Synergistic Allostery, a Sophisticated Regulatory Network for the Control of Aromatic Amino Acid Biosynthesis in Mycobacterium tuberculosis*

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The shikimate pathway, responsible for aromatic amino acid biosynthesis, is required for the growth of Mycobacterium tuberculosis and is a potential drug target. The first reaction is catalyzed by 3-deoxy-D-arabinose-7-phosphate synthase (DAH7PS). Feedback regulation of DAH7PS activity by aromatic amino acids controls shikimate pathway flux. Whereas Mycobacterium tuberculosis DAH7PS (MtDAH7PS) is not inhibited by the addition of Phe, Tyr, or Trp alone, combinations cause significant loss of enzyme activity. In the presence of 200 μM Phe, only 2.4 μM Trp is required to reduce enzymic activity to 50%. Reaction kinetics were analyzed in the presence of inhibitory concentrations of Trp/Phe or Trp/Tyr. In the absence of inhibitors, the enzyme follows Michaelis-Menten kinetics with respect to substrate erythrose 4-phosphate (E4P), with Hill coefficients of 3.3 (Trp/Phe) and 2.8 (Trp/Tyr). Structures of MtuDAH7PS/Trp, MtuDAH7PS/Tyr, and MtuDAH7PS/Phe complexes were determined. The Phe homotetramer binds four Trp and six Phe molecules. Binding sites for both aromatic amino acids are formed by accessory elements to the core DAH7PS (β6α8) barrel that are unique to the type II DAH7PS family and contribute to the tight dimer and tetramer interfaces. A comparison of the liganded and unliganded MtuDAH7PS structures reveals changes in the interface areas associated with inhibitor binding and a small displacement of the E4P binding loop. These studies uncover a previously unrecognized mode of control for the branched pathways of aromatic amino acid biosynthesis involving synergistic inhibition by specific pairs of pathway end products.

The shikimate pathway is the biosynthetic route that is responsible for the production of essential aromatic compounds (1). These include the aromatic amino acids tryptophan, tyrosine, and phenylalanine, folic acid, an essential cofactor for many enzymatic processes, and salicylate, used for the biosynthesis of the siderophores through which bacteria acquire iron (2). The pathway is found in microorganisms and plants and has more recently been discovered in apicomplexan parasites (3, 4). The pathway is absent in higher organisms, making the enzymes of this pathway attractive as targets for the development of antimicrobial agents. Recent gene disruption studies have shown that operation of the shikimate pathway is essential for the viability of Mycobacterium tuberculosis (5), the causative agent of tuberculosis, a disease that remains a significant world-wide health risk (6). Although effective anti-tuberculosis drugs exist, the long treatment times required, the problems of latent or persistent tuberculosis (7), and the proliferation of multidrug-resistant strains of M. tuberculosis (8) have all created an urgent need for the development of new antimycobacterial agents.

The first committed step in the shikimate pathway is the stereospecific aldol reaction between phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to produce 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAH7P), catalyzed by the enzyme DAH7P synthase (Fig. 1). DAH7P is converted into chorismate, the product of the main shikimate pathway, via six further enzyme-catalyzed reactions. At this point the pathway to the aromatic amino acids branches, with chorismate converted either to anthranilate by anthranilate synthase or to prephenate by chorismate mutase. Anthranilate ultimately produces Trp, whereas prephenate is converted into Phe and Tyr.

As the first enzyme, DAH7P synthase, is a major control point for shikimate pathway flux. Several organisms express two or more isozymes of this enzyme that show different sensitivity to the pathway end products. Escherichia coli and Neurospora crassa each produce three isozymes, with each enzyme individually inhibited by either Phe, Tyr, or Trp (9, 10). For Saccharomyces cerevisiae there are two differentially regulated DAH7P synthases sensitive to either Phe or Tyr (11).

4 The abbreviations used are: PEP, phosphoenolpyruvate; Bistris, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; DAH7PS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; MtuDAH7PS, M. tuberculosis DAH7PS; E4P, o-erythrose 4-phosphate.

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DAH7P synthases have been classified into two distinct types (12). Type I enzymes have molecular masses less than 40 kDa, and this group can be further divided into two sequence subfamilies: Iα and Iβ (13, 14). The type Iα and Iβ DAH7P synthases have been well characterized both structurally and functionally. The crystal structures of the type Iα enzymes from E. coli (15–17) and S. cerevisiae (11, 18) and the type Iβ enzymes from Thermotoga maritima (19) and Pyrococcus furiosus (20) reveal a common (β/α)₅ triose phosphate isomerase barrel fold. Both the E. coli (Phe-sensitive) and S. cerevisiae (Phe- and Tyr-sensitive) enzymes have additions to their core barrels that have been shown to be associated with the binding of a single regulatory aromatic amino acid (11, 21, 22). Regulated type I enzymes show one addition to the core barrel that is associated with allosteric regulation. This covalently attached regulatory domain appears to be either ferredoxin-like (characterized by the enzyme from T. maritima) (19) or a functional chorismate mutase fused either to the N or C terminus (DAH7P synthases from Thermotoga maritima, Mycobacterium tuberculosis, and several Mycobacterium species) supports the role of type II DAH7P synthases enzymes in aromatic amino acid biosynthesis. Type II enzymes are larger (>50 kDa) than their type I counterparts, and the two types share very low sequence identity (<10%). However, the core structure and catalytic machinery of MtuDAH7P synthase is remarkably similar to those of the type I enzymes, revealing that despite their low sequence similarity these two DAH7P synthase types are evolutionarily related (24). Along with a distinct quaternary structure, MtuDAH7P displays a distinctly different decoration of the core catalytic barrel to the type I enzymes, with two additional independent subdomains that were predicted to be linked to allosteric regulation of this enzyme.

