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Current Developments and Challenges in the Search for a Naturally Selected Diels-Alderase

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Abstract

Only a very few examples of enzymes known to catalyze pericyclic reactions have been reported, and presently no enzyme has been demonstrated unequivocally to catalyze a Diels-Alder reaction. Nevertheless, research into secondary metabolism has led to the discovery of numerous natural products exhibiting the structural hallmarks of [4+2] cycloadditions, prompting efforts to characterize the responsible enzymatic processes. These efforts have resulted in a growing collection of enzymes believed to catalyze pericyclic [4+2] cycloaddition reactions; however, in each case the complexity of the substrates and catalytic properties of these enzymes poses significant challenges in substantiating these hypotheses. Herein we consider the principles motivating these efforts and the enzymological systems currently under investigation.

Introduction

The Diels-Alder reaction is a specific type of [4+2] cycloaddition in which a diene and dienophile react through a single pericyclic transition state to generate a cyclohexene containing product [1]. Biological catalysts of the Diels-Alder reaction have been recognized for the past two decades and realized through the artificial selection (i.e., directed evolution) of catalytic ribozymes [2, 3], the immunological selection of catalytic antibodies [4–6] and the recent computational selection of “theozyme” catalysts [7]. At present, however, a *bona fide* Diels-Alderase that has evolved through the process of natural selection remains to be identified, despite a number of promising candidates having been overproduced and characterized *in vitro*. In this current opinion, we describe the impetus behind the search for such an enzyme and briefly consider the leading candidates along with the key challenges involved.

Do Diels-Alderases exist?

Enzyme-catalyzed organic transformations can typically be categorized into two broadly defined classes depending on whether their catalyses are characterized by ionic mechanisms (2-electron processes) or radical-induced conversions (1-electron processes). The Diels-Alder reaction, however, belongs to neither of these classes and is instead a pericyclic reaction involving the concerted reorganization of a system of six electrons via a single

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electrocyclic transition state [1]. Pericyclic reactions in general are extremely rare within the enzymological context despite their prevalence in synthetic applications in organic chemistry [8, 9]. Among the naturally selected enzymes, the sigmatropic rearrangements catalyzed by chorismate mutase [10–11], isochorismate pyruvate lyase [12–13], precorrin-8x methyl mutase (CobH) [14–15] and dimethylallyltryptophan synthase [16] represent the small number of such reactions described thus far.

There is, however, reason to believe that enzymes specific for catalyzing a Diels-Alder reaction do indeed exist. Research into secondary metabolism has led to the discovery of numerous natural products such as lovastatin, cytochalasins, solanapyrones, and spirotetronates among others that contain one or more cyclohexene rings [17–18]. Such a ring system frequently results from the [4+2] cycloaddition of a dienophile to a diene in organic synthesis and thus its presence is highly suggestive of a Diels-Alder reaction. The fact that the cyclohexene moieties of these natural products are found with defined stereochemical configurations suggests a biosynthetic process of either accelerated cyclization leading to the observed configuration or decelerated cyclization in alternative configurations is likely at work. These observations have prompted investigations into the biosynthesis of a number of such compounds in the hope of finding the putative Diels-Alderase and unraveling the origin of the cyclohexene ring in each case.

How might a Diels-Alderase operate?

The search for a “Diels-Alderase” is in many ways a study of how cyclohexene rings are constructed biosynthetically. One possibility is that no catalyst is actually needed at all [19]. This suggestion stems from the observation that most of the cyclization reactions studied previously also proceed at appreciable rates in the absence of any putative enzyme [18]. Instead, the enzymes responsible for generating the immediate precursors to the cyclization product may simply serve as a chiral template to prevent formation of the undesired stereoisomers, whereas the desired cyclization effectively proceeds at the uncatalyzed rate prior to release from the enzyme active site. However, addressing this question is difficult, because the “uncatalyzed” cyclization reaction will invariably be tied to the enzymatic transformation leading to the immediate precursor.

