Remote Control by Inter-Enzyme Allostery: A Novel Paradigm for Regulation of the Shikimate Pathway

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ABSTRACT

DAHP synthase and chorismate mutase catalyze key steps in the shikimate biosynthetic pathway en route to aromatic amino acids. In *Mycobacterium tuberculosis*, chorismate mutase (MtCM; Rv0948c), located at the branch point towards phenylalanine and tyrosine, has poor activity on its own. However, it is efficiently activated by the first enzyme of the pathway, DAHP synthase (MtDS; Rv2178c), through formation of a non-covalent MtCM-MtDS complex. Here, we show how MtDS serves as an allosteric platform for feedback regulation of both enzymes, using X-ray crystallography, SAXS, SEC, and MALS. Crystal structures of the fully inhibited MtDS and the allosterically down-regulated MtCM-MtDS complex, solved at 2.8 and 2.7 Å, respectively, reveal how effector binding at the internal MtDS subunit interfaces regulates the activity of MtDS and MtCM. While binding of all three metabolic end products to MtDS shuts down the entire pathway, the binding of phenylalanine jointly with tyrosine releases MtCM from the MtCM-MtDS complex, hence suppressing MtCM activation by 'inter-enzyme allostery'. This elegant regulatory principle, invoking a transient allosteric enzyme interaction, seems to be driven by dynamics and is likely a general strategy used by nature.

Keywords: enzyme catalysis/metabolic pathway/multi-enzyme complex/*Mycobacterium tuberculosis* Rv2178c and Rv0948c/X-ray crystal structure

INTRODUCTION

Control of enzyme activity in metabolism is of utmost importance for cells to efficiently respond to altered environmental conditions and to avoid wasting resources. Many different modes of modulating catalytic activity are known, both at the gene and protein level. A common regulatory strategy used in biosynthetic pathways is feedback inhibition of enzymes, which often involves allosteric control. Thereby, allosteric effector molecules (inhibitors or activators) bind at locations distant from the substrate binding sites and often induce considerable changes in the tertiary and/or quaternary structure resulting in altered activity. Classical examples are hetero- or homomeric proteins, such as hemoglobin, aspartate transcarbamoylase and the Lac repressor [1-5], which interact with or chemically transform substrates. Allosteric ligands typically bind at subunit interfaces, along symmetry axes, promoting changes in subunit arrangement [6]. More recently, allostery has also been observed in monomeric proteins [7-9] and RNAs and ribozymes [10]; and the role of dynamics in allosteric regulation was recognized [11, 12]. It has further been acknowledged that ligand binding is a thermodynamic phenomenon, which in its most extreme form can be governed purely by entropy, with no associated macroscopic changes in structure [13, 14]. An important and until recently under-recognized factor is the influence that allosteric effectors may have on the relative energies of an ensemble of conformational states [15-18]. This implies that allosteric regulation does not necessarily involve the transmission of distinct structural alterations to distant sites, but instead can simply be a consequence of the selection of conformations associated with a specific function upon effector binding.

An important advantage of allosteric regulation of metabolic pathways, in contrast to transcriptional control, is the possibility to receive an immediate response to elevated or depleted metabolite pools [19]. Allosteric control usually acts at the first enzyme of a metabolic pathway or at branch point enzymes that channel metabolites toward different end

products. A particularly well-studied example of a rigorously regulated metabolic cascade is the shikimate pathway. It represents an essential biosynthetic route in bacteria, fungi, and plants, which links carbohydrate metabolism to the synthesis of important aromatic compounds, such as the amino acids L-tyrosine (Tyr)¹, L-phenylalanine (Phe) and L-tryptophan (Trp), as well as coenzymes and vitamins [20, 21]. The shikimate pathway starts out with the aldol-like condensation of phosphoenolpyruvate (PEP) **1** and D-erythrose-4-phosphate (E4P) **2** to 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) **3** and inorganic phosphate, catalyzed by the enzyme DAHP synthase (DS) (Fig. 1). DAHP is subsequently transformed in six enzymatic steps to the branch point compound chorismate **4**, which is further processed to Tyr, Phe, and Trp, and other aromatic compounds.

Bacteria are usually equipped with a tightly controlled shikimate pathway that is subject to direct metabolite feedback inhibition and/or repression at the genetic level [21, 22]. In addition to DS, regulation occurs at the key branch point enzymes chorismate mutase (CM; responsible for channeling chorismate towards Tyr and Phe), and anthranilate synthase (AS; initiating the branch towards Trp). Feedback inhibition by the specific pathway end products Tyr, Phe, and Trp is typically exerted through allosteric binding to dedicated regulatory protein domains (*e.g.*, the ACT domain of an *Escherichia coli* CM [23]) or to different enzyme isoforms (exemplified by the three *E. coli* DSs [21, 24, 25]). The CM-DS of *B. subtilis* is another example for a fusion protein in which the DS is covalently linked to a regulatory domain that is, in this case, homologous to a CM of the α -helical AroQ class [26]. Even though the fusion protein exhibits some (poor) CM activity, it is believed to be just an

¹ *Abbreviations:* AS, anthranilate synthase; CM, chorismate mutase; BTP, (1,3-bis[tris(hydroxymethyl)methylamino]propane); DAHP, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (**3**); DS, DAHP synthase; EcCM, *Escherichia coli* chorismate mutase domain of the bifunctional CM-PDT (PheA) protein; E4P, D-erythrose-4-phosphate (**2**); MALS, multi-angle light scattering; MtCM, *Mycobacterium tuberculosis* chorismate mutase encoded by Rv0948c; MtDS, *Mycobacterium tuberculosis* DAHP synthase; PDH, prephenate dehydrogenase; PDT, prephenate dehydratase; PEP, phosphoenolpyruvate (**1**); Phe (or F), L-phenylalanine; r.m.s.d., root mean square difference/deviation; SAXS, small-angle X-ray scattering; SEC, size-exclusion chromatography; TCEP, (tris[2-carboxyethyl]phosphine hydrochloride); Trp (or W), L-tryptophan; Tyr (or Y), L-tyrosine

evolutionary relict; instead, the main purpose of the CM domain is to provide the DS with feedback regulation by chorismate and prephenate. Remarkably, the DS activity in this bacterium is not regulated by aromatic amino acids [27]. Recent reports on shikimate pathway enzymes in *Mycobacterium tuberculosis* have shown that nature also uses an ingenious alternative strategy for allosteric control of the flux through the shikimate pathway [28-30].

In *M. tuberculosis*, there is only a single DS (MtDS; Fig. 1a) encoded in the genome (*i.e.*, there are no isozymes specific for a particular end product). The bacterium has two CMs; a highly active one of unknown function that is secreted from the cell [31, 32] and a cytoplasmic one (MtCM) that lacks a regulatory domain [28]. Unlike other CMs, MtCM is a poor catalyst on its own, but it can be activated >100-fold by associating with MtDS. The activated non-covalent complex between MtDS and MtCM has been characterized crystallographically to 2.35 Å resolution [28]. Two MtCM dimers dock on opposite sides to an MtDS tetramer to form a heterooctamer (Fig. 1c). This association remodels two of MtCM's structural elements (a surface loop and a C-terminal extrusion) to catalytically competent conformations, whereas MtDS residues are not directly involved in MtCM catalysis.

