A single amino acid substitution uncouples catalysis and allostery in an essential biosynthetic enzyme in *Mycobacterium tuberculosis*

Wanting Jiao\(^{1,2,*}\), Yifei Fan\(^{1,2,*}\), Nicola J. Blackmore\(^1\), and Emily J Parker\(^{1,2*}\)

From \(^1\)Maurice Wilkins Centre for Molecular Biodiscovery, Biomolecular Interaction Centre, University of Canterbury, Christchurch, New Zealand; \(^2\)Ferrier Research Institute, Victoria University of Wellington, Wellington, New Zealand

Running title: *Uncoupling dynamic allostery in DAH7PS*

*To whom correspondence should be addressed: Emily J Parker: Ferrier Research Institute, Victoria University of Wellington, Wellington, New Zealand; emily.parker@vuw.ac.nz; Tel. (64) 4-4639055.

\(^*\)Both authors contributed equally to this work

**Keywords**: Allosteric regulation, enzyme catalysis, enzyme kinetics, mutagenesis, *Mycobacterium tuberculosis*, 3-deoxy-d-arabino-heptulosonate 7-phosphate synthase (DAH7PS), protein dynamics, statistical coupling analysis, aromatic amino acid biosynthesis, shikimate pathway, ligand binding

**ABSTRACT**

Allostery exploits the conformational dynamics of enzymes by triggering a shift in population ensembles towards functionally distinct conformational or dynamic states. Allostery extensively regulates the activities of key enzymes within biosynthetic pathways to meet metabolic demand for their end products. Here, we have examined a critical enzyme, 3-deoxy-d-arabino-heptulosonate 7-phosphate synthase (DAH7PS), at the gateway to aromatic amino acid biosynthesis in *Mycobacterium tuberculosis*, which shows extremely complex dynamic allostery: three distinct aromatic amino acids jointly communicate occupancy to the active site via subtle changes in dynamics, enabling exquisite fine-tuning of delivery of these essential metabolites. Furthermore, this allosteric mechanism is co-opted by pathway branch-point enzyme chorismate mutase upon complex formation. In this study, using statistical coupling analysis, site-directed mutagenesis, isothermal calorimetry, small-angle X-ray scattering and X-ray crystallography analyses, we have pinpointed a critical node within the complex dynamic communication network responsible for this sophisticated allosteric machinery. Through a facile Gly to Pro substitution, we have altered backbone dynamics, completely severing the allosteric signal yet remarkably, generating a non-allosteric enzyme that retains full catalytic activity. We also identified a second residue of prime importance to the inter-enzyme communication with chorismate mutase. Our results reveal that highly complex dynamic allostery is surprisingly vulnerable and provide
further insights into the intimate link between catalysis and allostery.

Proteins are dynamic molecules that exert functionally important motions over both long (milli-seconds to seconds) and short (femto-seconds to micro-seconds) timescales. Protein structures and conformational dynamics are tuned for the delivery of complex protein roles. Allostery, where a signal initiated by ligand binding is communicated to elicit a remote functional response, exploits conformational dynamics. Such ability to communicate between sites and deliver control on activity is essential for biosynthetic enzymes. Most of these enzymes utilize allostery as a mechanism to control metabolic flux and enable delivery of metabolites in response to metabolic demand.

The enzyme 3-deoxy-d-arabinohexulonate 7-phosphate synthase (DAH7PS) catalyzes the first step in the shikimate pathway and is feedback regulated by the pathway end products, the aromatic amino acids Phe, Tyr and Trp. The DAH7PS family is categorized into subfamilies including type Iα, Iβ, and type II DAH7PSs, which display a wide range of mechanisms for allosteric regulation. The common structural feature among all subfamilies is the core catalytic unit, which adopts a (β/α)8 barrel fold and hosts the active site. The subfamilies differ in both quaternary structure and in the types of structural elements decorating the catalytic core, and these decorations deliver the binding sites for allosteric ligands. Type Iα DAH7PS, including those from *Escherichia coli*, *Saccharomyces cerevisiae*, and *Neisseria meningitidis* contains an N-terminal extension and extended loops to the core barrel, which host the binding site for a single allosteric ligand (Figure 1A). Whereas type Iβ contains a group of DAH7PS which has a discrete allosteric do main covalently linked to the catalytic barrel, such as the those from *Thermotoga maritima* or *Geobacillus sp* (Figure 1B).

The first fully characterized type II DAH7PS was from *Mycobacterium tuberculosis* (MtuDAH7PS, Figure 1C). MtuDAH7PS contains both an N-terminal extension (three helices) and two other inserted helices to the core catalytic barrel, which constitute distinct allosteric binding sites specific to three different allosteric ligands (Trp, Phe and Tyr). The N-terminal extension (helices α0a-α0c) contributes to binding sites for Phe and Tyr, whereas the inserted helices (α2a-α2b) contribute to the Trp binding site (Figure 1C). The additional structural elements are also associated with the formation of the dimer and tetramer interfaces to form the tetrameric quaternary structure of MtuDAH7PS (Figure 1D). MtuDAH7PS exhibits a complex internal dynamic communication network resulting in a highly sophisticated regulation mechanism. Binary combinations of aromatic amino acids that include Trp significantly inhibit catalytic activity of DAH7PS, and the presence of all three aromatic amino acids completely abolishes activity. Communication exists between allosteric sites as well, as binding of one ligand favors subsequent binding of other ligands. Binding of the allosteric ligands is not associated with any significant conformational change and the mechanism of the allosteric regulation in MtuDAH7PS is mediated by internal changes in protein dynamics.