Recently it has been shown that MtuDAH7P plays a role in activating and regulating catalysis by the branch point enzyme chorismate mutase (30). MtuDAH7P forms an association with the M. tuberculosis chorismate mutase, which substantially enhances the rate of the conversion of chorismate to prephenate. This enhanced activity is inhibited by a combination of the products for this branch of the pathway, Tyr and Phe.

Here we report several structures of MtuDAH7P in complex with aromatic amino acids and analyze the unusual synergistic inhibition of MtuDAH7P, which results only from specific combinations of aromatic amino acids. This study illuminates a complex regulatory mechanism in M. tuberculosis for both this enzyme and aromatic amino acid biosynthesis, exquisitely tuned to support differential rates of production of the multiple pathway end products.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—DAH7PS from M. tuberculosis (MtuDAH7PS) was cloned, overexpressed, and purified as described previously (31), except that Thesit (polyoxyethylene(9)-lauryl ether) was omitted during cell lysis for enzymes used for kinetic analyzes and all lysis and purification buffers included 1 mM tris(2-carboxyethyl)phosphine).**

**Enzyme Assays and Kinetic Measurements**—The activity MtuDAH7PS was determined as previously reported (31). The reaction mixtures to contained E4P (fixed at 300 μM or varied to determine E4P catalytic rate) (Sigma), MnSO₄ (100 μM) (Sigma), and PEP (fixed at 300 μM or varied to determine PEP catalytic rate) (Research Chemicals) in assay buffer (50 mM Bis-tris, pH 7.5, and 1 mM tris(2-carboxyethyl)phosphine)) treated with Chelex (Bio-Rad). PEP and E4P solutions were made up in assay buffer, and the MnSO₄ solution was made up in ultrapure water that had been pretreated with Chelex. The reaction was initiated by the addition of purified MtuDAH7PS (2 μl, 2.2 mg/ml). Initial reaction rates were determined by a least squares fit of the initial-rate data. One unit of activity is defined
Inhibition of M. tuberculosis DAH7PS

**TABLE 1**

Data collection and refinement statistics for Mtu-DAH7PS in complex with Trp, Phe, Trp, and Phe, and unliganded MtuDAH7PS without Thesit bound

| Structures | Trp-only | Phe-only | Trp+Phe | Thesit-free |
|------------|----------|----------|---------|-------------|
| Space group | P₃ᵥ2₁ | P₃ᵥ2₁ | P₃ᵥ2₁ | P₃ᵥ2₁ |
| Unit cell dimensions (Å) | | | | |
| a = b | 204.71 | 204.65 | 204.54 | 203.54 |
| c | 66.26 | 66.61 | 66.46 | 66.42 |
| Data collection resolution range (outer shell) (Å) | 50.0–2.5 (2.59–2.5) | 39.6–3.0 (3.08–3.0) | 39.5–2.0 (2.07–2.0) | 39.4–2.25 (2.33–2.25) |
| Wavelength (Å) | 0.97929 | 1.542 | 1.542 | 1.542 |
| No. measured reflections | 52,344 | 31,363 | 105,425 | 73,037 |
| No. unique reflections | 52,344 | 31,363 | 10,487 | 7,137 |
| Completeness (%) | 100 (100) | 97.2 (97.2) | 98.2 (87.2) | 97.2 (95.4) |
| Refinement resolution range (Å) | 38.7–2.5 (2.57–2.5) | 39.6–3.0 (3.08–3.0) | 32.7–2.0 (2.06–2.0) | 39.37–2.25 (2.305–2.247) |
| Number of reflections used in refinement (test set) | 52,324 (2,815) | 29,566 (1,574) | 105,110 (2,245) | 69,533 (3,648) |
| R factor | 0.189 | 0.212 | 0.163 | 0.180 |
| Rfre | 0.228 | 0.269 | 0.191 | 0.208 |

**Number of non-hydrogen atoms**

| | Protein atoms | Solvent atoms | Ligand atoms | Mean B value (Å²) |
| | 6871 | 297 | 121 | 28.2 |
| | 6756 | 10 | 85 | 32.7 |
| | 6976 | | 191 | 25.42 |
| | 7098 | | 69 | 36.9 |

**Root mean square deviations from ideality**

| | Bond lengths (Å) | Bond angles (degrees) | Residues in the most favored region of Ramachandran plot (%) |
| | 0.018 | 1.67 | 95.6 |
| | 0.012 | 1.160 | 92.1 |
| | 0.012 | 1.318 | 97.6 |
| | 0.008 | | 95.1 |

as the loss of 1 μmol of PEP/min at 30 °C. K_m and k_cat values were determined by fitting the data to the Michaelis–Menten equation using the program Kaleidagraph (Synergy).