MtDS-mediated MtCM activation is counteracted synergistically by the two metabolic end products Tyr and Phe [28]. Evidence has been accumulating over the last few years that feedback effector binding leads to the dissociation of the active MtCM-MtDS complex [28-30]. MtDS itself is affected by these two amino acids only to a minor extent [33, 34], whereas the simultaneous presence of all three aromatic amino acids, Tyr, Phe, and Trp, essentially knocks out DS activity [35, 36]. MtDS crystals containing either Phe, Tyr, or Trp alone, or the combinations Trp + Phe or Trp + Tyr were characterized and have revealed the structure of MtDS in a partially inhibited state [33-35]. The structures show that these amino acids bind to small regulatory domains that decorate the central catalytic TIM barrel domain and provide

the bulk of the inter-subunit contacts in the tetrameric MtDS structure [33, 34]. Phe and Tyr bind close to each other near the N-terminus of MtDS, at the tight dimer interfaces of the tetramer, whereas Trp binds near the tetramer interfaces (Fig. 1a). The differences observable between structures without, just with one, or with two of these three amino acids are relatively small. Essentially, variations are limited to the sites that are directly involved in feedback inhibitor binding and to exposed surface loops at a considerable distance from the allosteric sites [34]. Appreciation of the full extent of structural changes upon feedback inhibition has been frustrated, however, by the lack of a model for the completely inhibited state of MtDS in the presence of all three end products. Moreover, a molecular explanation for the mechanism of MtDS-mediated regulation of the CM activity in *M. tuberculosis* so far lacked structural support.

Here, we set out to fully elucidate the intricately linked mechanisms of allosteric regulation of the two key enzymes MtDS and MtCM of the *M. tuberculosis* shikimate pathway. We have structurally characterized the fully inhibited state of MtDS and compared it to its activated state. In addition, by using a variety of biophysical approaches, we provide evidence how MtDS controls the enzymatic activity of MtCM. The new crystal structures show that the feedback inhibitors of MtCM, Phe and Tyr, bind nowhere near MtCM in the MtCM-MtDS complex, but instead occupy the same positions as identified for MtDS alone. Intriguingly, however, we observe almost no conformational changes, even in the presence of MtCM, and the crystal structures of the MtCM-MtDS complex, with and without allosteric regulators, superimpose almost perfectly. Nevertheless, the binding of Phe and Tyr to their allosteric sites in MtDS not only inhibits MtDS activity; it also affects MtCM-MtDS interaction, promoting the dissociation of the non-covalent complex, thereby lowering CM activity >100-fold. Our results establish a novel paradigm for allosteric regulation of the shikimate pathway, which occurs only in the phylum *Actinobacteria*. These bacteria have

evolved a cleverly regulated version of a central metabolic route, which is stripped down to essentials.

RESULTS

Activity of Native MtDS and Inhibition by Phe, Tyr, and Trp

Previous studies on the regulation of MtDS activity were performed with non-native formats of MtDS (recombinant versions with an N-terminal Gly-Ala extension remaining after cleavage of a His₆-affinity purification tag [30, 33-35, 37]). Since the N-terminus is very close to the Phe allosteric site, we were concerned that changes in this region may affect the structure and kinetics. Therefore, we assessed the catalytic activity and the inhibition profile with authentic native wild-type MtDS in comparison to an N-terminally His₆-tagged form (His₆-MtDS), which was produced and assayed in parallel. While some differences were observed when the effect of only one or two of the aromatic amino acids was tested (discussed in the *Supplementary Information*), both the fully active and fully inhibited MtDS showed identical activity for both tested formats (Fig. 2, Fig. S1). We found that MtDS activity was effectively reduced to less than 5% when all three effectors were present at 100 μ M.

Impact of the Feedback Inhibitors Phe, Tyr, and Trp on MtDS-Mediated Activation of MtCM

As shown previously [28], binding of MtCM to MtDS leads to a dramatic (>100-fold) increase in CM activity and this boost can be counteracted by Phe and Tyr (Fig. 2). When probing the influence of the MtDS format (native MtDS vs. His₆-MtDS) on the activity and allosteric regulation of MtCM, we found no significant difference (Table S1). For both formats, we obtained the same response (within experimental error) to Phe, Tyr, and Phe + Tyr ($28 \pm 0\%$, $64 \pm 2\%$, and $5 \pm 1\%$ residual CM activity, respectively) at 200 μ M total effector concentration. Since native and His₆-tagged MtDS showed no difference with respect

to MtCM activation and regulation, all subsequent analyses were undertaken with His₆-MtDS rather than native MtDS.

Next, we examined whether MtCM-MtDS activity would also be subject to effective feedback regulation at effector concentrations typically encountered in the bacterial cytoplasm. The physiological levels of the aromatic amino acids in *Escherichia coli* were determined to be 18 μ M Phe, 29 μ M Tyr, and 12 μ M Trp [38]. Thus, we monitored residual CM activities after addition of 25 μ M effector instead of 200 μ M. The single additions showed only for Phe, known as the strongest effector [28], a modest reduction of activity, to 83%, whereas Tyr and Trp alone had no effect (Fig. 2). The combination Phe + Tyr resulted in a synergistic inhibition, with only 30% residual activity. However, in no case did we observe a response to Trp; for instance, the combination of all three aromatic amino acids showed the same CM activity level as with Phe + Tyr.

Crystal Structure of the MtCM-MtDS Complex with the Allosteric Regulators Phe and Tyr

To reveal the molecular basis for MtCM regulation, we solved the crystal structure of the MtCM-MtDS complex with the effectors Phe and Tyr, and the transition state analog **6** [39] of the CM reaction. The structure was determined to 2.7 Å resolution (Fig. 3a; Table 1). Remarkably, the two feedback inhibitors did not bind to MtCM, but to MtDS, in their previously characterized allosteric sites [33-35]; and no additional electron density attributable to Phe or Tyr was observed in direct contact with MtCM or anywhere close to the MtCM-MtDS interface. In general, a comparison with the structure of the effector-free MtCM-MtDS complex with **6** (PDB ID: **2W1A** [28]) revealed surprisingly few differences (Fig. 3b). Except for the immediate environment of Phe and Tyr (Fig. S2, Fig. S3), the whole structure, including the arrangement of the active site residues of MtCM, is virtually identical

for the two complexes, and also the quaternary structure seems unaffected (Fig. 3b). Upon more detailed scrutiny, the MtDS helices at the interface with MtCM show minor displacement for some of the residues (≈ 0.5 Å), which is close to the precision limit of a structure at this resolution, and the MtDS subunits might experience a small rotation by 1-2° (Fig. S4). Our structural data thus establish that the >100-fold activation of MtCM by MtDS can be efficiently counteracted by Phe and Tyr binding to MtDS, at a distance of approximately 45 Å and 55 Å, respectively, from the active site of MtCM.

In contrast to the minor structural changes upon Phe and Tyr binding, we observe differences in the dynamics at the MtCM-MtDS interface. While such differences are hardly reflected by differential crystallographic *B*-factors (Fig. 3c and 3d), altered dynamic behavior is suggested by time-averaged molecular dynamics refinement [40] (Fig. 3e and 3f). The changes in the protein dynamics upon effector binding are most noticeable for the MtDS regions interacting with the MtCM loop that connects helices H1 and H2, and for MtCM segments in contact with MtDS (Fig. 1c, 3e vs. 3f).

To confirm the overlap of the regulatory machineries of DS and CM activities at the level of function, we generated and tested the MtDS variant His₆-MtDS Arg256Ala, which displays impaired feedback regulation of DS activity [35, 36]. Residue 256 is located in the Tyr-binding site of MtDS [35]. When measuring DS activity, we found that the mutation increased the apparent K_i for Phe and Trp about 10 fold. It also abolished any inhibitory response upon titration of Tyr, testing concentrations of up to 100 μM (Fig. S1 and *Supplementary Information*; [36]), proving that the Tyr allosteric site on MtDS was rendered fully dysfunctional by the R256A exchange. We then assayed the CM activity of MtCM in the presence of His₆-MtDS-R256A as a function of Phe and Tyr addition. In the absence of the effectors, the mutant MtDS was still able to activate MtCM to the same extent as wild-type MtDS (Table S1). However, in contrast to the measurements with the wild-type

complex, Tyr did not show any inhibition of CM activity ($108 \pm 6\%$ residual activity), and Phe and Phe+Tyr showed a clearly reduced sensitivity ($70 \pm 1\%$ and $83 \pm 1\%$ residual CM activity, respectively, at 200 μM total effector concentration; Table S1). Together with the structural localization of the effector binding sites, the observation of a parallel loss of effector responsiveness of both DS and CM activities upon knocking out the MtDS Tyr binding sites proves that the tight feedback inhibition of MtCM is remote-controlled in an unusual way by the allosteric sites positioned within its partner enzyme MtDS.

