Mechanistic insights into Diels-Alder reactions in natural product biosynthesis

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Abstract

Natural enzymes that catalyze Diels-Alder reactions have long been sought after, yet few enzymes have been experimentally confirmed to perform this reaction. In the past 5 years, several stand-alone enzymes that can catalyze the Diels-Alder reaction had been identified and characterized. Among which, the crystal structures of SpnF, PyrI4 and AbyU have been determined. The structures of PyrI4 and AbyU, which are involved in spirotetronate/spirotetramate biosynthesis, are particularly informative since they shed light on how a natural catalyst captures the flexible substrate and facilitates the intramolecular Diels-Alder reaction through stabilization of the transition state in catalysis. These pioneering studies will inspire the design of artificial catalysts for Diels-Alder reactions.

INTRODUCTION

The Diels-Alder reaction is an elegant and perhaps the best-known pericyclic transformation in synthetic chemistry. In this reaction, a 1,3-diene and an alkene (dienophile) cyclize into a cyclohexene through a single transition state [1]. This [4+2]-cycloaddition reaction is a simple yet powerful tool for the construction of C–C bonds and is therefore often utilized for the chemical synthesis of natural products with a transannular cyclohexene ring(s) [2]. More than 400 compounds with this transannular ring structure have been identified among primary and secondary metabolites [3], leading to decades of speculation on the existence of enzymes capable of catalyzing Diels-Alder reactions in the biosynthesis of these compounds [4]. However, only a few such enzymes have been identified, which are often called “Diels-Alderase” [3-6].

Since the 1980s, some biological catalysts of the Diels-Alder reaction, such as ribozymes [7] and catalytic antibodies [8-10], have been developed using a hapten that mimics the transition state of the target reaction. *De novo*-designed artificial enzymes for Diels-Alder reactions have also been developed [11]. However, artificial catalysts of the Diels-Alder reactions often undergo product inhibition [12], because such catalysts recognize the reaction product as well as the hapten due to their structural similarity. In contrast, natural Diels-Alderase would avoid product inhibition to accomplish the efficient conversion of the substrate to the proper product. Therefore, mechanistic and structural studies of these natural enzymes are expected to reveal a more elaborate underlying mechanism.

Pioneering studies having sought natural Diels-Alderase identified macrophomate synthase (MPS) [4,13], which catalyzes the transformation of 2-pyrone derivatives into the corresponding benzoate analogues. The crystal structure of MPS complexed with pyruvate enolate and Mg^{2+} was solved [14], making MPS the first potential Diels-Alderase with its structure determined. However, later quantum mechanics/molecular mechanics (QM/MM) calculations on the MPS-catalyzed transformation indicated that a two-step Michael-aldol sequence is energetically preferred over a concerted Diels-Alder reaction [15]. Subsequent biochemical studies also supported this conclusion [16,17]. Thus, although MPS is not a *bona fide* Diels-Alderase, the series of studies on MPS have provided significant insights into the concertedness of two-bond formations in Diels-Alderase-catalyzed reactions.

Most enzymes known for their [4+2]-cycloaddition activity catalyze other reactions in addition to the Diels-Alder reaction (Figure 1). For example, solanapyrone synthase (SPS) catalyzes the oxidation of prosolanapyrone II, followed by a Diels-Alder reaction to yield solanapyrone A [18-21]. Lovastatin nonaketide synthase (LovB) is a highly reducing fungal iterative type I polyketide synthase that catalyzes modification reactions as well as carbon chain-elongation reactions. In addition, LovB catalyzes a Diels-Alder reaction during one of its carbon chain-elongation cycles [22-24]. Riboflavin synthase has also been predicted to be a possible Diels-Alderase. This enzyme catalyzes the deprotonation of 6,7-dimethyl-8-ribityllumazine and a subsequent Diels-Alder reaction to yield a

pentacyclic intermediate [25]. TcIM and its homologues, which are involved in the biosynthesis of thiopeptide antibiotics, catalyze a Diels-Alder reaction between nitrogen-containing diene and dehydroalanine [26-28]. First, an amide bond is epimerized into a diene, followed by a TcIM-catalyzed aza-Diels-Alder reaction. The subsequent dihydroxylation and aromatization are also catalyzed by this enzyme. The multiple functions of these enzymes complicate the detailed analysis of their reaction mechanisms.