A second possibility involves a direct enzymatic contribution to lowering the free energy of activation for cyclization, in other words, true catalysis. How this might be accomplished by a Diels-Alderase is of great interest, because in contrast to the activation of Lewis acids and bases and the generation of substrate radical intermediates, there is relatively little precedent for the enzymological binding and stabilization of pericyclic transition states. Rate enhancement of the Diels-Alder reaction in aqueous media is well known and believed to be a consequence of the hydrophobic packing of diene and dienophile into a low-volume configuration primed for cyclization [20, 21]. This suggests that a cyclase enzyme could simply function as an “entropic trap” for the cyclization reaction [18, 19]. Such a hypothesis implies that binding enthalpy facilitates the entropic destabilization of substrate in the Michaelis complex so as to effectively “freeze out” those conformers most conducive towards reaction [22]. This prediction has been corroborated by the measurement of greater reductions in activation entropy compared to enthalpy in the enzymatic (in terms of k_{cat}) versus the unimolecular nonenzymatic pericyclic reactions of chorismate mutase and the analogous antibody 11F1-2E11 [23–25].

An alternative view is that catalysis might be achieved via enthalpic reductions in the free energy of activation primarily through electrostatic effects [26]. Thus, rather than simply preorganizing substrate for cyclization, the charge distribution of the pericyclic transition state is specifically accommodated and stabilized within the enzyme active site. Under this

hypothesis, a greater reduction in activation enthalpy rather than entropy in the enzymatic (k_{cat}) versus nonenzymatic (k_{non}) reactions is expected. Consistent with this prediction are several examples of catalytic antibodies and enzymes. These include the catalytic antibodies 1E9 [27], which catalyzes a bimolecular [4+2] cycloaddition, and 1F7 [28], which acts as a chorismate mutase, along with isochorismate pyruvate lyase [29] and the chorismate mutase from *Bacillus subtilis* [30].

The reality would likely be some combination of these factors and lead to additional questions as to the detailed chemical mechanism by which they are realized. However, all of this is predicated on the assumption that a Diels-Alder mechanism of cycloaddition is indeed operant during the catalytic cycle. In addition to identifying true catalytic effects, it will also be necessary to establish the pericyclic nature of the cyclization. A [4+2] cycloaddition could in principle also proceed stepwise via Lewis acid/base or radical-mediated chemistry with dipolar or diradical intermediates, respectively [1]. In fact, it is only relatively recently that careful measurement of kinetic isotope effects and computational modeling have led to wider acceptance of a pericyclic mechanism as the defining feature of the Diels-Alder cycloaddition *per se* [31–33]. However, with respect to the catalytic cycle of an enzyme, this remains very much an open question, and the complex structures of many of the substrates along with the multifunctionality of the putative enzymes have thus presented a considerable challenge in this regard.

Putative Diels-Alderase currently known

Several candidate enzymes have been investigated for the mechanistic hallmarks of the Diels-Alder reaction. Macrophomate synthase is one of the first studied [34]. This enzyme catalyzes a net cyclization reaction between 2-pyrone and oxaloacetate to form macrophomic acid in the fungus *Macrophoma commelinae* (see Figure 1)[35]. An early structural study demonstrated that the active site of macrophomate synthase is organized to accommodate 2-pyrone and a dienophile such as pyruvate in a binding configuration compatible with that leading to an electrocyclic transition state [36]. While, a [4+2] cycloaddition mechanism is possible for this enzyme, there are also two decarboxylation events during the catalytic cycle consistent with a stepwise reaction involving a Michael addition followed by an aldol addition to afford the final product as shown in Figure 1 [37]. Such a stepwise pathway was later shown computationally by Jorgensen and coworkers to be energetically more reasonable in the macrophomate synthase active site compared to the pericyclic alternative [38]. Furthermore, Hilvert and coworkers also demonstrated experimentally that macrophomate synthase can operate as a promiscuous aldolase consistent with the second half of the stepwise mechanistic hypothesis [39]. Thus, current evidence suggests that macrophomate synthase is not a true Diels-Alderase.