The complexity of this dynamic allosteric mechanism is not limited to within the MtuDAH7PS enzyme. It has been shown that the allosteric machinery of MtuDAH7PS can be utilized by another small protein partner, chorismate mutase (MtuCM), by forming a non-covalent complex. CM catalyzes a downstream reaction in the shikimate pathway, converting chorismate to prephenate, and is at the start of the branchpoint of the shikimate pathway that leads to the production of Phe and Tyr. It has been shown that complex formation between MtuDAH7PS and MtuCM significantly enhances the catalytic activity of MtuCM. Furthermore, the normally unregulated MtuCM becomes sensitive to inhibition by Phe, which binds to the dimer interface on MtuDAH7PS. A similar scenario was also observed in another type II enzyme, i.e. the DAH7PS from *Corynebacterium glutamicum*, indicating this inter-enzyme communication may be a more common feature among the type II DAH7PS enzymes.

In this study, we aimed to pinpoint the precise molecular mechanism that governs the
communication networks that allow for such sophisticated allosteric control of the gatekeeper enzyme for aromatic biosynthesis. Using type II MtuDAH7PS as a reference, sequence correlations and key residue roles were explored, illuminating a crucial point in the internal dynamic pathway that is responsible for propagation of allosteric signal. We report a variant enzyme with interrupted signal at this crucial point that is completely lacking any allosteric response yet maintains full catalytic capacity.

RESULTS

Sequence analysis and protein sectors in type II DAH7PS. Type II DAH7PS utilizes internal dynamic networks to facilitate allostery, thus it is not possible to pinpoint residues involved in the network purely based on structural inspection. Due to the functional importance of allostery, such networks are expected to be conserved and thus to have coevolved, so that information regarding the internal dynamic communication pathway lies in the correlations between residues in the sequences of type II DAH7PS. Statistical coupling analysis (SCA) has been demonstrated to identify successfully groups of residues that have coevolved to preserve function or structural integrity of enzyme families.20-22 We employed SCA to investigate sequence co-variations within the type II DAH7PS family that may reflect important communication pathways that are preserved due to functional importance, such as allostery, through the course of evolution. In SCA, the correlation matrix of residue conservations is computed from a multiple sequence alignment of the protein family, and analyzed by spectral decomposition using independent component analysis (ICA).20 Independent components (IC) are then identified, which represent conserved, differentially evolving functional units in protein families. The ICs that show inter-correlations are further grouped into protein sectors, which in other enzyme families, have been shown to correspond to distinct functional roles.20

A total of 4217 amino acid sequences of type II DAH7PS were included in the initial multiple sequence alignment (MSA). Pairwise identities between the sequences in the alignment were analyzed and showed a distribution with an average identity value of 50% (Supporting Information Figure S1). The initial MSA was then processed by removing highly gapped positions and sequences, and mapping position numbers to the reference sequence (MtuDAH7PS in this case).20-23 The processed MSA contained 3345 sequences with 431 positions. A weighting factor was then applied to correct for the biasing effects of sequences with high identities, and the effective number of sequences in this processed MSA was calculated to be 510, which indicates the sequences in the processed MSA are sufficient to give good estimates of amino acid frequencies that are representative of the protein family.20

The correlations of positional conservations in the processed MSA of type II DAH7PS were analyzed by SCA. Eleven ICs were identified, which were further grouped into three protein sectors (Supporting Information Figure S2). In order to examine possible functional roles for these protein sectors, residues identified in each protein sector were mapped onto the structure of MtuDAH7PS, and the locations of protein sectors and ICs were examined.

Sector 1 contains 119 residues, and consists of ICs 1, 7 and 8 (Figure 2A). Residues in ICs 1 and 8 are mostly located near the center of the barrel hosting the active site (Supporting Information Figure S3). Interestingly, IC 1 contains a number of residues in the active site that are known to play a role in substrate binding, such as K306, R337, R126, R284 for PEP binding, and R135, S136, W280, E248 and K133 for E4P binding.14 IC 1 also contains residues of the Phe-specific binding site, including F91, P56, and Y173. Residues in IC 7 appear to form a potential pathway between known allosteric binding sites for Trp and Phe (Figure 2B), and contains residues of the Trp binding site (A192, L107, and L194). The locations and functional roles of residues of Sector 1 suggest that it likely contributes to the communication between the allosteric ligand sites, and to the active sites within the DAH7PS tetramer.

Sector 2 contains 136 residues, which include residues in the Tyr-specific binding site (R256, L271 and L259), the Trp binding site (K123, V111, A241 and V197), and the Phe-specific binding site
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(N175). It also includes a number of DAH7PS residues that contribute to the DAH7PS-CM interface (as observed in MtuDAH7PS-CM complex structure), such as R213, D389, R344 and E451 (Figure 3A). In contrast to Sector 1, residues in Sector 2 tend to be clustered on the exterior of the catalytic barrel. Sector 2 consists of ICs 2, 3, 4, 5 and 11. Residues in ICs 3, 4, 5, and 11 do not appear to form a linked pathway between any known functional sites of type II DAH7PS (Supporting Information Figure S4). However, residues in IC 2 appear to connect the allosteric ligand sites to the interface that is formed between DAH7PS and CM upon complexation (Figure 3B). It is possible that residues in Sector 2 play a role to facilitate the communication between type II DAH7PS and CM upon complex formation, with major contributions from IC 2.

Sector 3 contains 49 residues and consists of ICs 6, 9, and 10 (Supporting Information Figure S5), which are mostly located between helices and $\beta$-sheets. Most of the residues identified in Sector 3 do not belong to any known functional sites on DAH7PS, thus the functional role of sector 3 is yet unclear. Previous SCA studies on other protein families have also identified protein sectors with unknown apparent functional roles. In addition, co-evolved residues could be identified for structural or functional importance. Based on the observed locations of Sector 3, it likely contributes to maintaining of structural fold of type II DAH7PS family.