In 2011, the discovery of the enzyme SpnF transformed the study of Diels-Alderase, because SpnF was experimentally demonstrated to be the first stand-alone Diels-Aldrase to exclusively catalyze the Diels-Alder reaction. Although SpnF has significant sequence similarity to *S*-adenosylmethionine-dependent *O*-methyltransferase, the enzyme solely catalyzes a [4+2]-cycloaddition reaction during the biosynthesis of spinosyn. SpnF accelerates the reaction rate (k_{cat}/k_{non}) by 500-fold compared with non-enzymatic cycloaddition [29]. However, the reaction mechanism of SpnF remains elusive [30-32], although its structure has been solved [33[•]].

Since the discovery of SpnF, reports of other potential stand-alone Diels-Alderase have increased [34[•],35[•],36,37[•]]. In particular, studies on the biosynthesis of the spirotetronate/spirotetramate group in versipelostatin, pyrroindomycin, and chlorthiricin have led to the isolation and characterization of a new stand-alone Diels-Alderase VstJ, PyrI4, and ChlL, respectively [34[•],37[•]]. Each of these enzymes catalyzes the intramolecular Diels-Alder reaction between a 1,3-diene and a tetronate/tetramate-conjugated dienophile of an acyclic substrate to form a macrocyclic ring structure with a spirotetronate/spirotetramate moiety. Homologous proteins of VstJ/PyrI4/ChlL are conserved in the known spirotetronate/spirotetramate biosynthetic gene clusters, which presumably exhibit similar activities toward their corresponding substrates (Figure 2) [34[•],37[•],38-43]. This enzyme family seems to be an ideal model for investigating how enzyme-catalyzed Diels-Alder reactions proceed, as the reaction occurs in a fully enzyme-dependent manner. Furthermore, in this year, the crystal structures of two proteins in this family, PyrI4 and AbyU, were reported [44^{••},45^{••}]. The structures of these Diels-Alderase provide mechanistic insights into how natural Diels-Alderase accomplish carbocyclization within their active sites.

The structure of two Diels-Alderase involved in spirotetronate/spirotetramate biosynthesis

W. Liu and co-workers determined the crystal structure of PyrI4 alone and in complex with its product to 2.5-Å and 1.9-Å resolution, respectively, by X-ray crystallography [44^{••}]. The structure of PyrI4 mainly adopts a rigid β -barrel core composed of nine β -strands (Figure 2 and Figure 3A). The active site is located in the center of the barrel. The N-terminal 22-amino acids of PyrI4 were not visible in the crystal structure.

The crystal of PyrI4 with its reaction product was prepared by crystallization of the PyrI4 with the substrate. X-ray analysis showed that the reaction product generated from the complete conversion of

the substrate was bound to PyrI4. The spiro-conjugated moiety of the product is positioned toward the bottom of the active site pocket. Comparative analyses between the two PyrI4 structures revealed that the N-terminal 22 amino acids of PyrI4 form an α -helix ($\alpha 0$) lid in the binary complex with its product. This $\alpha 0$ helix tightly locks the product in the active site through strong interactions between $\alpha 0$ and the rim of the β -barrel core. Mutations that interfere with this interaction or the truncation of this N-terminal sequence result in the complete loss of PyrI4 activity.

The recognition of the product is mainly contributed from interactions with the hydrophobic residues in the active site pocket. Furthermore, the polar groups of the product, a ketone group and a hydroxyl group, directly interact with a glutamine residue (Q115). A single mutation at Q115 decreased the activity of PyrI4 by 60%. Therefore, this residue plays a key role in the interaction with the tetramate moiety of the substrate. However, a series of single mutations of the nearby residues adjacent to Q115 reduced PyrI4 activity by less than 30%. Even the quadruple mutant such as Q115A/Y85A/E65A/E87A still retained some activity for the cycloaddition reaction. Based on these results, the authors concluded that the catalysis of PyrI4 is accomplished not by a single residue in the active site but through a synergistic effect contributed from multiple amino acid residues.

A multiple sequence alignment of known VstJ homologues revealed that the N-terminal α -helical sequence is found only in PyrI4 and ChlL [37], but not in other homologues (Figure 2). Therefore, it has been speculated that a different mechanism may be operative for the homologues lacking the extended N-terminal sequence [46].