Riboflavin synthase catalyzes the final step in the biosynthesis of riboflavin in which two molecules of 6,7-dimethyl-8-ribityllumazine are disproportionated to produce riboflavin and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione as shown in Figure 2 [40]. The mechanism of this complex transformation involves the formation of a pentacyclic intermediary dimer of 6,7-dimethyl-8-ribityllumazine [41, 42]. A hypothesis as to the origin of the pentacyclic intermediate involves formation of a covalent enzyme-substrate complex to activate one of the monomers as an electrophile for nucleophilic attack by the conjugate base of the other [42, 43]. Subsequent rearrangement with elimination of the catalytic residue would yield the pentacyclic intermediate [42]. However, structural studies in conjunction with mutagenesis experiments failed to identify an enzymatic residue that might serve as the catalytic nucleophile, though a water molecule might serve this function [44–46]. This, and the observation that the *N*-terminal domain of riboflavin synthase binds the exomethylene anion, led to the proposal of an alternative mechanism involving a

Cannizzaro-type disproportionation followed by an inverse demand Diels-Alder reaction [43]. Presently, the hypotheses concerning riboflavin synthase are still very much speculative, and future work will be needed to clarify the origin of the pentacyclic intermediate and the role the enzyme plays in its formation.

Biogenesis of the “lynchpin” six-membered nitrogen heterocycles of thiopeptide antibiotics might also involve a Diels-Alder reaction. Early isotope feeding experiments demonstrated that dehydroalanine residues could serve as both diene and dienophile in cycloadditions to produce vinylogous carbinolamines that are subsequently aromatized to yield the pyridine lynchpins as depicted in Figure 3 [47]. Recent identification of a number of gene clusters responsible for thiopeptide biosynthesis and corresponding bioinformatic analyses resulted in the identification of a set of homologous genes, *tsrL*, *tpdD* and *tclM*, that are proposed to encode [4+2] cyclases [48]. While they appear to have no functional homology to known enzymes, their ubiquitous presence in all clusters is indicative of their importance for the biosynthesis of thiopeptides. To test this hypothesis, Walsh and coworkers created a *tclM* knockout mutant strain of *Bacillus cereus* to examine its effects on the biosynthesis of thiocillin *in vivo* [49]. Metabolites identified from the mutant strain culture were indeed devoid of the pyridine linkages though the dehydroalanine residues remained intact. This result strongly suggests an enzyme-catalyzed cyclization reaction in construction of the thiopeptide lynchpin motif. While an aza-Diels-Alder reaction is very much a possibility, alternative stepwise mechanisms have also been proposed [50].

Lovastatin nonaketide synthase (LovB) and solanapyrone synthase (Sol5) represent two enzymes involved in polyketide biosynthesis that have been proposed to catalyze [4+2] cycloadditions (see Figure 4). LovB is a member of the highly reducing class of polyketide synthases that together with LovC is responsible for the biosynthesis of dihydromonacolin L in the biosynthetic pathway of lovastatin [51, 52]. Solanapyrone synthase is a polyketide tailoring dehydrogenase that participates in the intramolecular [4+2] cycloaddition of prosolanapyrone II to yield solanapyrone A [53, 54]. Both enzymes are thus multifunctional. LovB catalyzes multiple reactions to construct the decalin ring system of dihydromonacolin L, of which the [4+2] cycloaddition is only one [51]. Likewise, solanapyrone synthase is a flavoenzyme responsible for the dehydrogenation of prosolanapyrone II to prosolanapyrone III, which is the species that formally undergoes the subsequent intramolecular cycloaddition [53]. In each case the immediate precursors to cyclization are known to react at appreciable rates even in the absence of enzyme [54, 55]. The strongest argument for cyclase activity has therefore rested on the observation of increased stereochemical specificity of the respective cyclizations in the presence of LovB [56] and solanapyrone synthase [53, 54]. Therefore, LovB and solanapyrone synthase indeed appear to play an important part in the respective cyclization reactions. However, questions still remain regarding the catalytic role of these enzymes during the cyclization events and the nature of the corresponding transition states.

Spinosyn A is an insecticide produced by the actinomycete bacterium *Saccharopolyspora spinosa* [57]. Biosynthesis of the tertacyclic polyketide core of spinosyn A has drawn considerable attention given the potential for involvement of Diels-Alder chemistry as shown in Figure 4 [57–59]. It was recently shown that the cyclohexene core is produced via the action of two enzymes known as SpnM and SpnF [60]. SpnM catalyzes a 1,4-dehydration reaction to produce a reactive intermediate that subsequently undergoes an intramolecular [4+2] cycloaddition to yield the tricyclic product. Though the cyclization was shown to proceed nonenzymatically with the same stereochemistry observed in the spinosyn product, the cyclization rate was significantly slower than that of dehydration and independent of SpnM concentration. This argued against any role being played by SpnM, catalytic or otherwise, on the cyclization process. Inclusion of SpnF into the reaction

mixture, however, led to a clear acceleration in the rate of cyclization that directly correlated with the SpnF concentration. Accordingly, a 500-fold rate enhancement (k_{cat}/k_{non}) was attributed to the SpnF-catalyzed cycloaddition. The apparent monofunctionality and specificity of SpnF for catalyzing the cyclization reaction makes it unique among the putative Diels-Alderase. Nevertheless, ambiguity remains as to whether it proceeds via stabilization of a single electrocyclic transition state or utilizes a stepwise mechanism involving zwitterionic intermediates.