A single amino acid substitution within Sector 1 completely annihilates allostery. SCA identified groups of residues that may play an important role in the communication pathways between the different functional sites in type II DAH7PS. In particular, both IC 7 in sector 1 and IC 2 in sector 2 form potential communication pathways connecting different sites, and interestingly, strong cross-sector correlations are also observed between IC 7 and IC 2 (Supporting Information Figure S6), indicating the cross-talk between different internal residue networks and the level of complexity of the whole signal propagation mechanism.

In order to further interrogate the communication pathways in MtuDAH7PS without physically changing any binding sites, we substituted G190 from IC 7 with a proline residue using site-directed mutagenesis, with the intention to alter the dynamic properties within the predicted allosteric communication pathway (Figure 4A). G190 is located in close proximity to the bound Trp ligand (~13 Å) but is not directly involved in the binding of Trp. G190 is remote from the active site with a distance of ~30 Å to the active site Mn$^{2+}$ ion (Figure 4B).

The G190P substitution did not alter the catalytic features of the enzyme. Kinetic assay results indicated $Mtu$DAH7PS$^{G190P}$ displayed highly similar kinetic characteristics in comparison to the wild-type enzyme, with comparable $K_M$ and $k_{cat}$ values (Table 1). Furthermore, $Mtu$DAH7PS$^{G190P}$ retained the ability to activate MtuCM, demonstrating that the inter-enzyme interaction was intact (Table 1). Both the wild-type enzyme and substituted MtuDAH7PS enhanced the activity of MtuCM over 100-fold in similar ways, by lowering the $K_M$ value for chorismate and enhancing turnover.

Whereas this G190P substitution had no effect on catalysis, the impact on allostery was far more striking. In marked contrast to the wild-type enzyme, which is synergistically inhibited by combination of aromatic amino acids involving Trp, the activity of $Mtu$DAH7PS$^{G190P}$ variant was completely unaffected by the presence of aromatic amino acids either alone or in combinations (Figure 5A). Full activity was retained even in the presence of all three amino acids, which was shown to completely inhibit the wild-type enzyme. These results indicate that this single amino acid substitution at G190 has completely abolished the allosteric response in the $Mtu$DAH7PS enzyme, while maintaining its full catalytic capacity.

The lack of allosteric response in $Mtu$DAH7PS$^{G190P}$ variant is due to severed communication pathways as a result of the G190P substitution, rather than to the attenuation of ligand binding. This was confirmed using differential scanning fluorimetry (DSF) experiments in the presence and absence of allosteric ligands, results of which showed large changes in $T_m$ associated with ligand binding. These changes in thermal stability, precisely paralleled those observed for $Mtu$DAH7PS$^{WT}$ (Table 2). As the G190P
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substitution is in close proximity to the Trp binding site, we further probed the binding of Trp to the MtuDAH7PS<sup>G190P</sup> variant using isothermal titration calorimetry (ITC) experiments. A $K_d$ value of 11.7 ± 0.7 μM was obtained (Supporting Information Figure S7). Albeit this measurement shows a slight increase compared to the wild-type enzyme ($K_d = 4.7 ± 0.1$ μM),<sup>15</sup> the result confirms binding of Trp to MtuDAH7PS<sup>G190P</sup> with comparable affinity, consistent with the DSF findings.

We also examined the effect of the G190P substitution on the allosteric response of MtuCM. Similar to that observed for the wild-type enzyme, in which Phe demonstrated the most pronounced inhibitory effect by single aromatic amino acid, Phe alone remained inhibitory on MtuCM activity in the presence of MtuDAH7PS<sup>G190P</sup> (Figure 5B). However, different responses were observed in the presence of all three aromatic amino acids. The wild-type MtuDAH7PS-MtuCM complex exhibits synergistic inhibitory response to ternary combination of aromatic amino acids resulting in only approximately 5% activity remaining. In contrast, combinations of aromatic amino acids do not deliver a greater inhibitory response on MtuCM activity in the presence of MtuDAH7PS<sup>G190P</sup> variant. The lack of synergy between Phe/Tyr and Trp in the presence of MtuDAH7PS<sup>G190P</sup> implies that the synergistic communication between allosteric sites has been severed by the G190P substitution. The inter-enzyme catalytic enhancement and allosteric regulation between MtuDAH7PS and MtuCM appears to be otherwise unaffected by the G190P substitution.

We then further interrogated the effect of the G190P substitution on the structure of the DAH7PS enzyme. The solution structure of MtuDAH7PS<sup>G190P</sup> variant was analyzed by small angle X-ray scattering (SAXS) and compared with wild-type enzyme (Figure 5C). SAXS data confirmed the quaternary structure of MtuDAH7PS<sup>G190P</sup> variant was maintained (Table 3). As for the wild-type enzyme, MtuDAH7PS<sup>G190P</sup> adopts a tetrameric subunit arrangement, with comparable $R_g$, $D_{max}$ and porod volume values. MtuDAH7PS<sup>G190P</sup> experimental scattering profile agrees well with the theoretical scattering generated from MtuDAH7PS wild-type crystal structure (PDB 3NV8). We also obtained the crystal structure of the MtuDAH7PS<sup>G190P</sup> variant (PDB 6PBJ), which showed high similarity to that of the wild-type structure (Figure 5D), with root-mean-square-deviation value of 0.35 Å (matching 1556 Cα atoms of the tetramer) compared to the wild-type crystal structure (PDB 3NV8), further confirming that the overall structure is undisrupted by the G190P substitution. Several regions are disordered in the structure of MtuDAH7PS<sup>G190P</sup>, including residues 264-266, 372-380, and 429-436 in chain A, and residues 11-15, 264-265, 372-381 and 413-443 in chain B. Clear electron density is observed around the Pro residue at the site of mutation (Supporting Information Figure S8A), with minimal change in conformations of local residues in close proximity to P190 (Supporting Information Figure S8B). Although a subtle shift in the inserted helix α2a consisting residues 194-209 can be observed (Supporting Information Figure S8C), the residues involved in the Trp binding site are mostly unaffected (Supporting Information Figure S8D), with the exception of L194, which shows a small change in sidechain position.

