Published in final edited form as:

Trends Biochem Sci. 2018 May ; 43(5): 342–357. doi:10.1016/j.tibs.2018.02.011.

Unraveling the structure and mechanism of the MST(ery) enzymes

Catherine L. Shelton and Audrey L. Lamb¹

¹Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045, United States

Abstract

The Menaquinone, Siderophore and Tryptophan (MST) enzymes transform chorismate to generate precursor molecules for the biosynthetic pathways defined in their name. Kinetic data, both steady state and transient state, and X-ray crystal structures indicate that these enzymes are highly conserved, both in mechanism and in structure. Because these enzymes are found in pathogens but not humans, there is considerable interest in these enzymes as drug design targets. While great progress has been made in defining enzyme structure and mechanism, inhibitor design has lagged behind. This review provides a detailed description of the evidence that begins to unravel the mystery of how the MST enzymes work, and how that information has been used in inhibitor design.

Keywords

chorismate; isochorismate; salicylate; aminodeoxychorismate; aminodeoxyisochorismate; anthranilate

Menaquinone, Siderophore, and Tryptophan Enzymes

The shikimate metabolic pathway, found only in plants, fungi, and bacteria, converts erythrose-4-phosphate and phosphoenolpyruvate to chorismate[1]. Chorismate, in turn, is the precursor for the production of the aromatic amino acids (tryptophan, tyrosine, and phenylalanine), siderophores, menaquinone (vitamin K), folate, ubiquinone, and phenazine-1-carboxylic acid. Of the seven chorismate-utilizing enzymes, five are structurally homologous, and are called the MST (menaquinone, siderophore, tryptophan) enzymes[2]. The core of MST enzymes is a β -sandwich made from orthogonal β -sheets surrounded by helices[2–7]. Historically, the protein fold is described as two domains: each domain comprises one β -sheet and the affiliated helices[4]. The active site cleft is between the two β -sheets, and harbors a catalytically required magnesium ion. Chorismate mutase and UbiC are chorismate-utilizing enzymes that are not in the MST structural family. The focus of this review is the structural and functional characterizations of the MST enzymes.

Corresponding Author: Audrey L. Lamb, lamb@ku.edu, phone: (785) 864-5075.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The chemistries performed by MST enzymes range from simple isomerizations to more complex, multi-step reactions. In the Key Figure, the MST enzymes are divided into two groups: those that use water as a nucleophile and those that use ammonia as a nucleophile. Within each of these groups the enzymes are arranged in order of increasing mechanistic complexity. The isochorismate synthases (Key Figure A) catalyze isomerization of the C4 hydroxyl group to C2. The salicylate synthases (Key Figure B) perform both the isomerization as well as a lyase activity that removes the pyruvylenol tail from C3. The aminodeoxyisochorismate synthases (Key Figure C) perform a similar reaction to the isochorismate synthases. The functional group from C4 is similarly isomerized to C2; however, because the nucleophile is ammonia, the hydroxyl group is replaced by an amine. Aminodeoxychorismate synthases (Key Figure D) replace the hydroxyl group at C4 with an amine group. Finally, anthranilate synthase (Key Figure E), catalyzes the same isomerization as aminodeoxyisochorismate synthase, and also catalyzes the lyase step to remove the pyruvylenol tail.

Isochorismate Synthase

Isochorismate synthases isomerize chorismate, moving the hydroxyl group from C4 to C2 (Key Figure A). In *Escherichia coli*, isochorismate is required for the production of the electron carrier menaquinone and also for the biosynthesis of the siderophore enterobactin. The respective isochorismate synthases for these pathways are MenF and EntC[2, 8, 9]. A third isochorismate synthase characterized from *Pseudomonas aeruginosa* is PchA, required for the production of the siderophore pyochelin[10].

Walsh *et al* showed that the isochorismate synthases establish an equilibrium between substrate and product that slightly favors the reverse reaction (~60:40) and proposed four possible mechanisms [11]. The first was a Michael addition/elimination reaction in which a nucleophilic attack at C2 resulted in an active-site stabilized dienolate followed by magnesium-assisted elimination of the hydroxyl group at C4. Two additional mechanisms involved covalent catalysis at C6, either by an active site nucleophile or by nucleophilic attack from the pyruvylenol carboxylate to form a bicyclic lactone intermediate. The final mechanism proposed that magnesium binds to both the hydroxyl leaving group at C4 and to the incoming hydroxyl to be added at C2. The two covalent catalysis hypotheses did not address the role of magnesium, while the first and fourth hypotheses required that the magnesium be positioned near the C4 of chorismate. However, subsequent structures of isochorismate synthases, and indeed all MST enzymes, demonstrate that the magnesium is instead liganded by the carboxyl group on C1 (Figure 1A)[2, 5, 6, 9, 12]. A new mechanistic hypothesis was developed in which the magnesium enhances the electrophilicity at C2, making this position more susceptible to nucleophilic attack[2, 7, 13].

After Walsh's initial proposals, the definition of the mechanism of the isochorismate synthases has been the cumulative work of several groups, frequently investigating several of the MST enzymes simultaneously. He & Toney suggested a general acid-general base mechanism in which a glutamic acid activated a water for nucleophilic attack at C2 concomitant with hydroxyl cleavage at C4, also assisted by an active site glutamic acid[14, 15]. Kolappan *et al* identified an active site lysine in MenF as the general base that activates