It is tempting to speculate as to how SpnF, solanapyrone synthase and LovB each might catalyze a Diels-Alder reaction despite the ambiguity surrounding their chemical mechanisms. In each of these cases the dienophile of the immediate precursor to cyclization is conjugated to a carbonyl moiety either directly or via an extended π -system. It is therefore possible that enthalpic reductions in the activation energy of cyclization could be introduced via specific substrate-enzyme interactions to facilitate the polarization of these conjugated enone systems [19]. Thus, while proximity effects may dominate the mechanisms of catalysis, chemical features are present by which the enzymes may participate in lowering the energy of the LUMO of the dienophile. In fact, the rational design of the Diels-Alder theozyme incorporated specific hydrogen-bonding interactions with the substrates to achieve this aim and increase the cyclization rate [7]. Likewise, the combination of a polarizing hydrogen-bond and tight approximation of the reacting species in a largely hydrophobic binding pocket is believed to be responsible for the rate acceleration of the 1E9 antibody [27]. Understanding the interplay of conformational constraints and direct modulation of the molecular orbital energies by a naturally selected Diels-Alderase would thus provide considerable inspiration for future investigations.

Conclusions

The class of enzymes utilizing pericyclic reaction mechanisms continues to grow, but it does not yet contain an enzyme unequivocally accepted to catalyze a Diels-Alder reaction. The discovery of such an enzyme would offer a new and important system for unraveling how enzymes have evolved to catalyze chemical reactions that involve neither ionic chemistry nor radical-mediated transformations. Such enzymes are believed to exist in large part due to tell-tale structural motifs observed in a mounting collection of secondary metabolites, in particular the cyclohexene rings with defined stereochemical configurations. This has led to the identification of a number of enzymes as putative Diels-Alderase. However, clarifying the role of each enzyme during the cyclization event as well as characterizing the nature of the transition state(s) represent considerable challenges in this endeavor. Nevertheless, the monofunctionality and specificity of SpnF for catalyzing the [4+2] cycloaddition reaction in spinosyn biosynthesis makes it the ideal system to address these issues.

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Highlights

- No enzyme has presently been identified unequivocally as a *bona fide* Diels-Alderase.
- Structural features of many natural products imply the existence of such an enzyme.
- Interest in these enzymes stems from uncertainty in how catalysis might be achieved.
- Several enzymes are currently under investigation as putative Diels-Alderaes.
- Complexity of the substrates and reactions involved has made their study challenging.

Figure 1. Macrophomate synthase

Macrophomate synthase produces macrophomic acid from oxaloacetate and 2-pyrone.

While the catalytic cycle may involve a Diels-Alder reaction, mounting evidence suggests a stepwise process of addition instead. Processes leading to C-C bond formation are highlighted in red.

Figure 2. Riboflavin synthase

Riboflavin synthase catalyzes the disproportionation of two molecules of 6,7-dimethyl-8-ribityllumazine (R denotes the ribityl moiety) to generate riboflavin and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione. Discovery of a pentacyclic intermediate has suggested a Diels-Alder reaction during the catalytic cycle, though other mechanisms are also possible. Processes leading to C-C bond formation are highlighted in red.

Figure 3. Thiopeptide biosynthesis

The pyrimidine lynchpin motif of thiopeptides such as thiocillin I could arise via an enzyme-catalyzed aza-Diels-Alder reaction. However, stepwise mechanisms of addition have also been proposed. Processes leading to C-C bond formation are highlighted in red.

Figure 4. LovB, SpnF and solanapyrone synthase

The enzymes LovB, SpnF, and solanapyrone synthase are involved in polyketide biosynthesis and have been shown either through direct kinetic characterization or stereochemical analysis to participate in [4+2] cycloaddition reactions (red).