Structural Insights from MtDS Co-Crystallized with the Three Allosteric Feedback Inhibitors Phe, Tyr, and Trp

Since MtDS exhibited hardly any structural differences in the MtCM-MtDS complex with or without Tyr and Phe, we wondered whether MtDS alone would reveal larger conformational changes when inhibited by all three of its allosteric regulators. We therefore crystallized His₆-MtDS in the presence of Phe, Tyr, and Trp, yielding the first fully inhibited MtDS-structure, determined to 2.8 Å resolution (Table 1). Again only subtle changes were observed compared to previously reported MtDS structures [33-35, 37] and MtDS in complex with MtCM [28], or the new complex structure determined here (PDB ID: **5CKX**; Fig. 3). Overall, there are two Tyr, four Phe and four Trp molecules bound to the MtDS homotetramer (Fig. 4a, Fig. S5), and the effector binding sites are virtually identical to those of unliganded MtDS (Fig. 4b) or MtDS co-crystallized [34] or soaked [33, 35] with just one or two of the feedback inhibitors.

When comparing the MtDS-MtCM interface with the corresponding surface on MtDS in the absence of MtCM, we observe the very same three structural adaptations in MtDS for accommodation of MtCM, regardless whether amino acid effectors are present (PDB ID: **5CKX** vs. **5CKV**; this work) or absent (PDB ID: **2W1A** vs. **3NV8**; [28, 33]). These

adaptations include (i) formation of a salt bridge by Arg213 with Glu458 to avoid interference with MtCM residues Glu59 and Val55, (ii) reorientation of the side chain of Gln392 from an H-bonding interaction with Glu396 to contact MtCM residues Leu54, Leu88 and Gly89 (at 3.5-3.8 Å distance), and (iii) restriction of Arg399 to one of two alternative side chain conformations to prevent a clash with His90 at the C-terminus of MtCM.

As observed for the MtCM-MtDS effector complex, fully inhibited MtDS exhibits increased flexibility at the MtCM docking site, as judged from slightly increased *B*-factors (Fig. 4c and 4d) and enhanced ensemble dynamics (Fig. 4e and 4f). In contrast, the immediate environment of Trp at the MtDS tetramer interface becomes more ordered upon effector binding, and also loop $\beta 7\text{-}\alpha 7$ (residues 368-385), which is close to the MtDS active site, becomes more rigid (Fig. 4c to 4f). This loop carries Lys380, which was shown to contribute to the binding of the E4P phosphate group [41] (mimicked by sulfate ions in the crystal structure). Another loop involved in the coordination of E4P ($\beta 2\text{-}\alpha 2$, containing residues 133-135), however, becomes considerably more flexible (Fig. 4c-4f). Analogous observations were made for residues that coordinate the active site Mn^{2+} . Together with the innocuous structural changes upon inhibitor binding, this strongly suggests that the loss of enzymatic activity may be linked to a change in protein dynamics, as first proposed by Jiao *et al.* [34]. Possibly, down-regulation of MtDS activity upon effector binding is due to suboptimal coordination of the substrates, including inadequate interactions with the active site metal cofactor, or to interference with “breathing” motions of the enzyme necessary for efficient substrate binding and product release.

Structure of the MtCM-MtDS Complex in Solution

The absence of prominent structural changes at the MtCM active site combined with the perceived increase in the dynamics at the MtCM-MtDS interface upon binding of the

feedback inhibitors suggested that the allosteric regulation of MtCM activity involves the reversible dissociation of the MtCM-MtDS complex. Evidence that the crystallographically observed complex also exists in solution came from previous MtCM activity assays showing activation upon the addition of MtDS and from band-shift assays on native polyacrylamide gels [28]. Furthermore, association of MtCM with MtDS was also observable by preliminary size-exclusion chromatography (SEC) experiments in the presence of chorismate [28] as well as by recently reported analytical ultracentrifugation experiments [30]. Here, we examined the quaternary structure of the MtCM-MtDS system by Small-Angle X-ray Scattering (SAXS) experiments, SEC, and Size-Exclusion Chromatography Multi-angle Light Scattering (SEC-MALS).

SAXS allows for the calculation of molecular weight and overall shape of the examined proteins in solution [42]. For MtDS alone, the experimental data are in good agreement with the simulated scattering curve based on the crystallographic coordinates of the MtDS tetramer, indicating that MtDS has the same overall tertiary and quaternary structures in solution as in the crystal (Fig. S6a). This matches the findings of Jiao *et al.* [34]. Similarly, MtCM SAXS data in the absence of MtDS are compatible with the dimer shape observed in the crystal structure (Fig. S6a). When MtDS and MtCM were measured together at concentrations of 1 mg/ml and 0.195 mg/ml, respectively, corresponding to a 1:1 molar ratio, the radius of gyration increased in comparison to MtDS alone. The SAXS data are consistent with the heterooctameric MtCM-MtDS crystal structure, but the exact occupancy of MtCM in the MtCM-MtDS complex is difficult to estimate due to its comparably smaller mass (Fig. S6a). Under the high protein concentration regime of the SAXS experiments, this association was observed even in the absence of a ligand in the active site of MtCM, such as chorismate or compound **6** (Fig. S6b).

Next, we performed SEC-MALS experiments, which provide real-time quantitative data for the molecular mass and size of a sample. MtDS and MtCM were first investigated separately in order to establish molecular mass peaks and oligomerization states for the individual complex partners (Fig. 5a, left panel), and subsequently for the MtCM-MtDS complex (Fig. 5a, middle panel). In this method, the molecular masses are obtained by combining information from retention times and multi-angle light scattering, which takes the molecular shapes into account. The elution profile of MtDS (highest peak) corresponded to a mass of 201 ± 5 kDa (calculated mass: 207,308 Da), consistent with tetramer formation, while the elution of MtCM confirmed the formation of a dimer (experimentally determined mass: 21 ± 2 kDa; calculated mass: 20,179 Da). When co-injected at 1:1 and 1:5 molar ratios, MtDS and MtCM co-eluted as a complex with an apparent mass of 229 ± 6 kDa and 230 ± 2 kDa, respectively, consistent with complex formation (Fig. 5a, middle panel). As expected, a peak for excess free MtCM dimer was observed only in the case of the 1:5 mixture. The observed mass for this species is somewhat smaller than calculated for an MtCM-MtDS heterooctamer (247,664 Da), but would fit to a heterohexamer (calculated mass: 227,486 Da), consisting of an MtDS tetramer with just one MtCM dimer bound (or 50% MtCM occupancy, as seen in some of the crystals; data not shown). Consistent with SEC experiments on an FPLC HiLoad Superdex 75 column (Fig. S7), the HPLC-SEC experiments described below, and with earlier observations [28], the new larger complex was only seen in the presence of chorismate.²

The MtCM-MtDS Complex Dissociates upon Addition of Feedback Effectors

SEC-MALS experiments with Phe and Tyr (each 500 μ M) added to the running buffer resulted in a shift of the mass of the main protein peak from 229 ± 6 kDa to 189 ± 3 kDa for a 1:1 molar ratio of MtDS:MtCM, and from 230 ± 2 kDa to 213 ± 2 kDa for a 1:5 molar ratio,

² No peak consistent with MtCM-MtDS complex formation could be observed, not even at a molar ratio of 1:10 MtDS:MtCM, unless the MtCM ligand chorismate was present in the buffer (data not shown).