the water for nucleophilic attack by mutational analysis[2]. In the same study, an active site glutamate in MenF was identified as important for catalysis because the enzyme was rendered inactive when this residue was mutated to glutamine. The apo-MenF structures were solved and the proposed general acid-general base residues were hypothesized to be likely candidates by comparison to holo-structures of anthranilate synthase (discussed later). The structure of EntC was solved in 2010 with isochorismate in the active site and the strictly conserved lysine and glutamate residues proposed for MenF were indeed ideally suited for acid-base chemistry (Figure 1B)[9]. The hypothesis was questioned again when Ziebart and Toney showed that mutation of the proposed lysine general base to glutamine did not result in a complete loss of activity [16]. However, in a study of the *P. aeruginosa* isochorismate synthase PchA, Meneely *et al* demonstrated that the general acid and general base residues are in reverse protonation states [13]. Typically, it is expected that glutamic acid will be in the deprotonated form (Glu-COO⁻) and that lysine will be in the protonated form (Lys-NH₃⁺). However, in the reverse protonation state both amino acids are in the neutral form (Glu-COOH and Lys-NH₂, respectively). The presence of reverse protonation states was described in the early 1970s in the enzyme enolase and has been described in other systems since then [17–21]. The demonstration of a small population of the enzyme in reverse protonation states was critical in understanding the mechanism of isochorismate synthases. These data resolve the apparent discrepancy raised by Ziebart and Toney, because the active site lysine of the wild type protein and the glutamine of the variant they described will present the same functional group in the same protonation state (NH₂), albeit at different lengths from the mainchain. Therefore, the glutamine variant may maintain some residual activity. The general acid – general base mechanism for isochorismate synthase is demonstrated by the scheme in Figure 1C.

A recent investigation of the role of magnesium in MST enzymes was carried out by Meneely *et al* and has refined the current understanding in the field[22]. Single turnover experiments indicated that the rate of isomerase chemistry is slow compared to the binding of substrate (chorismate) and cofactor (magnesium). Most importantly, this study demonstrated that magnesium inhibits product release in the isochorismate synthases at concentrations higher than 0.5 mM. This finding was in agreement with an early, but frequently overlooked, description of magnesium inhibition of MenF[23]. Meneely *et al* showed that at higher concentrations of magnesium, product release is inhibited and retention of isochorismate in the active site promotes the reverse reaction[22]. As a result of this study, the kinetic mechanism of the isochorismate synthases has been defined as ordered sequential, and that in the absence of inhibitory concentrations of magnesium, chemistry is rate limiting. Finally, the authors propose that the role of the magnesium is to exert an electrostatic effect that favors formation of the reverse protonation states necessary for catalysis [22]. The finding that high magnesium concentration inhibits the isochorismate synthases necessitates reevaluation of the previous work. Many of the studies were performed in the presence of 10 mM magnesium, an understandable experimental design given the increased catalytic efficiency obtained with higher concentrations of magnesium. However, the kinetic analysis in these studies is obfuscated by the inhibitory effect of magnesium. This may likely be true for all MST enzymes.

Salicylate synthases

The salicylate synthases catalyze both the isomerization reaction and removal of the pyruvyl enol tail (Key Figure B). Salicylate is used by bacterial non-ribosomal peptide synthetases as the first step in siderophore biosynthesis. The best characterized salicylate synthases are Irp9 from *Yersinia spp.* for the production of yersiniabactin (Figure 2A) and MbtI from *Mycobacterium tuberculosis* in the pathway responsible for mycobactin biosynthesis. However, a salicylate synthase from the methanol utilizing strain *Amycolatopsis methanolica* 239^T was recently described as the first enzyme in the biosynthesis of the siderophore amychelin[24].

Early NMR experiments that followed product formation showed that the mechanism of the salicylate synthases was two steps: first, isochorismate is generated, and then the pyruvyl enol tail is cleaved [25]. Like the isochorismate synthases, the isomerization reaction is catalyzed by a general acid – general base mechanism using lysine and glutamic acid residues strictly conserved in these enzymes (Figure 2B) [7, 12]. All MST enzymes have a glutamic acid at the general acid position and both the isochorismate synthases and salicylate synthases have a lysine at the general base position[2]. The lyase activity was originally suggested to be a pericyclic reaction, similar to that catalyzed by the non-MST enzyme, PchB (an isochorismate-pyruvate lyase), in which the hydrogen at C2 is transferred to C9 in the cyclic intermediate[7, 12]. Subsequent work by Ziebart and Toney suggested that the lysine general base for the isomerization reaction was also important for the lyase activity, promoting the idea of general acid-general base catalysis for the lyase reaction[16], which we will discuss again in the anthranilate synthase section below. Importantly, the mechanism of lyase function in the MST enzymes is still actively debated. The two possible mechanisms for lyase function (pericyclic and general acid – general base) are represented in Figure 2B. Indeed, a significant amount of effort has been directed toward interconverting lyase deficient and lyase active enzymes. Despite very high sequence homology in the active site, three non-conserved amino acids were identified that differ systematically between salicylate synthases and isochorismate synthases[26]. However, mutation of one or more of these isochorismate synthase residues to the cognate salicylate synthase residues failed to make the isochorismate synthases competent for lyase function[2, 9, 27]. It is clear that the functional difference between the two classes of MST enzymes is not solely due to variation in active site sidechain composition.

As part of the magnesium study mentioned in the isochorismate synthase section, the role of magnesium in the function of the salicylate synthase Irp9 was also analyzed[22]. The effect of magnesium was studied both when chorismate and when isochorismate were used as substrates. A global fit analysis of the kinetic data demonstrated that the binding constant for magnesium to the Irp9-chorismate complex was high micromolar. By contrast, the binding constant for magnesium to the Irp9-isochorismate complex was low nanomolar. These findings point towards a new understanding of the kinetic mechanism of salicylate synthases. The high affinity complex formed with the intermediate isochorismate indicates an ordered-sequential mechanism, in which the magnesium binds after the substrate and prevents egress of the intermediate by buttoning the active site closed and driving the lyase reaction forward. This mechanism is consistent with the finding that, unlike the

isochorismate synthases, salicylate synthases are not inhibited by high magnesium concentrations. In addition to evaluating the role of magnesium, Meneely *et al* also investigated how iron affects catalysis of isochorismate synthases and salicylate synthases. They showed iron inhibits catalysis, binding with a higher affinity than magnesium. This hypothesis was further confirmed when crystals of the isochorismate synthase EntC and the salicylate synthase Irp9 were soaked with ferrous ammonia sulfate: the catalytic magnesium binding site was instead populated with iron. Based on these findings, it was hypothesized that the magnesium binding site acts as an iron inhibition/regulatory site so that when iron levels are sufficient, siderophore biosynthesis is inhibited by iron binding to the first enzyme in the pathway.

