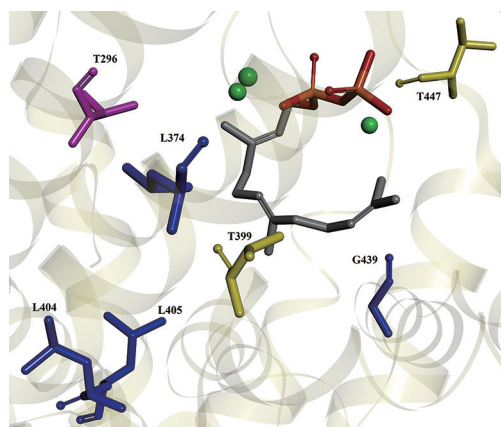


Figure 1 Enlarged section of the active site of ADS model showing FPP substrate in grey (pyrophosphate in red), magnesium ions in green and the functional residues identified by Fang *et al.* T296 involved in the first 1,6-ring cyclization in pink. L374, L404, L405 and G439 contributing to the second 1,10-ring closure in blue. T399 and T447 responsible for regioselective deprotonation in yellow. ADS, amorphadiene synthase; FPP, farnesyl pyrophosphate.

T447 are on the opposite side of the active site. Also, T374 and T399 are neighboring the DDXXD motif. Hence, they concluded that the side chains of these residues can affect the positioning of the substrate and/or intermediates in the active site in addition to their subsequent cyclization. This was confirmed by mutating these residues to different size amino acids.

Fang and co-authors aimed at improving ADS activity. It was previously published that T399S mutant had higher catalytic efficiency compared to the wild type (14). In a similar fashion, T447S ($K_{cat} = 0.334 s^{-1}$) showed two fold increase in K_{cat} compared to the wild type ($K_{cat} = 0.186 s^{-1}$). Since it was suggested that T399 and T447 act independently in the active site, the T399S/T447S double mutant should have further increased efficiency compared

to the single mutants. Indeed, the double mutant had a K_{cat} of $0.518 s^{-1}$ which is three times higher than the wild type ($K_{cat} = 0.186 s^{-1}$) but at the expense of four times elevation of the K_m . It is worthwhile to note that kinetic parameters of ADS reported in literature vary based on the method used and also show differences within articles using the same method (13-16) (Table 1).

In summary, Fang *et al.* effort to identify functional residues of ADS led to the discovery of the role of some residues in the mechanism of ADS. T296 is involved in the first 1,6-ring cyclization, four residues (L374, L404, L405 and G439) contribute to the second 1,10-ring closure and finally two residues (T399 and T447) are responsible for regioselective deprotonation to produce the major product amorpha-4,11-diene. Figure 1 highlights these identified residues in the active site of ADS. In addition, they suggested that mutation of residues around the metalion binding motifs can be the key for the evolution of their enzymes. Further investigation and mutations are required to examine the role of other residues in the active site of ADS since in-silico studies along with sequence alignments with different terpene synthases suggest the possibility of involvement of more residues in the function of ADS (9,10). In the future, accumulation of these studies will give a clear picture of the structure-function relations in ADS and help create a strategy to improve the catalytic activity of ADS.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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