**Feedback Inhibition Studies**—Solutions of L-Phe (Sigma), L-Tyr (Sigma), and L-Trp (Sigma) in ultrapure water pretreated with Chelex were added to standard assay reaction mixtures to give a concentration range of 0–200 μM in inhibitor studies. The assay solution contained PEP (150 μM), E4P (150 μM), and MnSO_4 (100 μM) in assay buffer. The reaction was initiated by the addition of MtuDAH7PS (2 μL, 2.16 mg/ml). Additional assays were performed using combinations of aromatic amino acids by fixing Trp, Phe, or Tyr at 200 μM and varying one of the alternate two amino acids from 0–200 μM. Steady-state kinetic experiments were also performed using combinations of aromatic amino acids: 100 μM L-Trp plus 100 μM L-Phe, 100 μM L-Trp plus 100 μM L-Tyr, and 100 μM L-Phe plus 100 μM L-Tyr. Where the assay conditions were identical to those described in the presence of no aromatic amino acid.

**Crystalization and Data Collection**—The crystal structures of MtuDAH7PS in complex with only Trp, only Phe, and both Trp and Phe were obtained by soaking native MtuDAH7PS crystals in mother liquor supplemented with the corresponding combination of amino acids. Native MtuDAH7PS protein was crystallized as described (31), and the crystal was soaked in Tris-HCl (pH 7.0, 0.1 M), ammonium sulfate (1.5 M), glycero1 (15% (v/v)), and 2 mM either Trp (for the Trp-only structure) or Phe (for the Phe-only structure).

For the Trp-only structure, crystals were left overnight at 10 °C then flash-frozen in liquid nitrogen with cryoprotectant consisting of mother liquor with an increased concentration of glycero1 (25% (v/v)). The data were collected at a wavelength of 0.97929 Å at the Stanford Synchrotron Radiation Laboratory. The data were processed using DENZO and SCALEPACK.

For the Phe-only structure, crystals were left soaking for 7 days at 10 °C. For data collection the crystals were soaked in a cryoprotectant composed of mother liquor plus an increased concentration of glycero1 (25% (v/v)). The crystal was then flash-frozen in a cold stream of gaseous nitrogen at 110 K. Data were collected at a wavelength of 1.542 Å (rotating copper anode) using a Rigaku MicroMax007 generator with Osmic Blue optics and an RAxis IV++ detector. Data were processed using d*TREK as part of CrystalClear Version 1.3.6 (Rigaku).

For the Trp+Phe structure, the native crystal was soaked in a cryoprotectant solution containing Tris-HCl (0.1 mM, pH 7.5), ammonium sulfate (1.4 mM), Trp (2.2 mM), Phe (2.2 mM), and glycero1 (25% (v/v)), for ~30 min. The crystal was then flash-frozen in a stream of gaseous nitrogen at 110 K. The data collection and process method was the same as that used for the Phe-only structure. Full details of the data collection statistics are in Table 1. Crystallization and data collection method for the Thesit-free structure of MtuDAH7PS was the same as previously described, except that an AXCo PX50 capillary optic was used for monochromation and focusing of the x-ray beam (31).

**Structure Determination and Refinement**—For all three liganded MtuDAH7PS crystal structures (Trp-only, Phe-only, and Trp+Phe), the soaking of the native crystals did not alter the crystal packing in any way in that the space group and cell dimension parameters are essentially identical to the original unliganded Se-Met MtuDAH7PS dataset. As all structures were isomorphous, we were able to use phase information from the Se-Met structure to determine the crystal structures of MtuDAH7PS ligand complexes. Measured intensities were converted to amplitudes using TRUNCATE as part of Scalepack2mtz or dtrek2mtz (CCP4). The structure was then solved by performing two rounds of rigid body refinement (CCP4) using calculated phases from the selenomethionine structure. Optimization of the model consisted of repetitive cycles of rebuilding using COOT and refinement with REFMAC5 (CCP4). Water molecules were added automatically in COOT and verified using 2Fo–Fc and Fo–Fc maps by their potential to hydrogen bond to at least one protein atom or water molecule.
For the Thesit-free crystal structure, twinning was observed at fractions of 0.622 and 0.378. Intensity-based twin refinement was carried out with REFMAC5 (CCP4) after each cycle of structure modification from COOT. All other refinement methods are the same as stated above for the three liganded crystal structures. The final refinement statistics for these three crystal structures are given in Table 1. The set of reflections for calculation of $R_{\text{free}}$ was transferred from the original structure to the data sets for all subsequent structures and augmented in the case of the higher resolution Trp/H11001 Phe and the Thesit-free structures.