Of all the MST enzymes, the salicylate synthase from *Mycobacterium tuberculosis* (MbtI) has undoubtedly received the most attention for inhibitor development, with hopes of finding a lead compound for drug discovery: tuberculosis infects 1.7 billion people worldwide and is becoming increasingly antibiotic resistant[28]. A high-throughput screen to identify potential MbtI inhibitors was reported in 2010 utilizing a library of 104,802 compounds[29]. From this library, sixteen compounds were selected as being both chemically tractable and having IC₅₀ values between 1–100 μ M against MbtI. Of these compounds, two were found to be from one structural class, benzimidazole-2-thiones, which the authors recommended as a good scaffold on which to develop new inhibitors. A subsequent study produced similar findings. A high-throughput screen against the isochorismate-pyruvate lyase PchB from *P. aeruginosa* was conducted in 2014[30]. PchB is not an MST enzyme, but is a lyase only enzyme that removes the pyruvylenol tail from chorismate, like the salicylate synthases do. Because of these functional similarities, the screen was extended to include the salicylate synthase Irp9 from *Yersinia spp.* The compound library comprised over 100,000 compounds. Interestingly, the two most effective inhibitors against Irp9 were similar to the benzimidazole-2-thiones compounds described in the MbtI high-throughput screen. This lends further credence to the use of this structure as a scaffold for future inhibitors.

Inhibitors of MbtI have also been generated by rational design, with varying success. Inhibitors were designed to mimic the isochorismate intermediate, but with an aromatized ring and a range of hydrocarbon groups attached to C9 of the pyruvylenol tail[31]. The inhibitors that were most effective *in vitro* had a methyl, ethyl, isopropyl or cyclopropyl group on C9 with low micromolar K_i values [31]. Subsequently, the same group solved the X-ray crystal structures of MbtI with each of these inhibitors[32]. Interestingly, only one of the inhibitors bound the active site in the same orientation as the salicylate; the remaining inhibitors were present in the active site but in an alternate binding pose (Figure 2C–E)[12, 32]. The inhibitor that is oriented with a binding pose consistent with product binding is the only one that is unsubstituted at C9 (Figure 2C). This inhibitor exhibited a 10–20-fold increase in K_i compared to the inhibitors that are substituted at C9[32]. Bulky R-groups on C9 may prevent the inhibitor binding in the active site in its native orientation. Additionally, the authors describe a disrupted active site with more disordered residues when inhibitors with C9 substituents are bound (Figure 2D–E). Unfortunately, when these compounds were tested against bacteria, they were found to be ineffective with minimal inhibitory concentrations (MIC₅₀) of > 1 mM. The authors speculated that the compounds were unable to traverse the bacterial cell membrane[33]. Attempts to design membrane soluble

compounds met with better success against bacterial culture (mid micromolar MIC₅₀), but there was no correlation between lower MICs and a lower K_i , suggesting an off-target effect[33].

More recently a series of potential transition state inhibitors has been developed for MbtI[34]; however, the transition state structure has never been formally determined using kinetic isotope effects. The designed cyclohexene inhibitor showed little inhibition of MbtI, and the compound was acknowledged as a poor transition state mimic [34]. Additional attempts to develop a transition state inhibitor with modifications to the pyruvylenol tail or to the substituents on C1 have also failed to significantly inhibit MbtI[35]. Since approaches to develop inhibitors based on hypothetical transition states have not provided the desired results, future studies in which the transition state is determined experimentally using rigorous enzyme kinetics provide a path forward.

Aminodeoxyisochorismate Synthase

Aminodeoxyisochorismate (ADIC) synthase catalyzes removal of the C4 hydroxyl and addition of an amino group at C2 (Key Figure C). Therefore, ADIC synthase is analogous to the isochorismate synthases, with the exception that the nucleophile is an ammonia instead of water (Figure 3A). To generate the ammonia, ADIC synthase requires an amidotransferase domain [36]. Also like isochorismate synthase and salicylate synthase, the hydroxyl leaving group is protonated by a conserved glutamic acid (Glu201)[36]. ADIC synthase is known by the gene name PhzE, and catalyzes the first committed step in production of phenazine-1-carboxylic acid [37]. This molecule is itself a potent antibiotic that strains such as *Pseudomonas fluorescens* deploy against fungal root pathogens and it is also the precursor for the virulence factor pyocyanin produced by *P. aeruginosa* [37, 38].

Each PhzE monomer contains both an MST and an amidotransferase in a single polypeptide chain (Figure 3B) [36]. However, the fused MST and amidotransferase domains do not form a functional unit [36]. Instead, the MST domain from one monomer forms a functional unit with the amidotransferase domain from the second monomer, a finding that was confirmed by small-angle x-ray scattering [36]. A common mechanism for shuttling ammonia from the glutamine amidotransferase to its target active site is by an internal channel between the two domains[39]. A 25 Å channel was identified whose outlet is aligned with the C2 of chorismate, positioning the ammonia for a nucleophilic attack[36]. In Figure 3C, two distinct binding cavities can be seen. The upper is the MST active site with benzoic acid, pyruvate and magnesium bound. The lower is the amidotransferase in which a glutamate-mimic highlights the active site. The channel appears closed because a glutamate and an asparagine form two hydrogen bonds, acting as gatekeepers for the release of ammonia. When either of these residues are mutated the enzyme becomes inactive[36].