A Sector 2 substitution affects inter-enzyme communications. Sector 2, identified by SCA, identifies residues with a likely role in inter-enzyme communication between DAH7PS and CM. To interrogate the functional connections within this sector, a second target, Y131, was selected (Figure 6A). Y131 is located between the Phe-specific binding site and the active site and is not directly involved in any interactions with the Phe ligands (Figure 6B). Y131 is ~11 Å away from the active site metal ion Mn<sup>2+</sup> and ~12 Å away from the bound Phe ligand. We generated the variant MtuDAH7PS<sup>Y131A</sup> to examine the effect of this residue on the communication pathways.

MtuDAH7PS<sup>Y131A</sup> variant was found to be catalytically active and demonstrates overall similar catalytic efficiencies compared to the wild-type enzyme (Table 1). While the $K_M$ values of MtuDAH7PS<sup>Y131A</sup> decreased compared to the wild-type enzyme, its $k_{cat}$ value also reduced by around 75%. The allosteric response of MtuDAH7PS<sup>Y131A</sup> by aromatic amino acids is somewhat impaired, in which the presence of all three aromatic amino acids caused moderate level of inhibition on the MtuDAH7PS<sup>Y131A</sup> activity with approximately 30% activity still remaining. The response of
MtDAH7PS\textsuperscript{Y131A} to Phe alone or in binary combinations is attenuated and appears to enhance the DAH7PS activity (Figure 7A).

Surprisingly, MtDAH7PS\textsuperscript{Y131A} is capable of enhancing the overall catalytic efficiency of MtUCM much more significantly, by over 400-fold, compared to the 100-fold activation observed under the same conditions with MtDAH7PS\textsuperscript{WT} or MtDAH7PS\textsuperscript{G190P} (Table 1). This significant rate enhancement for MtUCM observed in the presence of the MtDAH7PS\textsuperscript{Y131A} variant was largely contributed by the increased turnover number. The inhibitory effect of Phe on MtUCM upon complexation with MtDAH7PS\textsuperscript{Y131A} was largely reduced compared to that observed in the wild-type enzyme, with over 60 % remaining MtUCM activity in the presence of Phe alone (Figure 7B). Whereas the inhibitory responses of MtUCM-DAH7PS\textsuperscript{Y131A} to other aromatic amino acid combinations is similar to those observed in MtUCM-DAH7PS\textsuperscript{G190P}.

The Y131A substitution also resulted in some modifications in the structure of the enzyme as revealed by SAXS analysis (Figure 7C). The structural parameters generated from the SAXS scattering indicate that MtDAH7PS\textsuperscript{Y131A} may adopt a slightly larger and more loosely-packed overall shape compared to the wild-type enzyme (Table 3). We generated a rigid body model of MtDAH7PS\textsuperscript{Y131A} (chi\textsuperscript{2} = 1.9, Figure 7F), which shows a flattened tetramer interface with an altered relative position between the tight dimers compared to the wild-type enzyme (which does not fit well with the MtDAH7PS\textsuperscript{Y131A} SAXS data with chi\textsuperscript{2} value of 5.6, Figure 7E). The predicted loose tetramer interface in MtDAH7PS\textsuperscript{Y131A} is echoed by the observation of a much lower melting temperature measured in DSF experiments, in which a \( T_m \) of 44 °C was measured for apo MtDAH7PS\textsuperscript{Y131A} (Table 2) compared to that of ~53 °C in wild-type enzyme and MtDAH7PS\textsuperscript{G190P} variant. Interestingly, the flattened tetramer interface also impacts the regions that interact with MtUCM upon complex formation (Figure 7D), therefore, it is possible that the improved enhancement of MtUCM activity by MtDAH7PS\textsuperscript{Y131A} variant is partially contributed by its changed structure.

DISCUSSION

Allosteric regulation of known type II DAH7PS enzymes is dynamically driven\textsuperscript{15, 19, 26} Among these, MtDAH7PS shows the most complex dynamic allosteric response. MtDAH7PS utilizes four distinct functional sites, including one active site and three allosteric sites that are specific to each of the three aromatic amino acids (Trp, Phe and Tyr). Binding of allosteric ligands do not cause any observable structural changes on the enzyme. Instead, MtDAH7PS exploits complex internal dynamic communication pathways to deliver the allosteric signal.

By examining co-evolved residues across the type II DAH7PS subfamily, we identified a critical node, G190, in the internal dynamic network within MtDAH7PS. A single G190P substitution abolishes the allosteric response in MtDAH7PS, but unexpectedly leaves catalysis and ligand binding intact. Deciphering dynamic allosteric networks that deliver functional change can be challenging, and recent studies showed that amino acid substitutions of key residues in the allosteric networks usually also result in altered catalysis.\textsuperscript{27-30} For example, substitutions of two hydrophobic residues involved in the dynamically driven allosteric communication in protein kinase A lead to inactivation of the enzyme;\textsuperscript{27} in ATP-phosphoribosyltransferase, substitution of an Arg important for allosteric signal transmission to either Gln or Ala resulted in severely impaired catalysis.\textsuperscript{31} In contrast to the mutagenesis experiments in other reports, which modify the properties of the amino acid side chains, it should be noted that our G190P substitution only alters the dynamic property of the protein backbone. Given that no overall structural changes are observed and that allosteric ligand binding is fully maintained in MtDAH7PS\textsuperscript{G190P}, it appears that the loss of allosteric signal here is due to altered dynamic motions of the protein backbone. G190 is located at the start of the two inserted helices \( \alpha_2a \) and \( \alpha_2b \), which are responsible for the formation of Trp binding sites and the tetramer interface in MtDAH7PS. Interestingly, at the end of these two inserted helices is another Gly residue (Gly232), and the substitution of G232P changed the quaternary structure of MtDAH7PS, which altered catalysis and broke allostery.\textsuperscript{32} The drastic changes caused by these Gly to Pro mutations clearly indicate the functional importance of helices \( \alpha_2a \) and \( \alpha_2b \) in communicating the allosteric signal.
within \textit{Mtu}DAH7PS. To the best of our knowledge, the uncoupling of allostery and catalysis caused by G190P substitution is the most clear-cut example of dynamic communication network disruption to date.

