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# Allosteric inhibitor specificity of *Thermotoga maritima* 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase

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ABSTRACT

**3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAH7PS) catalyses the first step of the shikimate pathway for the biosynthesis of aromatic amino acids. Allosteric regulation of *Thermotoga maritima* DAH7PS is mediated by L-Tyr binding to a discrete ACT regulatory domain appended to a core catalytic $(\beta/\alpha)_8$ barrel. Variants of *T. maritima* DAH7PS (*Tma*DAH7PS) were created to probe the role of key residues in inhibitor selection. Substitution Ser31Gly severely reduced inhibition by L-Tyr. In contrast both L-Tyr and L-Phe inhibited the *Tma*His29Ala variant, while the variant where Ser31 and His29 were interchanged (His29Ser/Ser31His), was inhibited to a greater extent by L-Phe than L-Tyr. These studies highlight the role and importance of His29 and Ser31 for determining both inhibitory ligand selectivity and the potency of allosteric response by *Tma*DAH7PS.**
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## 1. Introduction

Aromatic metabolites are biosynthesised in plants and microorganisms by way of the shikimate pathway [1]. This pathway comprises a series of seven enzyme-catalysed reactions resulting in the formation of chorismate. Chorismate is the precursor of many important aromatic metabolites including the folates, quinones and the aromatic amino acids, L-Phe, L-Tyr and L-Trp. The enzyme, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAH7PS), catalyses the first committed step of the pathway. The regulation of entry into the pathway by controlling the catalytic activity of DAH7PS is an important mechanism for determining cellular levels of the aromatic amino acids (Fig. 1).

One of the striking differences between DAH7PSs from different sources is the variation in allosteric mechanisms and associated allosteric machinery [2]. Whereas all known DAH7PS proteins share the same core catalytic $(\beta/\alpha)_8$ barrel, this barrel is appended with different extensions for the purpose of allosteric control by pathway intermediates or by the aromatic amino acids. Type Iα enzymes from *Escherichia coli*, *Saccharomyces cerevisiae*, and *Neisseria meningitidis* have N-terminal and loop extensions that create a single ligand binding site for one of the aromatic amino acids [3–6]. The type II enzyme from *Mycobacterium tuberculosis* has three ligand binding sites, which operate synergistically to provide an allosteric response [7–9]. The recently structurally characterised type Iβ DAH7PS from *Listeria monocytogenes* has a fused chorismate mutase domain, which is thought to provide a binding site for the regulation of DAH7PS activity by chorismate or prephenate [10]. In contrast to these examples, for which the allosteric ligand binding site is pre-formed by peptide or domain insertions to the barrel, allosteric inhibition of the type Iβ DAH7PS from *Thermotoga maritima* (*Tma*DAH7PS) is mediated by a discrete N-terminal ACT-like domain [11–13].

The ACT domain, named after the first three proteins in which the domain was recognised (aspartate kinase, chorismate mutase and TyrA), is a βαββαβ structural motif involved in the regulation of amino acid metabolism [14–16]. *Tma*DAH7PS is allosterically inhibited by L-Tyr, and while L-Phe does inhibit the enzyme, it is considerably less potent an inhibitor [13]. The structural and functional characterisation of a truncated variant lacking the N-terminal ACT domain, and a L-Tyr-bound structure of *Tma*DAH7PS have shown that this domain is essential for allosteric regulation [12]. A significant change in the average conformation of the protein is observed upon ligand binding whereby L-Tyr is bound in a cleft formed between two diagonally opposing N-terminal ACT domains of the tetrameric protein, and contributions from

*Abbreviations:* BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; DAH7P, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DAH7PS, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase; E4P, D-erythrose 4-phosphate; PDB, protein data bank; PEG, polyethylene glycol; SAXS, small angle X-ray scattering

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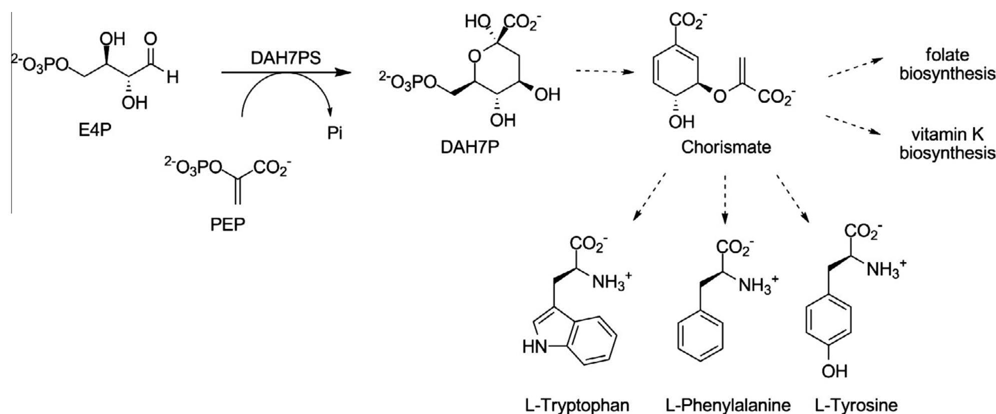


**Fig. 1.** The shikimate pathway detailing the first reaction, which is catalysed by DAH7PS. The pathway branches at chorismate to form a number of important aromatic and pre-aromatic products.