Culbertson & Toney have determined steady-state kinetic parameters for PhzE from *Pseudomonas aeruginosa* PAO1. The enzyme was 200-fold less active using free ammonia in place of the glutamine substrate of the amidotransferase domain[40], potentially providing kinetic evidence to corroborate the structural data that there is channeling of the ammonia from the amidotransferase domain to the MST active site. Culbertson and Toney provide

double reciprocal plots to justify an equilibrium random mechanism. However, a more refined mechanistic analysis should be acquired through pre-steady-state kinetics. Likely the ordered sequential kinetic mechanism of the isochorismate and salicylate synthases will hold for these MST enzymes as well. Finally, pyanocyanin and 1-hydroxyphenazine were not inhibitory, indicating that these compounds are not regulatory.

Aminodeoxychorismate Synthase

The chorismate transformation performed by aminodeoxychorismate (ADC) synthase is replacement of the hydroxyl group at C4 with an amino group, the first step in generating the folate precursor *p*-aminobenzoate. Unique to this MST enzyme, the result is functional group exchange at C4, as opposed to ring isomerization or migration of the ring functionalization from C4 to C2 (Key Figure D). ADC synthases use ammonia as a nucleophile, which is provided by an amidotransferase. The second step of *p*-aminobenzoate production is removal of the pyruvylenol tail; however, the lyase function is not catalyzed by ADC synthase, but by a downstream PLP-dependent aminotransferase.

The ADC synthase in *E. coli* is known by the gene name PabB. The structure of PabB was solved by Parsons *et al* [6]. In the structural analysis, a lysine residue was identified in the active site that in other MST enzymes is a highly conserved alanine. It was hypothesized that the lysine sidechain sterically blocks the ammonia nucleophile from attacking at C2, thereby promoting attack at C4 [6]. Alternatively, He & Toney proposed that the lysine *itself* performs a nucleophilic attack at C2 forming a covalent intermediate [14, 15]. In this proposed mechanism, the production of aminodeoxychorismate requires two steps: 1) nucleophilic attack by the active site lysine at C2 that promotes elimination of the hydroxyl at C4, and 2) nucleophilic attack by ammonia at C4 that promotes elimination of the lysine sidechain at C2. However, ADC synthases from *Bacillus subtilis* and *Stenotrophomonas maltophilia* have the more common alanine in place of the lysine, eliminating the possibility that this residue either occludes attack by ammonia at C2 or participates in a nucleophilic attack at C2 [41, 42]. Furthermore, no covalent intermediate could be demonstrated in the *B. subtilis* enzyme [41]. By comparison to the isochorismate synthases, which are known to be readily reversible, a likely explanation is that a first ammonia nucleophile attacks at C2, promoting elimination of the hydroxyl at C4. In a second step, a second ammonia attacks at C4, promoting elimination of the C2 amine added in the first step (Figure 4A) [31].

To date, no ADC synthase structures have been solved with the substrate in the active site. The structures that have been determined are highly mobile, with many residues disordered [6, 42]. Indeed, the lysine of debate above is undefined in most solved structures, and poorly defined where it could be modeled. The best *E. coli* ADC synthase structure has formate, derived from the crystallization conditions, bound in the active site, possibly mimicking the pyruvylenol tail (Figure 4B) [6]. The structure also includes a tryptophan as a ligand in a site that is akin to the allosteric site in anthranilate synthase (discussed below); however, here the role seems to be structural and not regulatory [6]. The structures also suffer from disorder in the region that putatively binds an amidotransferase. Structures in which substrate or product are bound in the active site would enhance our understanding of the PabB mechanism, and co-crystallization with an amidotransferase would provide evidence for domain interactions.

When the *S. maltophilia* PabB (SmPabB) structure was determined, the *S. maltophilia* amidotransferase had not been identified [42]. The SmPabB was quite inefficient when provided with exogenous ammonia; however, turnover was improved 20-fold with the amidotransferase from *E. coli* in the pathway for production of *p*-aminobenzoate[42]. The affinity between SmPabB and the *E. coli* amidotransferase was quite high (nanomolar K_D). Promiscuous use of amidotransferases between MST enzymes requiring ammonia as a nucleophile had been demonstrated previously[43]. Therefore, Bera & Parsons concluded that the *S. maltophilia* PabB must utilize the anthranilate synthase amidotransferase TrpG (described below)[42]. This hypothesis was strengthened by the demonstration that *E. coli* amidotransferase has 61% sequence identity overall for *S. maltophilia* TrpG and 81% sequence identity for residues that likely make up the MST:amidotransferase heterodimer interface.

In 2006, the one-bead one-compound approach[44] was used with the intent of identifying inhibitors for ADC synthase[45]. A library of compounds was generated that contained a chorismate analogue to target the inhibitor to the ADC synthase active site. The analogue was coupled to a spacer followed by a variable region, different motifs that may interact with the exterior of the protein, and finally attached to a bead. The protein itself was fluorescently labeled, and the screen was performed by determining which beads bound protein. Of the inhibitors tested the best K_i was high micromolar. Several years later the work was expanded to test the compounds identified in the bead screen against several members of the MST family[46]. In this case, the inhibitors were no longer attached to beads, and standard kinetic assays were used. When tested against ADC synthase, isochorismate synthase and anthranilate synthase, the compounds were still not effective, with K_i values ranging from high micromolar to low millimolar.

Anthranilate Synthase

Anthranilate synthase (AS) is the first enzyme in the tryptophan biosynthesis pathway, and is frequently called by the gene name TrpE. Like aminodeoxyisochorismate synthase, TrpE adds an amino group to C2 while removing the hydroxyl group at C4. Like the salicylate synthases, TrpE is lyase active and also removes the pyruvyl tail at C3 (Key Figure E, Figure 5A). The ammonia needed for nucleophilic attack is generated by the amidotransferase TrpG. The earliest crystal structures of the TrpE/G complex were solved from *S. marcescens*[5], *Sulfolobus solfataricus*[47], and *Salmonella typhimurium*[3]. More recently two structures have been solved with TrpE alone, one from *M. tuberculosis*[48] and one from *Streptococcus pneumoniae* (PDB:5KCK) which is unaffiliated with a publication. In most cases, TrpE and TrpG are expressed as separate polypeptides; however, a recent report described a fused TrpE/G monomer from *Streptomyces venezuelae* much like the fused PabA/B arrangement described above[49]. In this review descriptions of TrpE/G will be limited to the canonical two polypeptide form. Note that the orientation of the MST and amidotransferase domains in the PhzE (aminodeoxyisochorismate synthase, Figure 3) and TrpE/G (anthranilate synthase Figure 5) are consistent, suggesting common modes for delivery of the ammonia nucleophile.