Another complex feature of the allosteric regulation mechanism of \textit{Mtu}DAH7PS is the inter-enzyme communication to \textit{Mtu}CM.\textsuperscript{16-18} This small enzyme partner forms a non-covalent complex with \textit{Mtu}DAH7PS, which enables it to adopt the Phe binding sites of \textit{Mtu}DAH7PS to deliver allosteric inhibition of \textit{Mtu}CM by Phe binding. SCA highlighted Y131 as a part of the dynamic network that may be important for the inter-enzyme communications between \textit{Mtu}DAH7PS and \textit{Mtu}CM. Here we showed that modifying the side chain through Y131A substitution led to impaired inhibitory response of \textit{Mtu}CM and \textit{Mtu}DAH7PS to Phe, and Phe alone appears to enhance activity of \textit{Mtu}DAH7PS. Unexpectedly \textit{Mtu}CM activity was significantly more enhanced in the presence of the \textit{Mtu}DAH7PS\textsuperscript{Y131A} variant, compared to that in the presence of the wild-type enzyme or \textit{Mtu}DAH7PS\textsuperscript{G190P}. The Y131A substitution causes structural changes in DAH7PS, which likely resulted in the enhanced activation of \textit{Mtu}CM.

Dynamic allostery or entropically-driven allostery has been extensively studied in recent years, with its intriguing functional responses accompanied with the apparent lack of observable structural changes.\textsuperscript{33-42} Dynamic allostery is an intrinsic property of enzymes and is intimately linked with catalysis.\textsuperscript{34, 39, 42, 43} In this study, we identified a key node embedded in an intricate and extensive allosteric network, which is remote to any ligand binding site. We have broken the allosteric links through a simple change in backbone dynamics. Our results reveal that the highly sophisticated and complex dynamic allostery can be surprisingly vulnerable. This vulnerability may represent a glimpse of the evolution of such an important biosynthetic enzyme en route to more advanced regulations and provides further insight on the nature of the intimate link between catalysis and allosteric regulation.

EXPERIMENTAL PROCEDURES

\textbf{Statistical Coupling Analysis.} Full sequence alignment of type II DAH7PS was obtained from PFAM database\textsuperscript{44, 45} (release 32.0, accession code PF01474, total of 4217 sequences) and was subject to pre-processing with default settings as described.\textsuperscript{20} After processing, the alignment contains 3345 sequences and 431 positions. The number of effective sequences was 510. According to original publication of SCA,\textsuperscript{20} an effective sequence number of greater than 100 indicates the sequence alignment is large enough to give good estimates of amino acid frequencies. The pre-processed sequence alignment was then subject to the full SCA analysis using the pySCA toolbox, which consists of five steps as outlined.\textsuperscript{20}

\textbf{Site-directed mutagenesis of \textit{Mtu}DAH7PS mutants.} QuikChange\textsuperscript{®} site-directed mutagenesis technique was used to generate protein variants \textit{Mtu}DAH7PS\textsuperscript{G190P} and \textit{Mtu}DAH7PS\textsuperscript{Y131A} using wild-type \textit{pProExHTa-Mtu}DAH7PS (Rv2178c) plasmid as template.\textsuperscript{12} Primers for mutagenesis were designed with the point of mutation in the centre and sufficient overlaps (~15 base pairs) on both ends. Primers (5' - 3') GCTGACTTCGTCGCCCCTGGCGTCGCTG and CAGCGACGCCAGGGGCGACGAAGTCAGC were used for mutagenesis of \textit{Mtu}DAH7PS\textsuperscript{G190P}, CATCGCCGGTCAGGCGGCGAAGCCTCGG and CCGAGGCTTCCGCGCCCTGACCGGCGATG were used for \textit{Mtu}DAH7PS\textsuperscript{Y131A}. Mutagenesis was performed using a QuikChange II Site-Directed Mutagenesis Kit (Agilent) with reaction (50 µL) and cycling protocols recommended by the manufacturer. PCR products were treated with Dpn1 (New England BioLabs) for removal of methylated templates. The product was then transformed into Stellar\textsuperscript{TM} (Clontech) cells and sequence verified before transforming into \textit{E. coli} BL21(DE3) pGroESL cells for expression.

\textbf{Expression and purification of \textit{Mtu}CM and \textit{Mtu}DAH7PS variants.} \textit{Mtu}CM and \textit{Mtu}DAH7PS variants were expressed and purified following the previously described protocols for the wild-type \textit{Mtu}CM and \textit{Mtu}DAH7PS respectively.\textsuperscript{12, 17} Briefly, transformants containing the recombinant plasmids were preincubated in Luria-Bertani medium at 37 °C with the appropriate antibiotics with shaking.
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until mid-logarithmic phase (OD_{600} ≈ 0.4 – 0.6) before the protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside (Roche) (0.5 mM) and incubated overnight at 23 °C. Following cell harvest and lysis, MtuCM was purified by using a GSTrap HP column (GE Healthcare) before and after the TEV protease treatment. 17 MtuDAH7PS variants were purified by using a HiTrap HP column (GE Healthcare) before and after TEV protease treatment. The proteins were then further purified using size-exclusion chromatography (SEC).