which may predominantly represent the MtDS tetramer. Thus, the complex appears to dissociate in the presence of the feedback inhibitors. In the case of the 1:1 complex, a shoulder appeared in the broad MtDS peak, at the elution volume corresponding to the free MtCM dimer (Fig. 5a, right panel). This shoulder was not observed in the measurements of the complex without Phe and Tyr. In addition, the signal for excess MtCM is stronger in the MtCM-MtDS sample when the effectors are present, indicating that MtCM dissociates from the complex. We also note that in the presence of the feedback inhibitors, the MtDS-peak shifts to higher elution volumes and shows significant tailing, which appears to suggest that the MtDS-homotetramer might partially dissociate. However, the calculated mass values (Fig. 5a, right panel) do not support such a conclusion. Possibly the higher elution volumes for MtDS after dissociation are an artifact of the alignment of the MtCM peaks (Fig. 5a, legend). It is well known that the chromatographic behavior of heteromeric assemblies is complex and can depend on numerous factors, such as slow association rates and specific chromatographic parameters (*e.g.*, column type, material, and length) [43, 44], hence peak positions can be deceptive. Clearly, the signal for excess MtCM is stronger in the MtCM-MtDS sample when the effectors are present, as expected for complex dissociation.

Confirmation of MtCM-MtDS dissociation upon feedback effector addition was, however, not possible with SAXS experiments (Fig. S6, right panel). This is likely due to the high MtDS and MtCM concentrations required for signal detection (4-20 μ M each), which were about two orders of magnitude above the K_d value of 140 nM for the MtCM-MtDS complex calculated from activation of the CM reaction upon titrating MtDS [28]. In contrast, independent analytical SEC experiments, performed at lower enzyme concentrations, with or without the addition of Phe and Tyr using HPLC gave analogous results to the SEC-MALS studies (Fig. 5b, panels I-VI). Again, formation of the MtCM-MtDS complex required the presence of the strongly UV-absorbing and enzymatically consumed ligand chorismate in the

running buffer, precluding simple analysis of SEC column eluates by UV absorption. Analysis of eluted fractions by SDS-PAGE revealed that mixing MtCM and MtDS in approximately equimolar ratios (1:1.5) resulted in co-elution of the two proteins (Fig. 5b, panel III) and shifted MtCM to earlier (larger) fractions. However, if 100 μ M Phe and 100 μ M Tyr were added to the SEC buffer for the MtCM + MtDS sample, MtCM elution was again retarded (Fig. 5b, panel VI). The presence of the feedback inhibitors had no influence on the elution of the individual MtDS or MtCM preparation (compare Fig. 5b, panel I & IV or Fig. 5b, panel II & V). We also observed no difference in the quaternary structures between samples containing Phe and Tyr (Fig. 5b) and those additionally provided with 100 μ M Trp (Fig. S8).

In summary, we have established by several independent SEC experiments that the formation of the MtCM-MtDS complex is highly dynamic and that the feedback inhibitors can dissociate the heterooctameric quaternary structure that forms in solution in the presence of the substrate chorismate.

Discussion

The shikimate pathway is a well-known metabolic sequence, which provides bacteria, fungi, and plants with essential aromatic amino acids and vitamins [21]. To preserve energy and building blocks needed for the expensive synthesis of aromatic compounds, the pathway is tightly regulated [21, 22]. Obvious key sites for regulatory interventions are DS, the first enzyme of the pathway, and the branch-point enzymes CM (for Phe and Tyr production) and AS (for Trp production) (Fig. 6). The present investigation focuses on the DS and CM reactions of the shikimate pathway in *M. tuberculosis*, for which we report mechanistic details of a fascinating novel mode of regulation differing substantially from textbook examples, including the prototypic shikimate pathway from *E. coli* (Fig. 6a). In *E. coli*, this biosynthetic route comprises three DS isoenzymes and two CM fusion proteins. The DS-isoforms each have allosteric sites that bind preferentially either Tyr, Trp, or Phe [21]. Furthermore, *E. coli* has two bifunctional, homodimeric proteins with CM activity: the CM-prephenate dehydratase (CM-PDT), encoded by *pheA*, and the CM-prephenate dehydrogenase (CM-PDH), encoded by *tyrA*. CM-PDT consists of three distinct domains, with the N-terminal domain having CM activity (designated as EcCM), a central domain providing PDT activity, and the C-terminal domain (a so-called ACT domain, [23]) responsible for feedback inhibition by Phe [45, 46]. Similarly, *E. coli* CM-PDH features an N-terminal CM domain followed by the C-terminal PDH domain, however, CM-PDH does not comprise a dedicated domain for feedback control. Instead, the site for feedback inhibition by Tyr is integrated within the catalytic PDH domain [47].

In contrast to *E. coli*, *M. tuberculosis* possesses but a single DS, which demands a more ingenious mechanism of regulation (Fig. 6b). This enzyme has recently been shown to be synergistically inhibited by pairwise combinations of Trp, Phe, and Tyr [33, 34], with full

inhibition requiring the simultaneous binding of all three aromatic amino acids, as shown by us (Fig. 2) and others [35]. Our structural characterization of the fully inhibited MtDS suggests that regulation of MtDS by the allosteric effectors is achieved through changes in protein dynamics rather than structural rearrangements. This had initially been proposed by Parker and coworkers based on an MtDS structure with Phe + Trp [34]. They observed that an active site loop ($\beta 2$ - $\alpha 2$) involved in coordination of the substrate E4P became more mobile when the feedback inhibitors are present, possibly increasing the entropic penalty for substrate binding. Our investigation of the protein dynamics in the triply-inhibited MtDS structure confirms the mobility of that loop. Additionally, we found that the loop on the other side of the catalytic center ($\beta 7$ - $\alpha 7$; Fig. 4) stiffens up. Together, all three allosteric regulators seem to affect proper substrate coordination and alignment, an important requirement for the aldol-like condensation of E4P and PEP to take place.

The aromatic amino acids not only inhibit DS activity; two of them (the end products of the CM branch, Phe and Tyr) additionally prevent activation of MtCM [28]. We have shown earlier that this naturally sluggish enzyme can be activated >100-fold by interaction with MtDS, and that Phe + Tyr addition downregulates CM activity of the assembly back to non-activated levels. Moreover, mutational studies suggested that the catalytic and regulatory machineries of MtCM are spatially distinct [28, 29], however, the details of the molecular mechanism of this regulation were unknown until now. In contrast to expectations for typical allosterically controlled enzymes, we demonstrate here that Phe and Tyr do not bind to MtCM, but solely to its complex partner, occupying the same sites as in the fully inhibited MtDS structure with Phe + Tyr + Trp. In fact, the structure superimposes exceedingly well with that of the effector-free MtCM-MtDS complex. We also provide functional proof for the coupling between effector binding in MtDS and CM activity of the complexed MtCM by showing that mutating the Tyr binding site in His₆-MtDS-R256A also abolishes Tyr

sensitivity of the CM reaction (Table S1). A recently published study independently came to the same result; it furthermore demonstrated in a parallel experiment that knocking out the Phe effector site in MtDS by the R171A mutation eliminated MtCM feedback regulation by this aromatic amino acid [30].

The fact that the effector binding sites were neither at the MtCM-MtDS interface nor connected to the active site of MtCM suggested that feedback inhibition of MtCM may be controlled by modulating its association to the activator protein MtDS rather than by direct or indirect interference with its catalytic machinery. This hypothesis is confirmed by our analytical SEC and SEC-MALS experiments, in conjunction with the just recently reported complementary investigation by sedimentation velocity and sedimentation equilibrium ultracentrifugation, and native PAGE analysis by Parker and coworkers [30]. The presence of Phe and Tyr caused a shift from the MtCM-MtDS complex to dissociated MtCM. This implies that binding of the allosteric effectors to MtDS results in a reduced affinity for its complex partner MtCM leading to a smaller fraction of the >100-fold activated MtCM. As a consequence of the overall diminished intracellular CM activity, less chorismate flows into the Phe and Tyr branch of the shikimate pathway, in turn reducing the concentrations of the feedback effectors (Fig. 7).