It was known that TrpE was allosterically regulated by tryptophan binding before any structures of the enzyme were solved[50]. This was confirmed when Spraggen *et al* solved two structures of the *S. marcescens* TrpE/G complex: one bound to tryptophan in the allosteric site and the other bound to benzoic acid and magnesium in the active site (Figure 5B)[5]. When tryptophan is bound a previously ordered loop becomes disordered and allows adjacent structural elements to shift 7 Å away from their normal position, far enough that catalytic and magnesium-binding residues in the active site are no longer in position to coordinate the metal and/or substrate (Figure 5C)[5]. This conformational change is also communicated to TrpG, where disorder in the active site affects the catalytic machinery, inhibiting ammonia formation.

As with isochorismate synthase and salicylate synthase, the major difference between ADIC synthase and anthranilate synthase is that the latter is lyase active (able to remove the pyruvylenol tail) while the former is not. As with the isochorismate and salicylate synthases, how to convert a lyase inactive enzyme into a lyase active one (and vice versa) is an ongoing source of curiosity for researchers in the field. The group that solved the *B. latta* ADIC synthase structure also attempted to use active site mutations to convert ADIC synthase into a lyase active enzyme[36]. However, these attempts were unsuccessful as the mutations either reduced or obliterated the enzyme's native function without conferring lyase function[36]. However, Culbertson *et al* recently utilized the Janus bioinformatics approach to predict which residues must be altered to convert enzyme function and have met with some success[51]. They were able to generate ADIC synthase constructs containing 7–11 simultaneous mutations that showed some anthranilate synthase activity, but the turnover rate was dramatically less than wild type anthranilate synthase (~1000-fold less in k_{cat}). The authors propose that the chemical nature of the sidechains in the active site (for example, location of polar or charged residues) does not determine whether the enzyme is lyase competent or not, because the mutants required to generate lyase activity do not necessarily contact the substrate molecule. Instead, they suggest that the variant residues produce an active site pocket shape that forces the substrate into a conformation suitable to confer lyase activity. While this sounds very much like an argument in favor of the pericyclic reaction mechanism for lyase activity, the authors conclude that the mechanism is a synergy of both substrate conformational change and also general acid-general base, based on molecular dynamics experiments that indicated the C2 hydrogen is presented to a lysine general base (the same for the isomerase activity) for deprotonation (Figure 5A).

Recently, the *M. tuberculosis* TrpE was co-crystallized with a carboxyethelenyloxy-2-hydroxybenzoic acid/methyl-AMT designed for the salicylate synthase MbtI (described above)[48]. As with the salicylate synthase, methyl-AMT was bound in the reverse orientation in the active site, in the absence of magnesium, and caused significant disruption to active site residues[31, 48]. These inhibitors (including methyl-AMT) were tested against the *M. tuberculosis* TrpE and were found to have IC₅₀ values on the same order of magnitude as the K_i values reported for salicylate synthase, indicating this series of compounds is equally ineffective in all MST enzymes.

Concluding Remarks

The MST enzymes all perform a variation of the readily reversible isomerization reaction of the isochorismate synthases, with slight variations to account for the nucleophile: water or ammonia. In four out of the five cases, the nucleophile attacks at C2, allowing elimination of the hydroxyl at C4. In the fifth case (aminodeoxychorismate synthase), this may be the first half reaction, and subsequently the reaction runs in reverse to generate the product with ammonia at C4. We hypothesize that the recently defined ordered sequential kinetic mechanism of the isochorismate and salicylate synthases is conserved in all five MST enzymes. Similarly, the lyase activity of the salicylate and anthranilate synthases will likely be found to be the result of a conserved mechanism; however, how this chemistry is performed is not well-determined to date. Indeed, the structural features that differentiate the lyase deficient from the lyase active MST enzyme remain unresolved.

MST enzymes are an attractive antibiotic target because they produce essential precursors that pathogens require. These are pathways that do not exist in humans, so the potential for side effects is minimal. For these reasons, the MST enzymes have been the subject of a considerable scientific study over the last 30 years. In a recent review, Holdgate *et al* have reiterated the necessity for rigorous mechanistic detail prior to development of enzyme inhibitors as potential drugs[52]. Indeed, they argue that determining steady state kinetic parameters is one of the first steps in enzyme inhibitor development, and argue that understanding the transition state structure accelerates drug design. We have presented the progress toward determining the chemical and kinetic mechanisms, but there is still much work to do (see Outstanding Questions). The lack of success in the development of inhibitors to these enzymes highlights the need for mechanistic approaches prior to the onset of inhibitor development.

Outstanding Questions

The past three decades have uncovered exciting and important information about the MST enzymes, however, several important questions remain.

1. What is the mechanism for lyase activity?
2. What features allow some of the MST enzymes to be lyase competent while others are not?
3. What is the interplay between the catalytic magnesium and the reverse protonation states of the general acid and base residues in the active site?
4. What are the experimentally determined transition state structures for each of the MST enzymes?

Acknowledgments

This publication was made possible by funds from National Science Foundation (CHE-1403293). C.L.S. was supported by the National Institutes of Health Institutional Research and Academic Career Development Award (IRACDA) Postdoctoral Program (K12 GM63651). Special thanks to Jeffrey S. McFarlane and Trey Ronnebaum for critically reading the manuscript.

