Domain cross-talk within a bifunctional enzyme provides catalytic and allosteric functionality in the biosynthesis of aromatic amino acids

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Because of their special organization, multifunctional enzymes play crucial roles in improving the performance of metabolic pathways. For example, the bacterium Prevotella nigrescens contains a distinctive bifunctional protein comprising a 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (DAH7PS), catalyzing the first reaction of the biosynthetic pathway of aromatic amino acids, and a chorismate mutase (CM), functioning at a branch of this pathway leading to the synthesis of tyrosine and phenylalanine. In this study, we characterized this P. nigrescens enzyme and found that its two catalytic activities exhibit substantial hetero-interdependence and that the separation of its two distinct catalytic domains results in a dramatic loss of both DAH7PS and CM activities. The protein displayed a unique dimeric assembly, with dimerization solely via the CM domain. Small angle X-ray scattering (SAXS)-based structural analysis of this protein indicated a DAH7PS-CM hetero-interaction between the DAH7PS and CM domains, unlike the homo-association between DAH7PS domains normally observed for other DAH7PS proteins. This hetero-interaction provides a structural basis for the functional interdependence between the two domains observed here. Moreover, we observed that DAH7PS is allosterically inhibited by prephenate, the product of the CM-catalyzed reaction. This allostery was accompanied by a striking conformational change as observed by SAXS, implying that altering the hetero-domain interaction underpins the allosteric inhibition. We conclude that for this C-terminal CM-linked DAH7PS, catalytic function and allosteric regulation appear to be delivered by a common mechanism, revealing a distinct and efficient evolutionary strategy to utilize the functional advantages of a bifunctional enzyme.

Multifunctional enzymes, bearing two or more catalytic activities, provide exceptional contributions to the efficient and coherent function of metabolic pathways. Two main benefits of multifunctional enzymes have been clearly described: first, linked catalytic modules can enhance the overall catalytic rate for consecutive reactions due to substrate channeling (1), and second, fusion can impart allosteric control, such that catalytic function is altered by a ligand binding to another linked enzymatic moiety (2, 3). The shikimate pathway is responsible for the biosynthesis of the aromatic amino acids, folic acid, salicylate and a variety of aromatic secondary metabolites in microorganisms and plants. The pathway has a number of multifunctional enzymes and enzymatic complexes that organize the catalytic entities for efficient substrate channeling or deliver allosteric functionality (2–4). Here we examine the role of multifunctionality in a system joining two nonconsecutive catalytic functionalities, linking the start of the pathway to a pathway branch.

The first committed step in this pathway is the aldol-like reaction between a phosphoenolpyruvate (PEP) and an erythrose 4-phosphate (E4P) to produce 3-deoxy-d-arabino-heptulosonate-7-phosphate (DAH7P) catalyzed by DAH7PS synthase (DAH7PS) (5). As the initiator and regulatory checkpoint of an important biosynthetic pathway, which supports both primary and secondary metabolism in plants and microorganisms, DAH7PS enzymes from various sources have been examined and some bifunctional DAH7PS enzymes linking another enzymatic capability have been found (2, 3).

Structures of DAH7PS enzymes from a variety of sources have shown their core catalytic unit, a (β/α)8 barrel, can be adorned by a variety of extra structural elements, such as loop insertions and extensions (6). These extra-barrel elements confer control of the pathway through allosteric regulation; rendering DAH7PS catalytic activity sensitive to pathway end prod-

The abbreviations used are: PEP, phosphoenolpyruvate; DAH7PS, 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase; CM, chorismate mutase; KDO8PS, 3-deoxy-d-manno-octulosonate-8-phosphate synthase; PniDAH7PS, DAH7PS from P. nigrescens; PniDAH7PSCM, the DAH7PS domain of PniDAH7PS; PniDAH7PS/CM, the CM domain of PniDAH7PS; PgiDAH7PS, DAH7PS from P. gingivalis; E4PDAH7PS, the DAH7PS domain of E4PDAH7PS; PgiDAH7PS/CM, the CM domain of E4PDAH7PS; GspDAH7PS, DAH7PS from Geobacillus sp.; BspDAH7PS, DAH7PS from B. subtilis; TmaDAH7PS, DAH7PS from T. maritima; E4P, erythrose 4-phosphate; SEC, size exclusion chromatography; CLANS, Cluster ANalysis of Sequences; AUC, analytical ultracentrifugation; SAXS, small angle X-ray scattering; TEV, tobacco etch virus; Bistris propane, 1,3-bis[(tris(hydroxymethyl)methylamino)propylene; DOPE, disaturated phosphatidylcholine.

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ucts or intermediates. Allostery allows the activity of this gateway enzyme to the pathway to be regulated by cellular metabolic demand for the pathway products (6). Based on the structural diversity of these extra-barrel decorations and their impact on overall protein sequence and size, DAH7PS proteins have been classified into two main groups, denoted type I and type II (7). The type I DAH7PS group is further divided into type Iα and type Iβ subgroups (8).

The subgroup comprising the type Iβ enzymes demonstrate the largest variation in extra-barrel decoration. The simplest type Iβ DAH7PS proteins comprise the core barrel alone, and thus only support basic enzymatic function (9, 10). More complex type Iβ DAH7PS proteins have the simple uninterrupted barrel also, but this bears an additional discrete N- or C-terminal domain (2, 11). This overall architecture differs rather markedly from that of the type Iα and type II DAH7PS proteins, where the barrel is interrupted by regulatory elements (12, 13).