Kinetic measurements. Steady-state kinetics were measured for DAH7PS activity by monitoring the consumption of PEP at 232 nm (ε = 2.8 x 10^3 M^-1 cm^-1 at 303 K) and for CM activity by monitoring the consumption of chorismate at 274 nm (ε = 2.63 x 10^3 M^-1 cm^-1 at 303 K) using a Varian Cary 100 UV–visible spectrophotometer. 12, 17 For the DAH7PS assays, initial velocity values were determined at varying concentrations of each substrate, when the concentration of the other substrate was fixed at saturating concentrations (defined as at least 5-fold higher than the K_M value). The reaction mixture contained 0.05 µM protein, varying or saturating concentrations of PEP (for determination of K_M^{E4P}), varying or saturating concentrations of E4P (for determination of K_M^{PEP}), 100 μM of Mn^{2+} and 0.5 mM tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) in 50 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP) buffer at 30 °C (pH 7.5). Reactions were initiated by addition of E4P. For CM assays, the reactions contained 9 to 1500 µM chorismate for standard measurements or saturating chorismate for fold activation measurements, 15 nM MtuCM, 0 to 3 µM MtuDAH7PS variants in the same buffer environment as for the DAH7PS assays. Each reaction was incubated for 10 min prior to initiation with chorismate to allow formation of the CM:DAH7PS complex. Apparent kinetic parameters were determined by fitting triplicate data to the Michaelis-Menten equation using GraphPad Prism 7.

Inhibition studies. Inhibition studies for DAH7PS and CM activity in response to aromatic amino acids alone or in combinations were performed using the same method as described for kinetic measurements. The DAH7PS reactions contained 0.05 µM enzymes with 260 µM PEP, 160 µM E4P for MtuDAH7PS WT and MtuDAH7PS V131A assays, and 143 µM E4P for MtuDAH7PS V131A assays in the absence or presence of 200 µM aromatic amino acids alone or in combinations. The CM reactions contained 7.5 nM MtuCM, 1.5 mM MtuDAH7PS variants, 40 µM chorismate, and total concentration of 200 µM aromatic amino acid(s). For the normalized data, the mean of each apo-dataset was defined as 100%.

Differential Scanning Fluorimetry (DSF). DSF was performed using a Thermo Fisher Quant Studio 3 Applied Biosystems. Samples were prepared in a 96-well plate to give a final volume of 25 µL. Each well contained 20 µL of buffer [(10 mM BTP, pH 7.5, 150 mM NaCl, 200 µM MnSO_4, 200 µM PEP, 200 µM TCEP with 1 mM of ligand(s)], 4 µL of protein at 0.9 mg/mL and 1 µL of 20x SYPRO Orange dye. The plate was subjected to a thermal cycling program that heated the samples from 20 to 85 °C in 0.02 °C/sec increments. Each protein sample was measured in triplicate and compared to a negative control in which no protein sample was present. Melting temperatures were calculated using Protein Thermal Shift Software 1.3 at the temperature at which the maximum inflection occurred along the generated melting curve of each protein sample. Errors were calculated based on standard deviation from triplicate measurements.

SAXS data collection, analysis and modelling. SEC-SAXS co-flow setup was used for collection of small angle X-ray scattering (SAXS) data at the Australian Synchrotron SAXS/WAXS beamline equipped with a Pilatus detector (1 M, 170 × 170 mm, effective pixel size, 172 × 172 µm). 46 The X-ray wavelength was 1.0332 Å. The sample-detector distance was 1.6 m, which delivered a s range of 0.01 – 0.40 Å^{-1} [where s is the magnitude of the scattering vector, which is related to the scattering angle (2θ) and the wavelength (λ) as follows: s = (4π/λ) sin(θ)]. Scattering data were collected at 25 °C following elution of the protein samples (6 mg·mL^{-1}) from a SEC column (Superdex 200, Increase 5/150), pre-equilibrated with buffer containing 10 mM BTP (pH 7.5), 150 mM NaCl, 200 µM PEP, 200 µM TCEP and 3% (v/v) glycerol. Raw data were processed and background-subtracted using Scatterbrain. 9 Scatterings from A280nm peaks were
summed and averaged for SEC experiments. Scattering intensity ($I$) versus $s$ of each protein was generated with Guinier plots representing a linear range for $sR_g < 1.3$, and plots were scaled for comparison using Primus.\textsuperscript{47} Theoretical scattering profile of $Mtu$DAH7PS was generated from the model coordinates (PDB 3NV8), compared and fitted with corresponding experimental scatterings with Crysol.\textsuperscript{48}

Rigid body modelling was performed for $Mtu$DAH7PS\textsubscript{Y131A} using SASREF.\textsuperscript{49} Monomer coordinates were extracted from PDB 3NV8 and used for SASREF along with the SAXS scattering data for $Mtu$DAH7PS\textsubscript{Y131A} with no symmetry applied. The following constrains were applied: dist 25, 1 1 1 3 1 1; dist 25, 2 1 1 4 1 1; dist 70, 1 467 467 3 467 467; dist 70, 2 467 467 4 467 467; dist 30, 1 467 467 2 467 467; dist 30, 3 467 467 4 467 467; dist 65, 2 467 467 3 467 467; dist 65, 1 467 467 4 467 467; dist 10, 1 181 181 3 181 181; dist 10, 2 181 181 4 181 181.

\textit{Crystallization and Refinement.} Crystal structure of $Mtu$DAH7PS\textsubscript{G190P} variant was obtained in condition that contains 20 mM BTP, 150 mM NaCl, 0.5 mM TCEP, 0.2 mM PEP, 0.1 mM MnCl\textsubscript{2}, 2 M Li\textsubscript{2}SO\textsubscript{4}, and 2% PEG 400. Diffraction data were collected at the Australian Synchrotron.\textsuperscript{50} Data were processed in ImosfIm\textsuperscript{51} and initial model obtained by molecular replacement using the ligand free crystal structure of $Mtu$DAH7PS wild-type enzyme as the search model (PDB 3NV8). The structure was further refined using REFMAC5.\textsuperscript{52} Details of the data collection and refinement statistics are in Supporting Information Table S1.