References

1. Bentley R. The shikimate pathway--a metabolic tree with many branches. *Crit Rev Biochem Mol Biol.* 1990; 25(5):307–84. [PubMed: 2279393]
2. Kolappan S, et al. Lysine 190 is the catalytic base in MenF, the menaquinone-specific isochorismate synthase from *Escherichia coli*: implications for an enzyme family. *Biochemistry.* 2007; 46(4):946–53. [PubMed: 17240978]
3. Morollo AA, Eck MJ. Structure of the cooperative allosteric anthranilate synthase from *Salmonella typhimurium*. *Nat Struct Biol.* 2001; 8(3):243–7. [PubMed: 11224570]
4. Knochel T, et al. The crystal structure of anthranilate synthase from *Sulfolobus solfataricus*: functional implications. *Proc Natl Acad Sci USA.* 1999; 96(17):9479–84. [PubMed: 10449718]
5. Spraggon G, et al. The structures of anthranilate synthase of *Serratia marcescens* crystallized in the presence of (i) its substrates, chorismate and glutamine, and a product, glutamate, and (ii) its end-product inhibitor, L-tryptophan. *Proc Natl Acad Sci USA.* 2001; 98(11):6021–6. [PubMed: 11371633]
6. Parsons JF, et al. Structure of *Escherichia coli* Aminodeoxychorismate Synthase: Architectural Conservation and Diversity in Chorismate-Utilizing Enzymes. *Biochemistry.* 2002; 41:2198–2208. [PubMed: 11841211]
7. Zwahlen J, et al. Structure and mechanism of MbtI, the salicylate synthase from *Mycobacterium tuberculosis*. *Biochemistry.* 2007; 46(4):954–64. [PubMed: 17240979]
8. Parsons JF, et al. Structure of isochorismate synthase in complex with magnesium. *Acta Crystallogr.* 2008; D64(Pt 5):607–10.
9. Sridharan S, et al. Crystal structure of *Escherichia coli* enterobactin-specific isochorismate synthase (EntC) bound to its reaction product isochorismate: implications for the enzyme mechanism and differential activity of chorismate-utilizing enzymes. *J Mol Biol.* 2010; 397(1):290–300. [PubMed: 20079748]
10. Gaille C, et al. Isochorismate synthase (PchA), the first and rate-limiting enzyme in salicylate biosynthesis of *Pseudomonas aeruginosa*. *J Biol Chem.* 2003; 278(19):16893–8. [PubMed: 12624097]
11. Walsh CT, et al. Molecular studies on enzymes in chorismate metabolism and the enterobactin biosynthetic pathway. *Chem Rev.* 1990; 90:1105–1129.
12. Kerbarh O, et al. Crystal structures of *Yersinia enterocolitica* salicylate synthase and its complex with the reaction products salicylate and pyruvate. *J Mol Biol.* 2006; 357(2):524–34. [PubMed: 16434053]
13. Meneely KM, et al. Lysine221 is the general base residue of the isochorismate synthase from *Pseudomonas aeruginosa* (PchA) in a reaction that is diffusion limited. *Arch Biochem Biophys.* 2013; 538(1):49–56. [PubMed: 23942051]
14. He Z, et al. Conservation of mechanism in three chorismate-utilizing enzymes. *J Am Chem Soc.* 2004; 126(8):2378–85. [PubMed: 14982443]
15. He Z, MDT. Direct Detection and Kinetic Analysis of Covalent Intermediate Formation in the 4-Amino-4-deoxychorismate Synthase Catalyzed Reaction. *Biochemistry.* 2006; 45:5019–5028. [PubMed: 16605270]
16. Ziebart KT, Toney MD. Nucleophile specificity in anthranilate synthase, aminodeoxychorismate synthase, isochorismate synthase, and salicylate synthase. *Biochemistry.* 2010; 49(13):2851–9. [PubMed: 20170126]
17. Dinovo EC, Boyer PD. Isotopic Probes of the Enolase Reaction mechanism. *J Biol Chem.* 1971; 246(14)
18. Shen TY, Westhead EW. Divalent cation and pH dependent primary isotope effects in the enolase reaction. *Biochemistry.* 1973; 12(17):3333–7. [PubMed: 4581789]
19. Nowak T, et al. Nuclear relaxation and kinetic studies of the role of Mn²⁺ in the mechanism of enolase. *Biochemistry.* 1973; 12(9):1690–1701. [PubMed: 4572991]
20. Cleland WW. Determining the chemical mechanisms of enzyme-catalyzed reactions by kinetic studies. *Adv Enzymol Relat Areas Mol Biol.* 1977; 45:273–387. [PubMed: 21524]