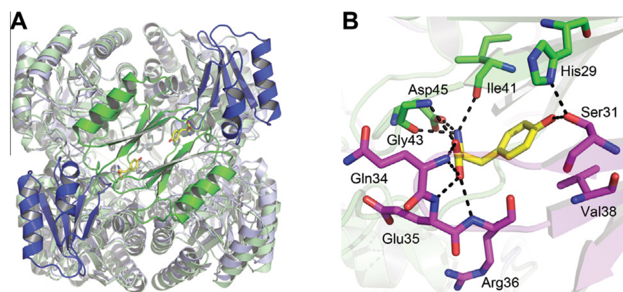


**Fig. 2.** (A) Comparison of the position of the regulatory ACT domain of *Tma*DAH7PS in the unbound (blue) and L-Tyr-bound (green) conformations. (B) The L-Tyr (yellow sticks) binding site with residues contributed from ACT domains from opposing monomers represented as either magenta or green sticks. Hydrogen bonding interactions are shown by black dashes.

the regulatory domains of the two opposing monomers contribute directly to the interactions with L-Tyr (Fig. 2). Placement of the regulatory domains in this way physically blocks substrate entry into the active site and alters the conformation of the active site loops, impeding the catalytic activity of the enzyme.

Herein we describe the characterisation of three variants of *Tma*DAH7PS in which substitutions were made to key residues of the ACT-like domain located in the Tyr-binding site. These studies demonstrate the importance of these residues in both the selectivity for L-Tyr as allosteric effector of this enzyme, and for controlling the sensitivity of the enzyme towards inhibitors.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, media, growth conditions

Site directed mutagenesis was performed using the QuikChange® Lightning site-directed mutagenesis kit (Stratagene), and pT7-7 *Tma*DAH7PS as the double stranded plasmid template to create the His29Ala (*Tma*H29A), Ser31Gly (*Tma*S31G) and His29-Ser/Ser31His (*Tma*H29S/S31H) variations of *Tma*DAH7PS. Mutagenic primers TmaDH29AFor 5′-GAGAGTTACAACTTGAAGTGT**GCC**ATTTCCAAAGGTCAGGAAAG and TmaDH29ARev 5′-CTTTCCTGACCTTTGGAAAT**GGC**ACACTTCAAGTTGTAACTCTC were used to convert histidine to alanine, TmaDS31GFor 5’-ACAACTTGAAGTGTCACATT**GGC**AAAGGTCAGGAAAGAACGG and TmaDS31GRev 5′-CCGTTCTTTCCTGACCTTT**GCC**AAAGGTCAGGAAAGAACGG for serine to glycine conversion and TmaDH29S/S31HFor 5′-GGCCGAGAGTTACAACTTGAAGTGT**AGC**ATT**CAC**AAAGGTCAGGAAAGAACGGTTATT and TmaDH29S/S31HRev 5′-AATAACCGTTCTTTCCTGACCTTT**GTG**AAT**GCT**ACACTTCAAGTTGTAACTCTCGGCC (mutations shown in bold) to interchange the serine and histidine residues. The sequences of the variants were verified, after which the vectors were transformed into *E. coli* (DE3) Star cells. Cultures were grown, overexpressed and purified as previously described for wild type *Tma*DAH7PS [12].

### 2.2. Determination of molecular mass

The masses of the purified proteins (diluted to 1 mg ml$^{-1}$ using milliQ water) were measured by electrospray ionisation using a Bruker maXis™ 3G (Bruker Daltonics).

### 2.3. Michaelis Menten kinetics

Enzyme activity was monitored and kinetic parameters determined as previously described [12]. Assays used to determine the kinetic parameters of all variants of *Tma*DAH7PS contained 100 μM $MnSO_4$, 2 μl of 0.5–1.2 mg ml$^{-1}$ enzyme in 50 mM BTP, pH 7.3 buffer. To determine the $K_m^{E4P}$, the PEP concentration was fixed at 80 μM (except for *Tma*H29S/S31H for which PEP was fixed at 200 μM), while the E4P concentration was varied (11–220 μM). For determination $K_m^{PEP}$, E4P concentration was fixed at 160 μM while the PEP concentration was varied (10–200 μM).

### 2.4. Response to inhibitors

The assay mixtures to determine the effect of L-Tyr and L-Phe on the activity of the variants contained PEP (120–200 μM, 5–9 × $K_m$), E4P (200–220 μM, 7–8 × $K_m$) and $MnSO_4$ (100 μM) in 50 mM BTP, pH 7.3. Reaction mixtures have either no amino acids or 0–1 mM L-Tyr or L-Phe. Triplicate assays were performed for all reaction conditions.

### 2.5. Small angle X-ray (SAXS) scattering data collection

Measurements were performed at the Australian Synchrotron SAXS/WAXS beamline with a Pilatus detector (1 M, 170 × 170 mm effective pixel size, 172 × 172 μm). The wavelength of the X-rays was 1.0332 Å. The sample detector distance was 1576 mm, which provided a $q$ range of 0.0126–0.400 Å$^{-1}$ (where $q$ is the magnitude of the scattering vector, which is related to the scattering angle ($2\theta$) and the wavelength ($\lambda$) as follows: $q = (4\pi/\lambda)\sin\theta$. Data were collected from a 1.5 mm glass capillary at 27 °C at 2 s intervals. Scattering data were collected from the *Tma*DAH7PS variants (2 mg ml$^{-1}$) with or without L-Phe or L-Tyr (1 mM).

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