This unprecedented mode of allosteric feedback inhibition of CM activity in the MtCM-MtDS enzyme complex is a unique mechanism for implementing tight regulation of biosynthetic routes. In contrast to the *E. coli* enzymes, MtCM is not part of a feedback-controlled multi-domain or fusion protein; it is a stand-alone catalyst with mediocre activity. For full activity, MtCM requires the activation by its complex partner. Feedback regulation can now be exerted simply by allowing or disallowing productive MtCM-MtDS complex formation. In this scheme, the solitary MtDS acts as the crucial regulatory platform, and nature can spare a dedicated regulatory domain for MtCM. For instance, if only Phe and Tyr

are abundant, MtDS will dissociate from MtCM, thereby essentially shutting down the CM branch of the shikimate pathway, while the unoccupied Trp binding sites in MtDS still ensures provision of sufficient precursors for Trp biosynthesis.

What qualifies MtDS to act as such a versatile regulatory platform? Interestingly, at the molecular level, only very subtle structural adaptations are observed upon binding of the different aromatic amino acids, typically confined to the immediate environment of the effectors. We mainly note changes in the molecular dynamics. In particular, time-averaged molecular dynamics refinement revealed increased local molecular vibrations at the interface to MtCM. It thus seems that MtDS allosteric regulation is mainly entropy driven. Already previous MD simulations by Parker and coworkers indicated that binding of aromatic amino acids causes very specific changes in dynamic fluctuations within MtDS [34], even though these findings have to be taken with a grain of salt³. Nevertheless, the driving force for allosteric regulation could have its origins in the effector-induced structural adaptations at the internal MtDS subunit interfaces, transmitted to MtCM at the periphery of the complex over a significant distance (approximately 50 Å). Upon reexamining all available structures, we noticed that the observed small shifts in the atomic coordinates not only concern some of the MtDS residues next to the effector binding sites, but also extend into the neighboring MtDS subunit and to MtCM. Even though such small shifts (≈ 0.5 Å) are at the precision limit for a structure at this resolution, the fact that they propagate through the entire subunit suggests that they could reflect a modest, but critical realignment of the subunits (Fig. S4). Such a scenario is reminiscent of the regulation of yeast CM, even though the realignment in the *M. tuberculosis* system would be significantly less obvious (relative subunit rotation by approximately 1-2° compared to 15° in yeast CM [48]). Considering that the MtCM

³ The authors removed the Phe residues from the less occupied secondary Tyr sites prior to the MD simulations, which may have artificially increased the flexibility of these loops. Furthermore, the Phe-only crystal structure used (PDB ID: **3NUD** [33]) does not contain Mn²⁺ (even though the ion was shown in the publication by Jiao *et al.* [34]) This will most certainly increase mobility, since according to our experience a damaged manganese center correlates with lower occupancy of MtCM in the MtCM-MtDS complex structures.

homodimer has two anchoring sites on different subunits of MtDS, a small realignment may be all that is needed to weaken binding to the point where MtCM is expelled. At the high protein concentrations required for SAXS and crystallization, the equilibrium would nevertheless be pushed towards complex formation, although the so enforced complex would not be very stable. It is thus also conceivable that the increased dynamics that we observe is a consequence of the compromised MtCM-MtDS interaction rather than the origin of the lower binding affinity for MtCM. In contrast, we observed stabilization of the complex by the presence of chorismate. This might be interpreted as quenching of some of the dynamics by substrate-induced preorganization of MtCM, thereby offsetting the entropic penalty accruing during MtCM-MtDS interaction. Indeed, a comparison of published MtCM structures without ligand or with malate in the active site (PDB ID: **2QBV** and **2VKL**; [28]) revealed strongly decreased *B*-factors in the liganded case (not shown), in the loops interacting with MtDS (H1-H2 and H2-H3; Fig. 1).

Despite attempts to determine inhibitory effects at physiological effector concentrations and calculate apparent K_i values in our experiments (*e.g.*, for Fig. 2 and *Supplementary Information*), we are aware that their relevance in the cellular context may be rather qualitative than quantitative. For instance, it is clear that MtCM and MtDS interact *via* a highly dynamic equilibrium that is dependent on many factors, including the concentration of substrates and feedback effectors that are expected to fluctuate widely as a function of the metabolic needs of the organism. The intracellular concentrations of the two enzymes affect the equilibrium, too. Recently, Schubert and coworkers determined the absolute abundance of *M. tuberculosis* proteins in the intracellular proteome using the SWATH MS technique [49]. MtDS (Rv2178c) was listed as an abundant protein (calculated intracellular concentration ≈ 3 μ M) at different stages of the life cycle, whereas the low abundance of the smaller MtCM (Rv0948c) did not allow assignment with certainty. However, from the data set of the closely

related non-pathogenic *Mycobacterium bovis* (BCG strain) [49] we calculated the DS and CM concentrations to be 3 μM and 0.3 μM , indicating that the 10-fold excess of MtDS over MtCM employed in our kinetic CM activity assays coincides with the available proteomic data. An excess of DS is metabolically sensible, as the DS embodies CM-independent feedback control, whereas the CM requires the DS for regulation. That intracellular DS concentration is high in widely different physiological states (exponential growth, hypoxia-induced dormancy, and resuscitation) of *M. tuberculosis* and *M. bovis* underscores the importance of rigorous activity regulation at the enzyme level.

Considering the intracellular enzyme concentrations, the MtCM-MtDS complex must exist *in vivo*, given the apparent affinity constant $K_{d,\text{app}}$ of 140 ± 30 nM as derived in previous kinetic assays [28]. Parker and coworkers recently found a similar value in the absence of feedback effectors ($K_{d,\text{app}} = 300 \pm 80$ nM), and they could show that addition of 200 μM Tyr or Phe decreased the affinity by two or seven fold, respectively, whereas Trp had no effect on $K_{d,\text{app}}$ of the complex [30]. Taken together, the data suggest that the MtCM-MtDS assembly dissociates also under physiological conditions in response to the metabolic state of the cell, and in particular to increasing Tyr and Phe concentrations. Thus, despite the interdependence of several relevant equilibria with the fluctuating intracellular conditions, the available data allow for an elucidation of the molecular mechanisms and regulatory principles that operate in the MtCM-MtDS system.

That a DS is recruited to serve as a switchboard for regulation is not limited to *M. tuberculosis*. DS-associated CMs have also been described in other species from the bacterial class of *Actinobacteria* including *Corynebacterium glutamicum* [28, 50] and *Amycolatopsis methanolica* [51]. For instance, a CM-DS complex in *Brevibacterium flavum* was already mentioned in the 1980s as a ‘two-component’ CM with both CM and DS activity, dissociating in the presence of Tyr and Phe [52-55]. Also one of the two CMs described in *A. methanolica*

forms a complex with a DS, resulting in activation of the CM and rendering it sensitive to feedback inhibition by Phe and Tyr [51, 56]. In none of these anecdotally described CM-DS complexes has the detailed mechanism of allostery been elucidated; however, it is probably based on similar molecular principles as the one we characterized for *M. tuberculosis*. Furthermore, a comprehensive search for sequence homologs of MtCM and MtDS (Fig. S9) predicts that, based on the simultaneous presence of an AroQ_δ subclass CMs and an AroA_{II} type DS, this regulation mechanism occurs only in the phylum *Actinobacteria*, in about half of the taxonomic orders of the class *Actinobacteria*. Interestingly, these orders clearly show a closer phylogenetic clustering than the ones where the complex is absent, suggesting its evolutionary origin at the branch point of the *Propionibacteriales* (Fig. S9).