21. Sims PA, et al. Reverse protonation is the key to general acid-base catalysis in enolase. *Biochemistry*. 2003; 42(27):8298–306. [PubMed: 12846578]
22. Meneely KM, et al. An Open and Shut Case: The Interaction of Magnesium with MST Enzymes. *J Am Chem Soc*. 2016; 138(29):9277–93. [PubMed: 27373320]
23. Daruwala R, et al. Menaquinone (vitamin K2) biosynthesis: overexpression, purification, and characterization of a new isochorismate synthase from *Escherichia coli*. *J Bacteriol*. 1997; 179(10):3133–8. [PubMed: 9150206]
24. Xie F, et al. A new salicylate synthase AmS is identified for siderophores biosynthesis in *Amycolatopsis methanolica* 239(T). *Appl Microbiol Biotechnol*. 2015; 99(14):5895–905. [PubMed: 25586582]
25. Kerbarh O, et al. Salicylate biosynthesis: overexpression, purification, and characterization of Irp9, a bifunctional salicylate synthase from *Yersinia enterocolitica*. *J Bacteriol*. 2005; 187(15):5061–6. [PubMed: 16030197]
26. Lamb AL. Pericyclic reactions catalyzed by chorismate-utilizing enzymes. *Biochemistry*. 2011; 50(35):7476–83. [PubMed: 21823653]
27. Meneely KM, et al. Redesign of MST enzymes to target lyase activity instead promotes mutase and dehydratase activities. *Arch Biochem Biophys*. 2013; 539(1):70–80. [PubMed: 24055536]
28. Organization, W.H. Global Tuberculosis Report 2017. 2017.
29. Vasan M, et al. Inhibitors of the salicylate synthase (MbtI) from *Mycobacterium tuberculosis* discovered by high-throughput screening. *ChemMedChem*. 2010; 5(12):2079–87. [PubMed: 21053346]
30. Meneely KM, et al. Expanding the results of a high throughput screen against an isochorismate-pyruvate lyase to enzymes of a similar scaffold or mechanism. *Bioorg Med Chem*. 2014; 22(21):5961–9. [PubMed: 25282647]
31. Manos-Turvey A, et al. Inhibition studies of *Mycobacterium tuberculosis* salicylate synthase (MbtI). *ChemMedChem*. 2010; 5(7):1067–79. [PubMed: 20512795]
32. Chi G, et al. Implications of binding mode and active site flexibility for inhibitor potency against the salicylate synthase from *Mycobacterium tuberculosis*. *Biochemistry*. 2012; 51(24):4868–79. [PubMed: 22607697]
33. Manos-Turvey A, et al. Synthesis and evaluation of *M. tuberculosis* salicylate synthase (MbtI) inhibitors designed to probe plasticity in the active site. *Org Biomol Chem*. 2012; 10(46):9223–36. [PubMed: 23108268]
34. Liu Z, et al. Stereocontrolled Synthesis of a Potential Transition-State Inhibitor of the Salicylate Synthase MbtI from *Mycobacterium tuberculosis*. *J Org Chem*. 2015; 80(13):6545–52. [PubMed: 26035083]
35. Zhang XK, et al. Synthesis of Transition-State Inhibitors of Chorismate Utilizing Enzymes from Bromobenzene cis-1,2-Dihydrodiol. *J Org Chem*. 2017; 82(7):3432–3440. [PubMed: 28282140]
36. Li QA, et al. Ligand binding induces an ammonia channel in 2-amino-2-desoxyisochorismate (ADIC) synthase PhzE. *J Biol Chem*. 2011; 286(20):18213–21. [PubMed: 21454481]
37. Mavrodi DV, et al. A seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2–79. *J Bacteriol*. 1998; 180(9):2541–8. [PubMed: 9573209]
38. Look DC, et al. Pyocyanin and Its Precursor Phenazine-1-Carboxylic Acid Increase IL-8 and Intercellular Adhesion Molecule-1 Expression in Human Airway Epithelial Cells by Oxidant-Dependent Mechanisms. *The Journal of Immunology*. 2005; 175(6):4017–4023. [PubMed: 16148150]
39. Zalkin H, Smith JL. Enzymes utilizing glutamine as an amide donor. *Advances in Enzymology*. 1998; 72:87.
40. Culbertson JE, Toney MD. Expression and characterization of PhzE from *P. aeruginosa* PAO1: aminodeoxyisochorismate synthase involved in pyocyanin and phenazine-1-carboxylate production. *Biochim Biophys Acta*. 2013; 1834(1):240–6. [PubMed: 23099261]
41. Schadt HS, et al. 2-Amino-2deoxyisochorismate is a key intermediate in *Bacillus subtilis* p-Aminobenzoic Acid Biosynthesis. *JACS Communications*. 2009; 131:3481–3483.
42. Bera AK, et al. Structure of aminodeoxychorismate synthase from *Stenotrophomonas maltophilia*. *Biochemistry*. 2012; 51(51):10208–17. [PubMed: 23230967]

43. Kane JF, et al. Metabolic interlock. The dual function of a folate pathway gene as an extra-operonic gene of tryptophan biosynthesis. *J Biol Chem.* 1972; 247(5):1587–96. [PubMed: 4622231]
44. Lam KS, et al. The “One-Bead-ON3-Compound” Combinatorial Library Method. *Chem Rev.* 1997; 97:411–448. [PubMed: 11848877]
45. Dixon S, et al. Aminodeoxychorismate synthase inhibitors from one-bead one-compound combinatorial libraries: “staged”s inhibitor design. *J Med Chem.* 2006; 49(25):7413–26. [PubMed: 17149871]
46. Ziebart KT, et al. Targeting multiple chorismate-utilizing enzymes with a single inhibitor: validation of a three-stage design. *J Med Chem.* 2010; 53(9):3718–29. [PubMed: 20359225]
47. Knochel T, et al. The Crystal Structure of Anthranilate Synthase from *Sulfolobus solfataricus*: Functional Implications. *Proceedings of the National Academy of Sciences.* 1999; 97(17):9479–9484.
48. Bashiri G, et al. Structure and inhibition of subunit I of the anthranilate synthase complex of *Mycobacterium tuberculosis* and expression of the active complex. *Acta Crystallogr D Biol Crystallogr.* 2015; 71(Pt 11):2297–308. [PubMed: 26527146]
49. Ashenafi M, et al. The fused anthranilate synthase from *Streptomyces venezuelae* functions as a monomer. *Mol Cell Biochem.* 2015; 400(1–2):9–15. [PubMed: 25355158]
50. Caligiuri MG, Bauerle R. Identification of amino acid residues involved in feedback regulation of the anthranilate synthase complex from *Salmonella typhimurium*. Evidence for an amino-terminal regulatory site. *J Biol Chem.* 1991; 266(13):8328–35. [PubMed: 2022650]
51. Culbertson JE, et al. Conversion of aminodeoxychorismate synthase into anthranilate synthase with Janus mutations: mechanism of pyruvate elimination catalyzed by chorismate enzymes. *Biochemistry.* 2015; 54(14):2372–84. [PubMed: 25710100]
52. Holdgate GA, et al. Mechanistic enzymology in drug discovery: a fresh perspective. *Nat Rev Drug Discov.* 2017
53. Dundas J, et al. CASTp: computed atlas of surface topography of proteins with structural and topographical mapping of functionally annotated residues. *Nucleic Acids Res.* 2006; 34(Web Server issue):W116–8. [PubMed: 16844972]

Highlights

- The catalytic mechanisms used for chorismate transformation, isomerization and lyase activities, are conserved within the family.
- Recent findings define the kinetic mechanism for two MST enzymes, providing a specific role for the catalytic magnesium, which will likely be evidenced in all family members.
- While MST enzymes are an excellent antibiotic target, development of inhibitors has stalled because transition state structures have not been determined for each enzyme.