_MtuDAH7PS Mutants_—MtuDAH7PS allosteric binding site mutants were produced using the QuikChange lightning site directed mutagenesis kit (Stratagene) following the protocol in the manufacturer’s instructions. Protein expression and purification were carried out as previously described for the wild type protein (primer information in supplemental Table S1). The activities of mutants in the presence of aromatic amino acids were determined using standard assay conditions in the presence of no amino acids and 200 $\mu$M L- Trp, 200 $\mu$M L-Phe, and a combination of 200 $\mu$M L-Trp plus 200 $\mu$M L-Phe. The assay solution contained PEP (150 $\mu$M), E4P (150 $\mu$M), and MnSO$_4$ (100 $\mu$M) in assay buffer.

**RESULTS**

_Inhibition of M. tuberculosis DAH7PS Requires Combinations of Trp and Phe or Trp and Tyr_—Purified MtuDAH7PS was treated with increasing concentrations of Phe, Tyr, or Trp. The presence of either Phe or Tyr in concentrations up to 200 $\mu$M gave no appreciable reduction of enzymatic activity, whereas the presence of Trp resulted in the slight decrease ($<5\%$) in the rate of the DAH7P synthase reaction (Fig. 2A). In distinct contrast, treatment of MtuDAH7PS with combinations of aromatic amino acids gave significant enzyme inhibition (Fig. 2B). Using increasing Trp concentrations in the presence of a fixed concentration of Phe (200 $\mu$M) gave the most significant inhibition, with 50% inhibition obtained with a concentration of only 2.4 $\mu$M Trp. Inhibition by Trp was slightly less effective in the presence of Tyr and required higher concentrations of Trp, indicating that the affinity of Trp for the Phe-MtuDAH7PS complex is greater than the affinity of Trp for the Tyr complex. Additionally, combinations of 200 $\mu$M concentrations of both Tyr and Trp resulted in some appreciable base-line DAH7PS activity, whereas the corresponding Phe-Trp combinations resulted in almost complete inhibition of enzyme activity. Strikingly, no inhibition was observed in the presence of both Tyr and Phe, a combination that has recently been reported to inhibit the activity of the M. tuberculosis chorismate mutase in combination with MtuDAH7PS (30).

_Combinations of Aromatic Amino Acids Alter the Enzymic Reaction Rate with Respect to E4P_—The steady-state kinetic parameters for the MtuDAH7PS-catalyzed reaction were examined in the presence of aromatic amino acid combinations (Fig. 3). In the absence of any effectors, both PEP and E4P profiles fitted well to the Michaelis-Menten equation and showed a standard hyperbolic response. In the presence of aromatic amino acid combinations the rate variation with PEP concentration also fitted the Michaelis-Menten equation, and although a marked $V_{\text{max}}$ reduction was observed,
$K_m^{PEP}$ only showed slight variation (from 29 to 47 μM, see supplemental Fig. S6).

In contrast, the effect of aromatic amino acid combinations on the reaction rate response with respect to E4P concentration was far more marked. With Tyr/Trp and Phe/Trp combinations, a sigmoidal response was evident (Fig. 3B). This was most significant for the Phe/Trp combination where a shift from $K_m^{E4P}$ of 37 μM in the absence of effectors to an E4P of 382 μM was observed. These data were fitted to the Hill equation yielding a Hill coefficient value of 3.3 ± 0.2. The Tyr/Phe combination had a more moderate effect, giving an E4P of 133 μM and a Hill coefficient of 2.8 ± 0.3. Without Trp, but in the presence of Phe and Tyr, clear cooperativity was not evident, although some change in affinity of the enzyme for E4P was observed ($K_m^{E4P}$ 145 μM).

**Structures of MtuDAH7PS in Complex with Aromatic Amino Acids**—Three new crystal structures for the liganded MtuDAH7PS were obtained by soaking Trp, Phe, or both Trp and Phe into crystals of wild type MtuDAH7PS. These structures describe MtuDAH7PS in complex with Trp (2.5 Å, $R_{free} = 0.228$), Phe (3.0 Å, $R_{free} = 0.269$), and Trp and Phe together (2.0 Å, $R_{free} = 0.191$). In addition, the structure for wild type MtuDAH7PS was determined (2.3 Å, $R_{free} = 0.208$) without the Thesit molecule (supplemental Fig. S3) that was bound in the original structure (24). The final model of the Trp-only structure contains one Thesit and two Trp ligands, the Phe-only structure contains five Phe ligands, the Trp + Phe structure contains three Phe and two Trp ligands, and the Thesit-free structure contains one PEP molecule. For all structures, the asymmetric unit contains two molecules with the same general ($β/α)_b$ triose phosphate isomerase barrel fold. Two major additions decorate the basic barrel structure in each monomer. The first (residues 1–76) is the extended N terminus and three additional helices (α0a, α0b, and α0c). The second consists of a pair of helices (α2a and α2b) and coil (residues 193–239) that extend the α2-β3 loop.