\textbf{DATA AVAILABILITY}

The coordinates and structure factors for $Mtu$DAH7PS\textsubscript{G190P} are deposited in the Protein Data Bank and assigned with accession code 6PBJ. All other data is contained in this manuscript.
ACKNOWLEDGEMENTS

This research was undertaken on the MX and SAXS beamlines at the Australian Synchrotron, part of ANSTO. This work was funded by the New Zealand Marsden Fund UOC-1105, and The Maurice Wilkins Centre for Molecular Biodiscovery.

CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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### TABLES

Table 1. Kinetic parameters of *Mtu*DAH7PS variants and *Mtu*CM.

| DAH7PS  | \( K_M^{\text{PEP}} \) (µM) | \( K_M^{\text{E4P}} \) (µM) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( k_{\text{cat}}/K_M^{\text{PEP}} \) (µM\(^{-1}\)s\(^{-1}\)) | \( k_{\text{cat}}/K_M^{\text{E4P}} \) (µM\(^{-1}\)s\(^{-1}\)) |
|---------|----------------|----------------|----------------|----------------|----------------|
| *Mtu*DAH7PS\(^{\text{WT}}\) | 27 ± 2 | 44 ± 4 | 4.5 ± 0.1 | 0.17 | 0.1 |
| *Mtu*DAH7PS\(^{G190P}\) | 29 ± 2 | 45 ± 4 | 5.8 ± 0.2 | 0.2 | 0.13 |
| *Mtu*DAH7PS\(^{Y131A}\) | 7 ± 0.6 | 14 ± 1.5 | 1.2 ± 0.02 | 0.17 | 0.09 |

| CM       | \( K_M^{\text{chorismate}} \) (µM) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( k_{\text{cat}}/K_M^{\text{chorismate}} \) (µM\(^{-1}\)s\(^{-1}\)) | Fold activation* |
|----------|----------------|----------------|----------------|----------------|
| *Mtu*CM alone | 759 ± 23 | 1.2 ± 0.1 | 1.6 x 10\(^{-3}\) | NA\(^{+}\) |
| *Mtu*CM-DAH7PS\(^{\text{WT}}\) | 40 ± 5 | 7.3 ± 0.5 | 0.18 | 114 |
| *Mtu*CM-DAH7PS\(^{G190P}\) | 42.2 ± 4 | 8.8 ± 0.3 | 0.21 | 131 |
| *Mtu*CM-DAH7PS\(^{Y131A}\) | 38.7 ± 4 | 27 ± 0.9 | 0.7 | 438 |

* Fold activation shows the activation of *Mtu*CM activity by *Mtu*DAH7PS variants;

\(^{+}\) NA: activity not applicable for the specified enzyme.
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Table 2. Thermostability of *Mtu*DAH7PS variants in the absence and presence of 1 mM aromatic amino acid(s).

| Added ligand | *Mtu*DAH7PS<sub>WT</sub> | *Mtu*DAH7PS<sup>G190</sup> | *Mtu*DAH7PS<sup>Y131</sup> |
|--------------|-------------------------|-----------------------------|-----------------------------|
| None         | 52.7 ± 0.1              | 54.4 ± 0.2                  | 44.1 ± 0.2                  |
| F            | 60.7 ± 0.1              | 62.4 ± 0.1                  | 49.4 ± 0.2                  |
| Y            | 55.1 ± 0.1              | 57.4 ± 0.1                  | 54.9 ± 0.4                  |
| W            | 68 ± 0.1                | 68.9 ± 0.1                  | 67.4 ± 0.1                  |
| FY           | 63.1 ± 1.2              | 64.3 ± 0.1                  | 53 ± 0.1                    |
| FW           | 66.1 ± 0                | 63.9 ± 0.1                  | 64.6 ± 0.1                  |
| YW           | 65.5 ± 0.1              | 64.4 ± 0.1                  | 65.3 ± 0.1                  |
| FYW          | 65.8 ± 0                | 64.3 ± 0.1                  | 63.3 ± 0.1                  |
Table 3. Structural parameters of *Mtu*DAH7PS variants from SAXS experiments.

| Structural parameters                           | *Mtu*DAH7PS<sup>WT</sup> | *Mtu*DAH7PS<sup>G190P</sup> | *Mtu*DAH7PS<sup>Y131A</sup> |
|-----------------------------------------------|---------------------------|------------------------------|-------------------------------|
| I(0) (cm<sup>-1</sup>) [from P(r)]           | 0.13                      | 0.13                         | 0.14                          |
| $R_g$ (Å) [from P(r)]                        | 40.91                     | 41.03                        | 41.68                         |
| I(0) (cm<sup>-1</sup>) (from Guinier)        | 0.13 ± 0.0001             | 0.13 ± 0.0007                | 0.14 ± 0.0001                 |
| $D_{max}$ (Å)                                | 131.23                    | 125.20                       | 148.5                         |
| $R_g$ (Å) (from Guinier)                     | 41.29 ± 0.18              | 41.39 ± 0.32                 | 41.99 ± 0.06                  |
| Porod volume estimate (Å<sup>3</sup>)         | 274,000                   | 277,000                      | 284,000                       |
| Dry tetrameric volume calculated from sequence (Å<sup>3</sup>) | 245,712                   | 245,904                      | 245,264                       |
| Calculated monomeric molecular mass from sequence (Da) | 50769.58                  | 50809.64                     | 50620.43                      |
| Molecular mass from Porod volume (Da) (from MoW) | 194393                    | 195991                       | 209903                        |
| Number of subunits (from MoW)                 | 4                         | 4                            | 4                             |
| Fit with crystal structure ($\chi^2$) (from CRYSOL) | 0.180                     | 0.171                        | 5.619                         |
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FIGURES