Figure 1. Isochorismate Synthase

A. Isochorismate synthase EntC (PDB:5JXZ) was crystallized with the substrate chorismate. The enzyme was active in the crystals, establishing an equilibrium mixture of chorismate (cyan) and isochorismate (magenta) in the active site with the catalytically required magnesium (orange). **B.** Stereo view of magnesium (orange) positioned at the C1 carboxylate of chorismate/isochorismate and the general base K147 and the general acid E197 (yellow). In MenF these residues are K190/E240 and in PchA they are K221/E269. A water molecule (blue) is positioned directly between K147 and C2 where it would be expected to perform a nucleophilic attack. **C.** Schematic representation of the general acid – general base mechanism utilized in the isochorismate synthase reaction.

Figure 2. Salicylate Synthase

A. Salicylate synthase Irp9 (PDB: 2FN1) from *Yersinia enterocolitica*. Active site products salicylate and pyruvate are shown in magenta and magnesium is shown in orange. (**B–D**) Irp9 (dark blue) is overlaid with MbtI (light blue). MbtI is the salicylate synthase from *M. tuberculosis*, for which inhibitor-bound structures, but not substrate/product/Mg²⁺-bound structures have been determined. These stereo images compare the binding pose of the products salicylate and pyruvate (magenta) and magnesium (magenta) in the Irp9 active site with inhibitors (yellow) in the MbtI active site. **B.** The isomerization step of salicylate synthase proceeds using a general acid – general base mechanism. There is active debate as to whether the lyase step proceeds via a pericyclic or a general acid – general base mechanism. **C.** Irp9 active site overlaid with the MbtI active site containing the AMT inhibitor (yellow, PDB: 3ST6) in very similar orientation to the salicylate and pyruvate seen in Irp9. **C.** Irp9 active site overlaid with the MbtI-bound methyl-AMT inhibitor (yellow, PDB: 3VEH). The methyl-AMT inhibitor is rotated 180° both in the horizontal and vertical axis compared to the salicylate and pyruvate in the Irp9 active site. **D.** Irp9 active site overlaid with the MbtI-bound phenyl-AMT (yellow, PDB: 3RV6). This is the only published structure of MbtI with magnesium in the active site (yellow sphere). However, like the methyl-AMT in the panel above, the carboxylate on the pyruvylenol tail is positioned to coordinate magnesium rather than the carboxylate at the C1 position of the ring.

Figure 3. Aminodeoxyisochorismate synthase

A. The aminodeoxyisochorismate synthase reaction utilizes ammonia for the nucleophilic attack but otherwise is quite similar to the isochorismate synthase reaction. **B.** The aminodeoxyisochorismate synthase PhzE (PDB: 3R76) from *B. lata* 383 with benzoic acid and pyruvate (cyan) in the active site of the MST domain (red). In the amidotransferase domain (grey), an active site cysteine (C526) was modified with a glutamyl moiety (purple) demonstrating the location of the amidotransferase active site. **B.** A putative 25 Å channel for transmission of ammonia from the amidotransferase to the MST domain was hypothesized in the original *B. lata* structure. However, the gatekeeper residues hold the channel closed in the determined crystal structures. The two active sites cavities are shown with the purple transparent surface. The proposed channel would connect them. Surface calculated using CASTp [53].

Figure 4. Aminodeoxychorismate synthase

A. The aminodeoxychorismate synthase requires two steps in which there is a nucleophilic attack by an amine first at C2 eliminating the hydroxyl at C4, and then at C4 eliminating the amine at C2. In this way, the hydroxyl group at C4 is replaced with an amine. **B.** Stereoview of aminodeoxychorismate synthase PabB structure (1K0E) showing formate (right side, magenta) in the active site and a non-regulatory tryptophan (left side, magenta).

Figure 5. Anthranilate synthase

A. The mechanism for anthranilate synthase is very similar to salicylate synthase with the exception that ammonia, rather than activated water, is utilized for the initiating nucleophilic attack. **B.** TrpE/G from *S. marcescens* in the active form (gold, PDB: 1I7Q) with benzoic acid and pyruvate (cyan) in the TrpE active site with magnesium (orange). The fully built loop (residues 41-49) in the TrpE tryptophan allosteric site is shown in cyan (left). The same loop is disordered in the tryptophan-bound structure (right) and is shown in purple. The amidotransferase TrpG (grey) contains the product glutamate (purple) in the active site. TrpE/G from *S. marcescens* in the allosterically inhibited form (salmon, PDB: 1I7S) with

tryptophan (purple) bound in the allosteric site and disruption of the loop in the allosteric site. **C.** Overlay of the active (gold) and inhibited (salmon) forms of TrpE/G from *S. marcescens*. The disorder induced in loop 41-49 upon binding of tryptophan in the allosteric site is accompanied by a shift of two helices away from the TrpE active site.

Key Figure. MST Enzymes

Chorismate-utilizing enzymes (CUEs) include the five MST enzymes that share similar structural features and mechanisms. **A–B.** Isochorismate synthase and salicylate synthase use water as a nucleophile. **C–E.** Aminodeoxychorismate synthase, aminodeoxyisochorismate synthase, and anthranilate synthase use ammonia as a nucleophile. **F.** The two non-MST CUEs are structurally dissimilar and utilize a different mechanism than the MST enzymes.