Very recently, P. Race and co-workers characterized the function of AbyU, another VstJ homologue that is involved in the biosynthesis of abyssomicin [45^{••}]. AbyU does not contain the N-terminal lid-like sequence found in PyrI4 (Figure 2). The investigators demonstrated that AbyU accepts a synthesized methyl-substituted substrate analog and converts it to the corresponding product. Non-enzymatic cyclization of this substrate analog was also detected, but the reaction was so slow that it showed little effect on the measurement of kinetic parameters. They determined the crystal structure of ligand-free AbyU to a resolution of 1.73-Å. The core of AbyU is a β -barrel structure, similar to PyrI4 (Figure 3B). In the AbyU structure, a loop (residues 26-36) between sheets $\beta 1$ and $\beta 2$ is located close to the active site in place of the N-terminal lid-like α -helical structure in PyrI4. Computational docking and molecular dynamics (MD) simulations of AbyU with its natural substrate suggested that the β -barrel core of AbyU is rigid and that structural change between the apo-form and the enzyme-substrate complex is almost indistinguishable. However, the loop seems to be very flexible and may function as a lid in the complex with the substrate, similar to the role played by the N-terminal sequence in PyrI4. The authors speculate that the active site cavity of AbyU would be covered by this loop accompanied with substrate binding. Almost all of the interactions between the substrate and the active site can be attributed to hydrophobic residues. The shape of the active site is a good fit to the substrate structure. Docking simulations suggested that Tyr76 in the active site interacts with the

carbonyl group of the substrate through a hydrogen bond. This interaction may be responsible for substrate selectivity.

Furthermore, the investigators probed the reaction process using QM/MM MD simulations to determine whether the reaction catalyzed by AbyU proceeds via a polar stepwise cycloaddition mechanism or a concerted Diels-Alder reaction mechanism. Their results suggested that bond formations catalyzed by AbyU occur in an asynchronous and concerted manner through a single transition state, with C13-C14 bond formation preceding C10-C15 bond formation. A similar conclusion was also reached for the SpnF-catalyzed [4+2]-cycloaddition by computation studies [30]. However, whether the AbyU reaction is a true Diels-Alder reaction remains to be experimentally verified. The free-energy profiles of the AbyU reaction were also simulated at the SCC-DFTB/ff14SB QM/MM level, in the reverse direction (product to substrate). The simulation indicated that the interactions between the reactant and the enzyme are the same as in both the forward and reverse reactions. It was thus proposed that the reaction by AbyU is catalyzed by the complementarity between the enzyme cavity and the transition state of the reaction, so the Diels-Alder cycloaddition can occur with a relatively low free-energy barrier.

Conclusions and perspectives

The structural analysis of PyrI4 and AbyU has revealed similarities and differences between these two closely related proteins, as well as how natural Diels-Alderase catalyze the Diels-Alder reactions (Figure 3). It is conceivable that the flexible N-terminus in PyrI4 and the flexible loop between sheets $\beta 1$ and $\beta 2$ in AbyU are both near the entrance of the active site, and participate in capturing the acyclic flexible substrate during catalysis. These flexible structures work as a lid to close the active site upon substrate binding and may stabilize the transition state of the reaction. Each active site, which is mainly composed of hydrophobic residues, may work as an “entropy trap” to facilitate the reaction. Mutational analyses of PyrI4 indicated that this lid-like N-terminal sequence is indispensable for activity. An analogous function may also be assigned to the loop structure in AbyU. Many artificial Diels-Alderase experience product inhibition because the product mimics the transition state of the reaction. In contrast, PyrI4 and AbyU appear to avoid this effect by using the lid-like sequences, enabling the reaction to progress efficiently. However, the molecular mechanism to avoid the inhibition effect remains to be elucidated.

Considering the large number of natural products containing a cyclohexene structure, the vast majority of Diels-Alderase still remain to be isolated and characterized [5,47-53]. Studies of the newly identified enzymes and structural characterization of these Diels-Alderase are expected to provide more insights into the essential features of these enzymes. Such knowledge will be useful for the design of novel artificial Diels-Alderase.