The two molecules in the asymmetric unit (Fig. 4b) form a tight dimer that further associates by application of crystallographic 2-fold symmetry to form an MtuDAH7PS homotetramer (Fig. 4a). The dimer interface is formed mainly by elements from the N-terminal extension to the core ($β/α)_b$ barrel, specifically residues 3–10, which form a two-stranded β sheet across the interface, and the α0b–α0c loop together with the long helix α2. The interface between the two dimers, through which the tetramer is generated, is less extensive and is formed by extra-barrel elements, helix α2b and the loop α2b–β3, with minor contributions from the core α1 helix. The ligands Phe and Trp bind at the dimer and tetramer interfaces, respectively, forming hydrogen bonds as well as hydrophobic contacts with the extra-barrel elements. One additional Phe per dimer is located between the α0a, α0b, and α3 helices and the α3-β4 loop in the Phe-only and Trp + Phe structures.

**The Phe Binding Sites**—The primary Phe binding sites in the Trp + Phe structure are located at the interface of the tight dimer, with binding involving interactions with residues from both monomers (Fig. 5a and supplemental Fig. S2a). There is one primary Phe binding site in each subunit. The α-carboxylate group of each Phe ligand forms hydrogen bonds with the side chain of Arg-171 and Asn-175, and the α-amino group interacts with the side chain of Asn-175 and the main-chain carbonyl oxygen of Phe-91. The hydrophobic ring of Phe is buried in a pocket formed by residues from both subunits including Ala-174, Ala-178, Pro-56*, Val-55*, Ala-174*, and Tyr-173* (where * indicates residues from the other subunit of the tight dimer). These binding pockets are “capped” by the extended N termini of both monomers, residues 3–10, with Trp-3 from subunit B positioned between the two Phe ligands and contributing to the binding pockets for both. Results from molecular modeling studies show that Tyr can also bind in the primary Phe binding site with the same binding mode as Phe (see supplemental Fig. S1), accounting for inhibition observed with Trp/Tyr combination.
In the Phe-only structure the primary Phe binding sites are essentially the same as in the Trp+Phe structure, with minor changes in side-chain conformations of Arg-171 and small shifts in the positions of the N-terminal residues. The interactions between the bound Phe ligands and the enzyme are retained.

In the Trp-only structure one Thesit molecule is bound in the tight dimer interface, occupying the primary Phe binding sites. The N-terminal residues have shifted in position in comparison with the Trp+Phe structure but correspond closely in position to those in the previously reported unliganded wild type structure, which also has a Thesit molecule bound at the same site (PDB code 2B70). This suggests that the shift in N-terminal residues only occurs upon Phe binding in the tight dimer interface. This shift in position may be caused by the positioning of Trp-3 of subunit B between the two bound Phe ligands.

A secondary Phe binding site was observable in subunit B of the tight dimer in both Trp+Phe and Phe-only structures (Fig. 5b). The binding pocket for this site is formed by residues from the additional α0a and α0b helices, the α3 helix, and the α3-β4 loop. The α-carboxylate group of the Phe ligand bound here interacts with the side chains of Arg-23 and Arg-256. The α-amino group is hydrogen-bonded to the backbone carbonyl oxygen of Arg-256 and the side chain of Glu-253. The hydrophobic ring sits in a pocket formed by nearby Leu residues 26, 18, 271, 261, and 259. The helices α0a and α0b, which make up the binding pockets of the secondary Phe binding sites, are slightly closer to each other in molecule A, and there is insufficient space for a Phe ligand to bind in this subunit. In subunit B, in contrast, the residues on the α0a helix (residues 16–27) have shifted in position so that the bound Phe can be accommodated. This difference between subunits A and B is also present in the unliganded wild type MtuDAH7PS struc-
Inhibition of M. tuberculosis DAH7PS

The Trp Binding Sites—The Trp binding sites in the crystal structure are located at the interface between the two tight dimers that make up the tetramer (Fig. 4a). Each molecule of Trp is bound in a cavity between the extra-barrel pair of helices (α2a and α2b) and the core barrel helix α1 in each monomer. In the Trp+Phe and Trp-only structures, the Trp binding site is predominately formed by residues of the α2b and α1 helices and the α2b-β3 loop, which is also involved in tetramer association. In subunit A (Fig. 5c and supplemental Fig. 2b), the α-carboxylate group of Trp forms a salt bridge with the Lys-123 side chain. The α-amino group interacts with the backbone carbonyl oxygen atoms of Asn-237 and Thr-240. The N° on the Trp indole ring is hydrogen-bonded to the backbone carbonyl oxygen of Ala-192. The hydrophobic ring sits in a relatively deep binding pocket formed by hydrophobic residues Val-111, Leu-107, Leu-194, Ala-241, and Val-197. Similar interactions are also observed for Trp bound in subunit B.