The confinement of the complex to specific orders within the *Actinobacteria* can be exploited to manipulate the shikimate pathway in an organism-specific manner. Discovery of small molecules that modulate the quaternary structure of protein complexes is in the focus of contemporary drug development [57-59]. A compound that disrupts and thus inhibits the CM-DS complex in pathogenic species from the *Corynebacteriales*, like *M. tuberculosis* or *Mycobacterium leprae*, may act as an anti-mycobacterial drug. At the same time, such an inhibitor would not harm beneficial gut bacteria from the *Bifidobacteriales*, as these do not possess the CM-DS complex (Fig. S9). Molecules that stabilize and thereby activate the CM-DS assembly in an organism-specific manner could be useful, too. For instance, a boost of the shikimate pathway in *Salinispora tropica* from the order *Micromonosporales*, known to incorporate aromatic building blocks into elaborate chemical structures, may facilitate the biotechnological production of important secondary metabolites [60].

In conclusion, this study elucidates a novel mode of regulation of the shikimate pathway machinery. All three aromatic amino acids synergistically inhibit the single initial DS enzyme, which in turn modulates the CM reaction in response to binding of the end

products of the Phe and Tyr specific branch. Thereby, the CM experiences indirect, inter-enzyme allostery, achieved mechanistically through the reversible dissociation of the CM-DS complex. The most remarkable feature of this set-up for controlling the metabolic flow through the shikimate pathway is the minimalistic but highly effective principle of packing all relevant allosteric sites onto a single regulatory platform allowing for a sophisticated command of more than a single enzyme. The regulation of the activities of entire enzyme assemblies through intricate but discrete regulatory changes in tertiary and quaternary structure, mediated by a minimal number of effector sites, is most likely not limited to the DS-CM system in *Actinobacteria*. It may in fact be a far more abundant general strategy of allosteric metabolic control than has been appreciated to date.

Materials and Methods

Materials and General Procedures

Chorismate (**4**) was produced by a published procedure [61]. All other chemicals were purchased from Sigma-Aldrich/Fluka. Bartlett's transition state analog (transition state analog **6** in Fig. 1) was prepared by R. Pulido according to [62].

Bacterial Strains and Plasmids

E. coli strains KA12 [63, 64] and KA13 [65, 66], and plasmids pKTDS-H, pKTDS-HN and pKTCMM-H [28] were previously described. The construction of plasmid pKTDS-HN-R256A is detailed in the *Supplementary Information*.

Protein Production and Purification

Native MtCM (encoded on plasmid pKTCMM-H), native MtDS (pKTDS-H), His₆-MtDS (pKTDS-HN), and His₆-MtDS-R256A (pKTDS-HN-R256A) were produced and purified by essentially following published protocols [28, 67], with the variations described in the *Supplementary Information* to optimize yields.

MtCM and MtDS Enzyme Assays

Continuous activity assays were performed at 30°C for MtCM and MtDS, as described previously [28], except that the MtCM assay was carried out in 50 mM BTP (1,3-bis[tris(hydroxymethyl)methylamino]propane), pH 7.5, 100 μ M MnCl₂, 0.2 mM PEP (**1**) and 1 mM TCEP (tris[2-carboxyethyl]phosphine hydrochloride) and that MtDS activity was measured in 50 mM BTP, pH 7.5, 1 mM TCEP, and 0.1 mM MnCl₂. For CM assays, the initial velocity of chorismate consumption (v_0) was monitored at 274 nm at 23 μ M of **4**, 300

nM His₆-MtDS, and 30 nM MtCM. For DS assays, PEP consumption was monitored at 232 nm at 25 nM MtDS (variant) concentration and 150 μ M of **1** and 150 μ M of E4P (**2**). The effector concentrations (Phe, Trp, and Tyr) were varied from 0 to 200 μ M.

Protein Crystallography

Crystals were obtained by the hanging drop vapor diffusion technique at 20 °C by mixing equivalent volumes of protein and reservoir solutions. For the MtDS-MtCM complex with Phe and Tyr, the protein solution consisted of 1.3 mg/ml MtDS, 0.5 mg/ml MtCM, 0.3 μ M transition state analog **6**, 200 mM (NH₄)₂SO₄, 20 mM BTP, pH 7.5, 150 mM NaCl, 0.2 mM PEP, and 0.1 mM MnCl₂, and the reservoir solution contained 0.1 M Tris-HCl, pH 7.9, 0.9 M (NH₄)₂SO₄, and 10% PEG400. Crystallization was triggered by streak seeding into drops equilibrated for 24 h, and crystals grew to dimensions of 40x40x900 μ m³ in the course of one week. Prior to data collection, crystals were soaked for <10 min in the reservoir solution “diluted” by the addition of 25% glycerol, the amino acids Phe and Tyr (5 mM each), and transition state analog **6** at 0.15 mM, and flash frozen.

The structure of fully inhibited MtDS was obtained by incubating His₆-MtDS with Phe, Tyr, and Trp (in an equimolar solution containing 1.6 mg/ml MtDS, 31 μ M each of Tyr, Trp, and Phe, in 20 mM BTP, pH 7.5, 150 mM NaCl, 0.2 mM PEP, and 0.1 mM MnCl₂) for 30 min on ice prior to co-crystallization, using a reservoir solution containing 0.1 M Tris-HCl, pH 8.2, 1.2 M (NH₄)₂SO₄, 12% glycerol, 0.5 mM MnCl₂, and 0.5 mM TCEP. Crystallization was triggered by microseeding into drops equilibrated for 24 h, and the crystals grew to 30x30x90 μ m³ in the course of one week. Prior to data collection, the crystals were soaked for <5 min in the reservoir solution additionally containing 25% glycerol and the amino acids at 1 mM, and flash-frozen in a nitrogen cryostream (100 K).

Data collection and refinement statistics are summarized in Table 1. Occupancies were refined by comparing the *B*-factors of the ligands with the interacting protein residues, and taking the difference Fourier maps into account. The ADP and occupancy refinement options in phenix.refine were used to optimize those parameters. Of the effector molecules in the MtDS structure, only the two Phe residues do not show full occupancy (0.87 and 0.93). For the MtCM-MtDS complex all effector molecules show full occupancy (Table S2). Further information regarding data collection, structure determination and refinement is provided in the *Supplementary Information*.

Small-Angle X-Ray Scattering (SAXS)

Small angle X-ray scattering data were collected at the ESRF bioSAXS beamline ID14-3 [68] at a wavelength of 0.931 Å. The buffer used for the SAXS experiments was 20 mM BTP, pH 7.5, 150 mM NaCl, 1 mM TCEP, 0.2 mM PEP, and 0.1 mM MnCl₂. MtDS and MtCM alone were measured at 1 mg/ml with three serial 2-fold dilutions (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml). To observe the complex, MtDS and MtCM were measured at 1 mg/ml and 0.195 mg/ml, respectively, which corresponds to 20 µM each and approximately half the concentrations of a typical crystallization experiment, and also in the corresponding 2-fold dilutions. Bartlett's transition state analog **6** was added at a concentration of 200 µM to 1 mg/ml MtCM (alone) or at 50 µM to the complex, and the aromatic amino acids Tyr, Phe, and Trp at 200 µM or 2 mM final concentration. A 25 µl protein sample was loaded into the test capillary and flowed at a constant rate for 10 exposures of 6 s each. Extra flow time was 35 s.