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Reference annotations

- 33 ●The authors determined the structure of SpnF, a monofunctional enzyme that catalyzes a [4+2]-cycloaddition. The revealed SpnF structure with *S*-adenosylhomocysteine bound adopts a *S*-adenosylmethionine-dependent methyltransferase fold.
- 34 ●The authors characterized the monofunctional Diels-Alderase VstJ *in vivo* and *in vitro*. The Diels-Alder reaction catalyzed by VstJ to generate spirotetronate is completely enzyme dependent.
- 35 ●The authors characterized the *fsa2* gene, which is required for the stereocontrol of a [4+2]-cycloaddition in the biosynthesis of equisetin and related tetramate-containing compounds.
- 37 ●The authors identified two monofunctional Diels-Alderases, PyrE3 and PyrI4, in the spirotetramate biosynthetic pathway.
- 44 ●●The authors revealed the structure of PyrI4, a Diels-Alderase involved in the synthesis of spirotetramate, in its apo-form and with its product. Mutational analyses indicated that the flexible N-terminal sequence of PyrI4 is required for the reaction.
- 45 ●●The authors characterized the function of AbyU in the construction of the spirotetronate moiety of abyssomicin using a synthetic analog of the natural substrate. They determined the crystal structure of the AbyU in its apo-form and discussed the binding of the substrate and the reaction mechanism as determined by simulations.

Figure legends

Fig. 1. Diels-Alderase-catalyzed reactions in natural product biosynthesis.

(A) Multi-function Diels-Alderases.

(a) Lovastatin synthase (LovB) is a highly reducing fungal type I polyketide synthase. KS: ketosynthase, MAT: malonyl-CoA acyltransferase, DH: dehydratase, MT: methyltransferase, Core: inactive enoyl reductase, KR: ketoreductase, ACP: acyl carrier protein, CON: condensation protein. LovB catalyzes carbon chain elongation. During elongation cycles, LovB catalyzes a single Diels-Alder reaction to yield dihydromonacolin L.

(b) Solanapyrone synthase (SPS) catalyzes the oxidation of prosolanapyrone II to yield prosolanapyrone III and subsequently catalyzes a Diels-Alder reaction to yield solanapyrone A.

(c) Riboflavin synthase has two lumazine-binding domains to bind the substrate 6,7-dimethyl-8-ribityllumazine. A pentacyclic intermediate suggests a possible Diels-Alder reaction during catalysis.

(d) Pyridine formation for thiopeptide antibiotics. Typically, thiopeptide antibiotics have a pyridine ring that acts as a linchpin for the macrocyclic structure. The reaction of TcIM during thiocillin biosynthesis is a representative example [27]. The amide bond of dehydroalanine is converted to an imine to generate a nitrogen containing diene. Then, a formal [4+2]-cycloaddition between two dehydroalanine residues occurs. Subsequent dehydroxylation and aromatization form the pyridine ring structure.

(B) Stand-alone Diels-Alderases.

(e, f, g, h, i) SpnF, PyrE3 [37], PyrI4, VstJ, and Fsa2 [35] were characterized as stand-alone Diels-Alderases. SpnF shows sequence similarity to methyltransferase, and PyrE3 shows sequence similarity to FAD-binding proteins. SPS and PyrE3 share no sequence similarity with each other, although both enzymes contain an FAD-binding domain.

Fig. 2. Multiple sequence alignment of VstJ homologues.

A multiple sequence alignment was constructed using a Clustal Omega software (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The secondary structure of PyrI4 with the product complex (PDB ID: 5bu3) was shown on the top of the alignment. The yellow star indicates an important residue in PyrI4 activity. The blue bar and the green bar represent the lid-like helix in PyrI4 and the lid-like loop in AbyU, respectively. Accession numbers: AFV71338.1 for PyrI4; AAZ77701.1 for ChlL; ACB37739.1 for TcaU4; AGI99498.1 for LobU2; AFI57012 for QmnH-N and QmnH-C; BAQ21945.1 for VstJ; EU301739.1 for KijCyc; WP_013733060.1 for AbyU. QmnH-N and QmnH-C show N-terminal (1 aa to 195 aa) and C-terminal (196 aa to 376 aa) domains of QmnH.

Fig. 3. Models of the reaction mechanisms of PyrI4 and AbyU.

(A) The Diels-Alder reaction catalyzed by PyrI4. The reaction occurs in the β -barrel catalytic core. The flexible N-terminal sequence forms a rigid, lid-like helix structure upon binding of the substrate. Q115 (yellow star) recognizes the ketone group and the hydroxyl group of (spiro)tetramate. (B) The Diels-Alder reaction catalyzed by AbyU. The reaction occurs in the β -barrel catalytic core, similar to PyrI4. In the case of AbyU, a flexible loop between sheets β 1 and β 2 acts as a lid-like structure to recruit the substrate.

Fig. 1. Diels-Alderase-catalyzed reactions in natural product biosynthesis.

Fig. 2. Multiple sequence alignment of VstJ homologues.

Fig. 3. Models of the reaction mechanisms of PyrI4 and AbyU.