In the Phe-only structure, Phe ligands are observed to occupy the Trp binding sites at the tetramer interface and show similar interactions (see supplemental Fig. S4). The α-carboxylate group of the Phe interacts with the side chain of Lys-123, and the hydrophobic ring sits in the same pocket as that of the Trp ligand when it is bound. Unlike Trp, the α-amino groups of the bound Phe ligands only show interaction with the backbone carbonyl oxygen of Asn-237 but not Thr-240. The loop comprising residues 237–242 is more disordered than in the Trp+Phe structure and shows at half-occupancy an alternative position similar to that observed in the unliganded structure.

Active Site—The active sites in the Trp+Phe, Trp-only, Phe-only, and Thesit-free MtuDAH7PS structures are very similar to that of the unliganded structure previously reported (PDB code 3B7O). In the Trp+Phe structure one phosphate ion binds in the active site of each monomer, where it interacts with the side chains of Lys-306 and Arg-337 and the backbone NH group of Glu-283 (see supplemental Fig. S8). This phosphate ion binds in the same position as the phosphate group of PEP when it is bound in the active site of unliganded MtuDAH7PS structure (Fig. 5d).

Comparison of the Trp+Phe Bound with the Unliganded Thesit-free Structure—The crystal structure of the Trp+Phe MtuDAH7PS complex was superimposed onto the new Thesit-free unliganded structure to identify any structural differences caused by ligand binding and to identify a possible mechanism for inhibition. Ligand binding was found to cause no significant change in the overall structure of the MtuDAH7PS enzyme (supplemental Fig. S7). The two structures match with a root mean square difference in Ca atomic positions of only 0.7 Å over the entire dimer. The greatest displacements were in the Phe and Trp binding sites, the N termini, and the disordered α3-β4 loop (residues 263–268). Upon binding of the Trp and Phe ligands, residues from β3 to α8 in both monomers (residues 273–462) show very little change in Ca positions, indicating these secondary structures are not affected significantly (supplemental Fig. S5).

Binding of both Trp and Phe ligands causes the extended β2-α2 loop (residues 135–144) in the active site that contains the highly conserved KPRS motif for type II DAH7PS enzymes to become more disordered. These residues have been shown to be associated with the binding of substrate E4P in other DAH7PS enzymes (18, 19). This change is more clearly observed in subunit A in which the loop shows alternative positions for residues 135–139 (Fig. 5d). This change in the active site loop is not observed in the Trp-only and Phe-only structures.

The solvent-accessible surface area of the tight dimer interface only changes slightly on Trp+Phe binding, from 1885 to 1858 Å². Binding of Phe at the tight dimer interface causes surprisingly little disturbance of the polypeptide chain conformation. The binding of Trp and Phe simultaneously or Trp alone results in a subtle movement of residues of the α2 helix. The Ser-189—Gly-190 peptide flips, and new interactions between Ser-189 on the α2 helix and Ser-62 on the α0c helix of the other monomer are formed. This flip is not observed in the Phe-only structure in which Ser-189 retained the ligand-free conformation. The side chains forming the salt bridge between Glu-96 and Arg-100 become disordered, with the new conformations packing more closely against the α0c helix. Another interaction gained at the dimer interface is a hydrogen bond between Arg-236 and Asn-237 of the other monomer. Asn-237 also contributes to the tetramer interface as described below. Only the N-terminal residues 1–10 have shifted significantly in position (supplemental Table S2), with displacements up to 6.2 Å in chain B and 3.1 Å in chain A. The displacement is greater in chain B because in the liganded structure Trp-3 of chain B sits in between the two Phe ligands, near where Val-5 is in the unliganded structure. In the unliganded structure the N-terminal β-strand consists of residues 5–7 from both subunits. However, in the Phe-only and Trp+Phe structures, this β strand becomes extended and consists of residues 3–9 from subunit A and residues 1–7 of subunit B. Upon binding of Phe, the side chain of Arg-171 in chain B becomes disordered and shows one alternative conformation that interacts with the Phe α-carboxylate group. This displaces the side chain of Asp-10 in chain A, which occupies the same site in the unliganded structure and results in the loss of a salt bridge between Arg-171 in chain B and the Asp-10 in chain A. Due to the shifts of N-terminal residues in subunit B, Arg-171 in chain A gains a salt bridge with Asp-6 and does not show any alternative conformation.