Size-Exclusion Chromatography-Multiangle Light Scattering (SEC-MALS)

In a typical experiment, 20 µl of protein solution were injected. MtCM was concentrated to 16.75 mg/ml, MtDS to 1.5 and 1.6 mg/ml. MtCM and MtDS were incubated on ice for 15 to

30 min in 1:1, 1:5 and 1:10 molar ratios. All samples were centrifuged for 15 min at 13,200 rpm, 4°C in order to remove aggregates. The running buffer consisted of 20 mM BTP, 150 mM NaCl, 1 mM TCEP, 0.2 mM PEP, and 0.1 mM MnCl₂ at pH 7.5. For individual experiments, chorismate (final concentration: 500 µM) and/or Phe and Tyr (final concentration: 500 µM each) were added. The experimental setup comprised an Agilent SEC-5 column (5 µm, 4.6 x 150 mm), an Agilent 1100 series HPLC system, including an online degasser (G1379A), an isocratic pump (G1310A), a thermostated column compartment (G1316A), a fluorescence detector (G1321A), a Diode Array Detector (G1315B), a refractive index detector (G1362A) a Dual Loop Autosampler (G2258A), a quaternary pump (G1311A) and a manual injector. Light scattering was measured with a triple-angle light scattering detector MiniDAWN™ TREOS® from Wyatt Technologies. The SEC-column was cooled to 25°C during the individual runs and the flow rate was set to 0.4 ml/min. The average molecular weight was established with the ASTRA® software from Wyatt Technologies (V 5.3.4.10) applying the Zimm light scattering model model (Wyatt Technology Corp.) and using a dn/dc value of 0.185 ml/g. The individual molecular masses of MtCM, MtDS and the MtCM-MtDS complexes with and without effectors were determined as an average over the individual peaks in each measurement, and represent averages and standard deviations of two to four independent injections.

Analytical Size-Exclusion Chromatography (SEC) by HPLC and Detection by SDS-PAGE

Analytical SEC was performed using a Yarra™ 3u SEC-3000, 300 x 4.6 mm (Phenomenex) HPLC column. The buffer containing 20 mM BTP, pH 7.5, 150 mM NaCl, 1mM TCEP, 0.2 mM PEP, 0.1 mM MnCl₂, and 100 µM chorismate was used as the mobile phase. If necessary, 100 µM Tyr, 100 µM Phe, and 100 µM Trp was added. For column loading, 40-µl

samples containing 10 μ M MtCM and 15 μ M MtDS were injected and run for 20 min at a flow rate of 0.35 ml/min.

Successive fractions of 550 μ l were collected and the proteins were concentrated for analysis by precipitation upon adding an equal volume of 20% TCA. After incubating for 1 hour at RT the proteins were pelleted by centrifugation for 20 min at 12,000 rpm. The pellets were washed twice with acetone, dried, and dissolved in 15 μ l SDS loading buffer (50 mM Tris-HCl, pH 6.8, 0.1 M dithiothreitol, 2% sodiumdodecylsulfate, 0.1% Bromophenol Blue, 10% glycerol). Samples (1 μ l) were analyzed by 20% SDS-PAGE using the PhastSystem from GE Healthcare and their Low Molecular Weight Marker as the size standard. Elution volumes (fraction number) and the presence of individual protein species on the gels correlated well upon repeating the experiments.

Search for Homologous Sequences

To predict the occurrence of a non-covalent CM/DS complex, different taxa were searched by BLASTP [69] for homologs of MtCM (CM of the AroQ₈ subclass) and MtDS (DS of the AroA_{II} class), as defined previously [28]. Hits were considered significant if the expectation value E was 10^{-5} or lower. Representative AroQ₈ sequences were further checked for the presence of Arg46 and an Arg85-Gly86 dyad (numbering with respect to MtCM; [28]. All AroQ₈-containing taxa considered in Fig. S9 also contained AroA_{II} members.

The atomic coordinates have been submitted to the Protein Data Bank⁴ [70] with accession codes **5CKV** (MtDS-FYW) and **5CKX** (MtCM-MtDS-FY).

⁴ www.pdb.org

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Author Contributions

P.K. and U.K. conceived the study; S.M., K.R., M.Ö. and J.K. performed the experiments, supervised by A.v.E., P.K. and U.K.; S.M., K.R., M.Ö., J.K., S.S., A.v.E., P.K. and U.K. analyzed the data; and S.M., K.R., M.Ö., J.K., S.S., A.v.E., P.K. and U.K wrote the paper.

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Figure Legends

Figure 1: Structures of MtDS and the MtCM-MtDS complex, and catalyzed reactions.

(a) Structure of the MtDS tetramer (PDB ID: **3NV8** [33]), showing subunits A and B (green) as well as the dimer/tetramer interfaces. The accessories to the TIM-barrel fold are depicted in blue, and Mn^{2+} at the MtDS active sites as purple spheres. The nomenclature for the interfaces and the location of the effector binding sites is indicated. (b) DAHP-synthase reaction: Aldol-like condensation of phosphoenolpyruvate **1** and D-erythrose-4-phosphate **2** to 3-deoxy-D-*arabino*-heptulosonate-7-phosphate **3** and inorganic phosphate. (c) Structure of the MtCM-MtDS-complex (PDB ID: **2W1A** [28]). MtCM (yellow) bridges the tetramerization interface of MtDS (orange/green). The transition state analog **6** of the CM reaction is bound in the MtCM active sites (blue sticks). (d) CM reaction: Claisen-type rearrangement of chorismate **4** to prephenate **5**.

Figure 2: Inhibition of enzymatic activity by allosteric effectors Phe, Tyr, and Trp.

Columns in the top half of the bar graph show the relative remaining DS activity of native MtDS (grey) and His₆-MtDS (blue) in the absence and presence of Phe, Tyr, and Trp, alone or in different combinations. Effector concentrations were 200 μM for the single additions, and 100 μM for each compound in the combinations. Specific initial velocities ($v_0/[\text{DS}]$) of phosphoenolpyruvate consumption at 30°C were determined at 150 μM each of **1** and **2**. 100% corresponds to $2.8 \pm 0.4 \text{ s}^{-1}$ for native MtDS and $2.9 \pm 0.8 \text{ s}^{-1}$ for His₆-MtDS; error bars represent the standard deviations (σ_{n-1}) of ≥ 2 independently purified and assayed enzyme batches. Columns projecting downwards show the relative remaining CM activities of MtCM in the complex with His₆-MtDS upon addition of the aromatic amino acids and their combinations. Green columns show data collected at 25 μM of each effector to simulate

physiological concentrations in the bacterial cytoplasm [38]. The specific activity of chorismate consumption ($v_0/[MtCM]$) in the absence of effectors ($4.0 \pm 0.2 \text{ s}^{-1}$) was defined as 100%. All values represent the mean of four measurements, with the error bars showing the standard deviation. For comparison, data reported earlier [28] with effector concentrations as for the DS assays is indicated with purple columns for the single additions (200 μM) and the combination Phe + Tyr (100 μM each). Corresponding kinetic measurements conducted for MtCM activation by the native MtDS format gave identical results to His₆-MtDS (Table S1).

Figure 3: Crystal structure of the Phe and Tyr-inhibited MtCM-MtDS complex. (a) MtCM-MtDS complex with the feedback inhibitors Phe (black) and Tyr (red), shown in space-filling representations, bound to their allosteric binding sites in MtDS (green/ orange; MtCM dimers in yellow). Mn²⁺ in the active site of MtDS is represented by purple spheres and the transition state analog **6** of the CM reaction by blue sticks. (b) Superimposition of the complex structure without (PDB ID: **2W1A** [28]; grey) and with the amino acid feedback regulators bound (PDB ID: **5CKX**; MtDS: green/ orange, MtCM: yellow). The structural changes upon amino acid binding are small and generally limited to their binding regions. (c) and (d) *B*-factor representations of (c) MtCM-MtDS in complex with **6** and (d) the feedback-inhibited MtCM-MtDS in complex with **6**. A spectrum of colors from dark blue to red illustrates increasing *B*-factors. (e) and (f) Ensemble refinement results of the MtCM-MtDS complex with **6** (space-filling representation) in the absence (e) and presence (f) of the two allosteric regulators of the MtCM reaction. Whereas effector binding decreases mobility of the MtDS active site loop $\beta 7$ - $\alpha 7$ (black arrows), the adjacent MtDS-MtCM interface experiences an increase in dynamics (emphasized by the red arrows and circles). The upper part of the heterooctamer, as shown in the figure, is related to the lower part by

crystallographic symmetry. In the subunits on the right, the mobile loop $\beta 7\text{-}\alpha 7$ prevents a clear view of the MtDS-MtCM interface.