Figure 1. Structures of DAH7PS from different subfamilies. (A) Crystal structure of *E. coli* DAH7PS (PDB 4UC5), representing type Iα. (B) Crystal structure of *T. maritima* DAH7PS (PDB 3PG9), representing type Iβ. (C) Crystal structure of *Mtu*DAH7PS (PDB 3KGF), representing type II DAH7PS. (D) Tetrameric structure of *Mtu*DAH7PS. Decoration structural elements associated with allostery are shown as orange cartoons, corresponding allosteric ligands are displayed as spheres with green carbon atoms, active site metal ions (cyan) and phosphate (sulphate) are displayed as spheres if present in the crystal structure.
Figure 2. Sector 1 identified from SCA. (A) Sector 1 mapped onto crystal structure of *Mtu*DAH7PS. Ligand binding sites are indicated by displaying the Trp and Phe ligands as spheres with green carbon atoms. The active site Mn$^{2+}$ (cyan) and the phosphate ions observed in the crystal are displayed as spheres to indicate location of the active site. (B) Residues in IC 7 mapped onto structure of *Mtu*DAH7PS.
Figure 3. Sector 2 identified from SCA. (A) Sector 2 residues mapped onto crystal structure of MtuDAH7PS. The active site Mn$^{2+}$ (cyan) and the phosphate ions observed in the crystal are displayed as spheres to indicate location of the active site. (B) Residues in IC 2 mapped onto structure of MtuDAH7PS. Ligand binding sites are indicated by displaying the Trp and Phe ligands as spheres with green carbon atoms. Crystal structure of a single chain MtuCM is shown in magenta cartoon representation to indicate location of the MtuDAH7PS-CM interface.

Figure 4. Position of G190 on MtuDAH7PS. (A) G190 (yellow) on the monomer of MtuDAH7PS with other residues in IC 7 (dark blue spheres). Allosteric ligands Trp and Phe are shown as spheres with green carbon atoms. (B) Close-up view of the Trp binding site relative to the position of G190. Polar interactions formed between Trp and surrounding residues are shown as black dashed lines. G190 is clearly not involved in any direct interactions with Trp.
Figure 5. (A) Allosteric response of MtuDAH7PS<sub>G190P</sub> to aromatic amino acids. Assay contained 50 mM BTP, pH 7.5, 1 mM TCEP, 100 µM Mn<sup>2+</sup>, 260 µM PEP, 160 µM E4P in the absence or presence of total 200 µM aromatic amino acids alone or in combinations. For the normalized data, the mean of each apo-dataset was defined as 100%. (B) Activity of MtuCM in the presence of MtuDAH7PS<sub>WT</sub> and MtuDAH7PS<sub>G190P</sub> in response to aromatic amino acids. Assay contained 7.5 nM MtuCM, 1500 nM MtuDAH7PS variants, 40 µM chorismate, and 200 µM total ligand. Data were generated from independent triplicate measurements, the mean values were plotted with error bars showing the standard deviation. (C) SAXS scattering profiles of MtuDAH7PS<sub>WT</sub> (green dots) and MtuDAH7PS<sub>G190P</sub> (blue dots) and theoretical scattering of MtuDAH7PS<sub>WT</sub> (black line, PDB 3NV8). (D) Superimposed crystal structures of MtuDAH7PS<sub>G190P</sub> (blue, PDB 6PBJ) and MtuDAH7PS<sub>WT</sub> (grey, PDB 3NV8).
Figure 6. Position of Y131 on MtuDAH7PS. (A) Y131 (yellow) on the monomer of MtuDAH7PS with other residues in IC 2 (dark red spheres). Allosteric ligands Trp and Phe are shown as spheres with green carbon atoms. Active site Mn$^{2+}$ and phosphates are shown as spheres. (B) Close-up view of the Phe binding site relative to the position of Y131, showing this residue is not in any direct contact with Phe. Polar interactions formed between Y131 and surrounding residues are shown as black dashed lines.
Figure 7. (A) Allosteric response of $\text{MtuDAH7PS}^{Y131A}$ to aromatic amino acids. Assay contained 50 mM BTP, pH 7.5, 1 mM TCEP, 100 µM Mn$^{2+}$, 260 µM PEP, 143 µM E4P in the absence or presence of total 200 µM aromatic amino acids alone or in combinations. For the normalized data, the mean of each apo-dataset was defined as 100%. (B) Activity of $\text{MtuCM}$ in the presence of $\text{MtuDAH7PS}^{\text{WT}}$ and $\text{MtuDAH7PS}^{Y131A}$ in response to aromatic amino acids. Assay contained 7.5 nM $\text{MtuCM}$, 1500 nM $\text{MtuDAH7PS}$ variants, 40 µM chorismate, and 200 µM total ligand. Data were generated from independent triplicate measurements, the mean values were plotted with error bars showing the standard deviation. (C) SAXS profile of $\text{MtuDAH7PS}^{Y131A}$ (orange dots) and theoretical scattering from the rigid body model (black line). (D) Relative position of $\text{MtuCM}$ in $\text{MtuCM-MtuDAH7PS}$ complex shown in red carton (PDB 5HUD), aligned with PDB 3NV8 (surface). (E) $\text{MtuDAH7PS}^{\text{WT}}$ shown in shades of green (PDB 3NV8). (F) $\text{MtuDAH7PS}^{Y131A}$ SAXS model shown in shades of orange.
A single amino acid substitution uncouples catalysis and allostery in an essential biosynthetic enzyme in Mycobacterium tuberculosis
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J. Biol. Chem. published online March 26, 2020

Access the most updated version of this article at doi: 10.1074/jbc.RA120.012605

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