FIGURE 5. Ligand binding sites and active sites. a, shown are the primary Phe binding sites in the tight dimer interface. Residues 3–10 are excluded for clarity. b, shown is the secondary Phe binding site in subunit B. c, shown are the Trp binding sites. In all three figures residues on the basic barrel structure are shown with blue carbons, residues that are on the N-terminal extra-barrel addition are shown in red, and the residues on the α2a-α2b helices are shown with yellow carbons. The bound Trp and Phe molecules are shown with green carbons. Water molecules are displayed as red spheres. Hydrogen bonds are displayed as red dashed lines. d, superposition of the active site of the Trp+Phe structure and the unliganded Thesit-free structure of MtuDAH7PS. Residues from the Thesit-free unliganded structure are shown with cyan carbons, and residues from the Trp+Phe structure are shown with green carbons. The PEP bound in the active site of molecule A in the Thesit-free structure is shown in yellow for comparison with the position of the phosphate ion from the Trp+Phe structure (shown in red). The side chains of residues 134–139 are omitted for clarity. The alternative backbone position of residues 135–139 is shown with purple carbons.
Inhibition of M. tuberculosis DAH7PS

The solvent-accessible surface area at the tetramer interface increases from 954 to 1065 Å² on ligand binding. Considerable structural changes at the tetramer interface occur in the α2b-β3 loop, whereas the angle between the α2a and α2b helices widens about 3°. In the Trp-only and Trp+Phe-ligated structures, substantial changes in orientations of hydrophobic side chains between α2a and α2b were observed. In contrast, in the Phe-only structure the changes were smaller and midway between the unliganded and the Trp+Phe-ligated structures. Upon binding of the Trp ligands, residues 232–241 (in the α2b-β3 loop) show the most significant displacements in Cα atoms (supplemental Table S2), enabling the backbone carbonyl groups of Gln-237 and Thr-240 to interact with the Trp α-amino group. The side-chain conformations of residues Leu-194 and Val-111 change to form the hydrophobic pocket that accommodates Trp, and the side chain of Lys-123 moves slightly to interact with the α-carboxylate groups of the bound Trp ligands.

Mutational Analysis of Ligand Binding Sites—To assess whether the ligand binding sites identified from the structures of the complexes are responsible for the inhibitory effects exerted by the aromatic amino acids on the enzymatic activity of MtuDAH7PS, several mutants of MtuDAH7PS were prepared and analyzed (Fig. 6). In the primary Phe binding site mutation of Arg-171 (R171A) resulted in active enzyme that was insensitive to the addition of Phe and Trp when added either individually or in combination. This result confirms that occupation of this site is essential for inhibition. In contrast, a mutation (R256A) in the secondary Phe binding site, which is occupied in only one subunit, gave an enzyme with similar sensitivity to Trp and Phe to that of the wild type enzyme. This result indicates that this binding site is not required for the sensitivity of this enzyme at low Phe and Trp concentrations.

Mutation of a key residue in the Trp binding site, Leu-194, gave a mutant enzyme (L194A) that was no longer sensitive to added Trp. This enzyme was less active than wild type MtuDAH7PS, and its activity was only slightly reduced by the addition of Trp and Phe in combination, consistent with the importance of this binding site for the synergistic inhibition of MtuDAH7PS. The activity of this enzyme was found to be slightly sensitive to the presence of Phe alone. Given the reduced activity of this mutant, it is possible that the mutation may partially mimic changes induced by Trp occupancy in the wild type enzyme.

DISCUSSION

Allosteric interactions are important for the control of biological pathways, and there has been considerable interest in understanding the molecular mechanisms that allow the precise control of metabolism in this way (33, 34). Branch pathway such as the biosynthesis of aromatic amino acids present particular challenges for control. The main trunk of the shikimate pathway leads to chorismate, and branching occurs at this point to Trp, to Phe and Tyr via prephenate, and to a variety of other aromatic compounds. Although it is evident that there needs to be branch-point control, there is also a need to limit wasteful entry into the pathway and the accumulation of the relatively unstable pre-aromatic compound chorismate if all aromatic amino acids are in plentiful supply. Organisms such as E. coli express multiple type I isozymes to achieve this, and the Phe-sensitive enzyme from E. coli and the Tyr- and Phe-sensitive enzymes from S. cerevisiae have been studied in some detail (11, 21, 35, 36). For M. tuberculosis, which expresses a single DAH7P synthase, this study has uncovered a more sophisticated mechanism for the control of shikimate pathway metabolism.

This study shows that MtuDAH7PS simultaneously binds more than one aromatic amino acid and that only combinations that include Trp inhibit DAH7P synthase activity. The type II enzyme from M. tuberculosis differs from all other structurally characterized DAH7P synthases by having two distinct additions to the core barrel that are both remote from each other and crucial for forming quaternary associations (24). It is these additions that form the allosteric binding sites for Trp and Phe. Sequence analysis of type II enzymes suggests that these extr barrel additions are found in many other type II enzymes. Intriguingly, for some enzymes, particularly those that are likely to be responsible for secondary metabolite production, the α2-β3 loop extension, responsible both for tetramer formation and Trp binding, is missing. Enzymes of this group are yet to be characterized. In type II enzymes that possess this subdomain this region shows remarkable sequence variation, possibly reflecting the variation of feedback effectors that have been reported. The importance of quaternary structure to the allosteric inhibition is evident; no synergistic inhibition was observed for the dimeric type II enzyme from H. pylori (37).