Figure 4: Changes upon binding of Phe, Tyr, and Trp to MtDS. (a) MtDS structure in complex with Phe (black), Tyr (red), and Trp (cyan). The aromatic amino acids occupy the previously identified binding sites. Purple spheres identify the Mn^{2+} sites. (b) Superimposition of the ligand-free MtDS structure (PDB ID: 3NV8 [33]; grey) and the fully inhibited co-crystal structure of MtDS (PDB ID: 5CKV, green/ orange). Only minor changes close to the amino acid binding sites are observed. (c) and (d) *B*-factor representations (C_α atoms) of ligand-free MtDS (PDB ID: 3NV8) and the MtDS complex with Phe, Tyr, and Trp (PDB ID: 5CKV), respectively. (e) and (f) Ensemble refinement results of the ligand-free MtDS structure (PDB ID: 3NV8) and the fully inhibited MtDS structure (PDB ID: 5CKV), respectively. Considerable changes in the dynamics are observed in the substrate binding sites (loops $\beta 2\text{-}\alpha 2$ and $\beta 7\text{-}\alpha 7$; boxed) and at the MtCM docking site (arrow).

Figure 5: Influence of the allosteric effectors Phe, Tyr (and Trp) on the integrity of the MtCM-MtDS complex in solution. (a) SEC-MALS experiments. Detection of the MtCM-MtDS complex by SEC required addition of the CM substrate chorismate (500 μM) to the chromatography buffer. Since the presence of this strongly UV absorbing and enzymatically converted compound precluded calibration of the SEC-MALS data *via* UV signals, the curves were aligned by superimposing the MtCM peaks. Refractive index (RI; dotted lines), light scattering signal (LS; solid lines), and molecular mass (Mw, circles) are shown for MtDS (red, left panel), MtCM (black, left panel), and MtCM:MtDS in ratios of 1:1 (blue) and 5:1 (gray) (middle and right panels); vertical interrupted lines indicate the elution volumes of the uncomplexed oligomers of MtCM (1.89 ml) and MtDS (1.59 ml), as determined in the left

panel. The main protein fraction of the MtCM-MtDS injections eluted at 1.54 ml (middle panel), indicative of a larger molecular mass compared to uncomplexed MtDS. In the presence of Phe and Tyr (500 μ M each; right panel), the 1:1 MtCM:MtDS complex exhibits a shoulder corresponding to the MtCM dimer (black arrow next to the RI curve), and the signal for excess MtCM increased strongly in the 5:1 sample compared to the middle panel, while the main peaks are shifted to the right (1.64 ml), as expected for dissociation of the MtDS-MtCM complex. (b) Analytical SEC experiments. Samples containing MtDS alone (I and IV), MtCM alone (II and V) and MtDS together with MtCM (III and VI) were run on a SEC-3000 HPLC column in the presence of 100 μ M chorismate. Fractions from runs without feedback inhibitors (I-III) and with 100 μ M each of Tyr and Phe (IV-VI) were analyzed by SDS-PAGE. Numbers above lanes represent the successive fraction numbers; M, size marker with values in kDa.

Figure 6: Regulatory circuits of enzymes in the aromatic amino acid biosynthesis pathway of *E. coli* and *M. tuberculosis*. Feedback inhibition is shown as red lines, activation as a green arrow. Feedback inhibition of DS, CM, prephenate dehydratase (PDT), prephenate dehydrogenase (PDH) and anthranilate synthase (AS) is shown. (a) In *E. coli* (Ec), three isoforms of DS are present, each of which is inhibited (to some extent) by one of the three aromatic amino acids. CM fusion proteins CM-PDT and CM-PDH are feedback inhibited by the end product Phe and Tyr, respectively. AS is sensitive to Trp. (b) Even though *M. tuberculosis* (Mt) encodes fewer individually regulated enzymes, global regulation of metabolic flux through the shikimate pathway is still possible by a sophisticated network of allosteric feedback inhibition by the three aromatic amino acids. ACT, amino acid binding domain.

Figure 7: Cartoon of the shikimate pathway of *M. tuberculosis*, regulated by ‘inter-enzyme allostery’. The shikimate pathway yields the three aromatic amino acids Phe (black triangle), Tyr (red diamond), and Trp (light blue ellipse). The activity of the first enzyme of the pathway, DAHP synthase (MtDS, in orange and green), is shut down by the presence of all three feedback inhibitors, whereas efficient inhibition of the key branch point enzyme chorismate mutase (MtCM, in yellow) requires the presence of only the corresponding branch end products Phe and Tyr. Intriguingly, these effectors do not bind to MtCM, but to its complex partner MtDS, approximately 50 Å from the MtDS-MtCM interface. MtCM requires activation by complex formation with MtDS for efficient catalysis. Upon effector binding, the MtDS-MtCM complex dissociates and activation of MtCM by MtDS ceases, shutting down Phe and Tyr biosynthesis.

Table 1 Summary of data collection and refinement statistics

	MtCM-MtDS-FY	MtDS – FYW
<i>Data collection</i>		
ESRF beam lines	ID 14-4	ID 29
Space group	<i>P</i> 3 ₂ 2 1	<i>P</i> 3 ₂ 2 1
<i>Unit cell parameters</i>		
a, b, c [Å]	203.4, 203.4, 67.1	204.7, 204.7, 66.8
α, β, γ [deg]	90, 90, 120	90, 90, 120
Resolution [Å]	101.5 - 2.7 (2.80 - 2.70)	47.3 - 2.8 (2.89 - 2.79)
R_{merge}^a	0.08 (0.53)	0.16 (0.87)
$CC_{1/2}$	n.d.*	0.995 (0.803)
$I/\sigma(I)$	8.2 (1.8)	13.9 (2.8)
Completeness [%]	98.5 (99.4)	99.4 (94.9)
Multiplicity	2.2 (2.2)	8.0 (7.5)
<i>Refinement</i>		
No. of total reflections	43181 (6292)	321302 (28861)
No. of unique reflections	43148 (4330)	40042 (3796)
$R_{\text{work}}/R_{\text{free}} (\%)^b$	18.8 (27.1)/ 25.3 (31.9)	16.7 (27.5)/ 22.6 (30.1)
<i>No. of atoms</i>		
Protein	8162	6983
Ligand/Ion	107	59
Water	55	41
<i>B-factors [Å²]</i>		
Protein	63.4	49.0
Ligand/Ion	73.3	64.3
Water	45.0	40.8
<i>R.m.s.d. from ideal geometry</i>		
Bond lengths [Å]	0.015	0.015
Bond angles [deg]	1.43	1.32
<i>Ramachandran</i>		
Favoured [%]	89	94.4
Disallowed [%]	1.3	0.8
PDB ID	<u>5CKX</u>	<u>5CKV</u>

Values in parentheses refer to the highest resolution shell. n.d., not determined.

*This data set was collected more than a year before the publication by Karplus & Diederichs [71], where $CC_{1/2}$ was introduced as the main quality criterium, over R_{merge} .

^a $R_{\text{merge}} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i \langle I_h \rangle$ where $\langle I_h \rangle$ is the average intensity over symmetry related measurements

^b $R = \sum ||F_o| - |F_c|| / \sum |F_o|$ where F_o and F_c are the observed and calculated structure factors, respectively. R_{free} is R calculated for 5% randomly selected reflections, which were omitted from the refinement.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

(a) MtCM and MtDS reference profiles without effectors

MtCM-MtDS complex without effectors

MtCM-MtDS complex with Phe and Tyr

(b)

Figure 6

Figure 7

***Graphical Abstract**