In marked contrast, catalytic barrel additions associated with sensitivity to a single allosteric effector are characteristic of type I enzymes. Type Iβ enzymes with fused functional chorismate mutase domains show sensitivity to both chorismate and prephenate (23, 38). The Trp-sensitive E. coli isozyme is inhibited by relatively high concentrations of Trp (0.1–0.2 mM) to achieve
50% activity) and the maximally inhibited enzyme maintained around 20% residual activity (22). In contrast, the inhibition of MtuDAH7PS by Trp (in the presence of Phe) is far more potent and gives complete loss of enzymic activity. This may reflect the fact that the E. coli Trp-sensitive isozyme is responsible for only 1% of the cellular DAH7P synthase activity (9). A regulatory system where all DAH7P synthase activity can be employed for the biosynthesis of any aromatic amino acid in short supply appears far more elegant than a system using multiple isozymes.

We have shown here that combinations of aromatic amino acids that include Trp have a dramatic effect on the kinetic profile of the MtuDAH7PS -alyzed reaction with respect to E4P. The hyperbolic Michaelis-Menten kinetics observed in the absence of inhibitors shifts dramatically to sigmoidal kinetics, with clear homotropic cooperativity observed with respect to E4P. This shift in profile can be analyzed in terms of a basic Monod-Wyman-Changeux (39) model for allosteric regulation, in which the tetramer can exist in either low or high affinity states for E4P. In the absence of allosteric binders this equilibrium greatly favors the high affinity state of the tetramer; hence, no apparent cooperativity is observed, and the subunits appear to behave independently. Binding of Phe has little effect on the position of the equilibrium between high affinity and low affinity states for E4P, so only limited E4P cooperativity is observed. However, if Trp binds strongly to the Phe-bound enzyme in the low affinity form, significant disruption of the equilibrium occurs, in favor of low affinity state. As a consequence, large homotropic E4P cooperativity is observed. Support for this analysis comes from the structures obtained in this study. In the Phe-only structure, Phe binding partially alters the structure of the Trp binding site, as indicated by the altered conformation of the half-occupancy loops. Thus, when Phe binds to the enzyme, the Trp binding sites become partially assembled. The entropic cost of Trp binding may, thus, be reduced by the presence of Phe. Whereas there are many reports of enzymes that display sensitivity to more than one allosteric effector, our observation of synergistic binding by multiple pathway end products appears quite unusual. However, it is conceivable, of course, that many combinations of potential effectors have not yet been explored.

Traditional Monod-Wyman-Changeux model analysis predicts that distinct conformational changes are associated with high affinity and low affinity states. However, in this system, very small differences are observed between the Trp + Phe and unliganded structures. The distances between the allosteric binding sites and between the allosteric sites and the active site are large (the minimum distance between Trp and the Mn2+ ion is 23.9 Å, between Phe and Mn2+ is 20.5 Å, and between the Trp and Phe is 26.4 Å). How, therefore, is occupancy of one site communicated to the other, and how is dual occupancy of allosteric sites communicated to the active site to dramatically alter E4P binding? It appears likely that the binding of Trp + Phe changes the dynamics of the active site loops associated with E4P binding. The KPRS motif on the extended B2-α2 loop, although only slightly displaced, is more disordered in the Trp + Phe structure, disfavoring the binding of E4P. The role of dynamics in signal transmission and allostery is becoming more understood (40), and insignificant gross conformational change associated with allosteric effector binding has been noted for a number of systems (41–46).

Whereas the inhibition of MtuDAH7PS by the Phe + Trp combination is particularly effective, Tyr and Trp combinations also significantly inhibit the MtuDAH7PS-catalyzed reaction. Sensitivity to either Phe or Tyr makes sense in terms of the biosynthesis of aromatic amino acids, as Tyr and Phe are formed from the same branch from chorismate (Fig. 1). In the Trp-only structure, Trp is observed to bind only in the Trp binding site at the tetramer interface. As Tyr can be satisfactorily modeled into the Phe binding site, it is reasonable to predict that Tyr binds in place of Phe in the Trp + Tyr combination.

As well as catalyzing the first step of the pathway, MtuDAH7PS is also involved in control at the branch point. In line with observations made for the type II enzyme from Amycolatopsis methanolica (47, 48), it has been shown that MtuDAH7PS forms a complex with the M. tuberculosis chorismate mutase, resulting in significant activation of the latter enzyme (30). Phe and Tyr, the products of this branch of the pathway, inhibit this enhanced activity. Phe has been observed to bind in the Trp binding site, and Tyr can bind in place of Phe. This combination has little impact on MtuDAH7PS activity, but how it alters the chorismate mutase activity is unknown. Control of the other branch of the pathway, the biosynthesis of Trp, is achieved by direct feedback regulation of Trp on the M. tuberculosis anthranilate synthase (49).

This study has uncovered an intricate system of regulation that operates both at the start and at the branch point of a pathway that gives rise to multiple end products. MtuDAH7PS plays a pivotal role in control of pathway flux via its multiple ligand binding sites. This reveals a complex network of ligand interactions that underpin metabolic control and may inform the design of inhibitors that target protein and allosteric networks rather than a single enzyme active site.

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