In addition to showing heterogeneity with respect to the position of the regulatory elements, type Iβ DAH7PS also bear two very different regulatory domains, displaying either an ACT-like N-terminal domain (14) or an N- or C-terminal chorismate mutase (CM) domain, delivering a protein of dual catalytic function in the latter case (2). The trihelical CM domain catalyzes the conversion of chorismate to prephenate, which is a later step of the branch of the pathway leading to Phe or Tyr. The ACT-like domain on the other hand has no catalytic activity. However, this βββαα αααα ligand-binding domain is a common regulatory unit found on proteins of amino acid or purine metabolism (15–18).

The allosteric mechanisms by which N-terminal ACT or CM domains control the catalytic function of the barrel are relatively well understood (3, 14, 19). For the Thermotoga maritima (Tma) DAH7PS, the ACT domains dimerize upon the binding of Tyr, blocking the entrance of DAH7PS active site (14, 19). Similarly, prephenate binding to the CM domain in DAH7PS from Geobacillus sp. (GspDAH7PS) also results in occlusion of the DAH7PS active site (3). Both of these proteins, TmaDAH7PS and GspDAH7PS, share a common homotetrameric arrangement and this quaternary association is essential for allosteric function. As the unregulated Iβ forms of DAH7PS share this homotetrameric assembly, it has been proposed that an unregulated type Iβ DAH7PS tetrameric scaffold is the ancestor for adopting divergent allosteric machinery (2). This proposal is supported by domain fusion and swapping experiments that reveal how readily allosterly can be acquired or interchanged by type Iβ DAH7PS domains (20, 21).

There is, however, one distinct group of type Iβ DAH7PS proteins for which very limited information is available. Unlike other type Iβ DAH7PS proteins this group possesses a C-terminal CM domain. Functional studies of this group are limited to the DAH7PS from Porphyromonas gingivalis (PgiDAH7PS), which shows dual DAH7PS and CM activity and allosteric inhibition by prephenate (2). However, unlike the N-terminal-linked proteins, on splitting the full-length protein into its individual DAH7PS and CM components, both enzymatic activities were lost (2, 3, 14). This characteristic may indicate a unique architectural organization of DAH7PS and CM domains for these C-terminal–modified proteins. However to date, no structural information has been available for these DAH7PS proteins, and their mechanisms of catalysis and allostery are unknown.

To probe the properties of the C-terminal CM-fused DAH7PS group and to elucidate more fully the effects arising from the fusions of enzymes, we chose to examine the DAH7PS from Prevotella nigrescens. P. nigrescens is a pathogenic bacterium relevant to oral diseases and carotid atherosclerosis (22–24). Our studies reveal a distinct quaternary structure for this C-terminal CM-DAH7PS fusion protein and demonstrate a functional interplay between two catalytic parts for this protein that is exploited for allosteric function through the binding of prephenate.

### Results

**The C-terminal CM-linked DAH7PS are a distinct subgroup of type I DAH7PS**

To illustrate the subdivisions of type I DAH7PS based on sequence and to explore more fully the sequence relationships, we sorted all the candidate sequences using Cluster ANalysis of Sequences (CLANS). In this way, and as expected based on previous sequence analysis (7, 8), two distinct clusters were identified: type Iα subgroup and type Iβ (Fig. 1A). The type Iβ subgroup also includes the closely related enzyme 3-deoxy-β-manno-2-octulosonate 8-phosphate synthase (KDO8PS), which catalyzes a closely related reaction, as a part of lipopolysaccharide biosynthesis in Gram-negative bacteria (25) (Fig. 1A). Using this analysis, the type Iβ subgroup was shown to comprise one major cluster with an adjacent smaller grouping (Fig. 1B). All well-investigated type Iβ DAH7PS proteins to date reside in this main cluster grouping, including the GspDAH7PS, TmaDAH7PS, and PfuDAH7PS (an unregulated DAH7PS from Pyrococcus furiosus) (3, 6, 9, 11).

From the smaller grouping, PniDAH7PS was selected as a representative for more detailed investigation. Sequence alignments of PniDAH7PS with the known GspDAH7PS, TmaDAH7PS, PfuDAH7PS, and PfuCM (from P. furiosus) indicate PniDAH7PS comprises an N-terminal DAH7PS domain and a C-terminal CM domain, a feature that it shares with other proteins clustered into this smaller grouping (Fig. 2A). Although, the alignments do not show great similarity of either the DAH7PS or CM portion of PniDAH7PS to other aligned sequences (Table 1), the key active site residues for both functional moieties are conserved (Fig. 2B). It should be noted that the gene encoding the full-length PniDAH7PS includes the only predicted reading frames for DAH7PS or CM proteins in P. nigrescens.

**WT PniDAH7PS is bifunctional and its catalytic domains can be separated**

To validate the sequenced-based prediction of bifunctionality for PniDAH7PS and to probe the relationship between the domains, the full-length protein and two variants, each of which only consist of either the predicted DAH7PS or CM domain (denoted PniDAH7PS<sup>DAH7PS</sup> and PniDAH7PS<sup>CM</sup>), were expressed and purified to homogeneity (Fig. 2C). As was expected, both the DAH7PS and CM activities were found for the full-length WT protein (PniDAH7PS), and the characteris-
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Figure 1. CLANS clustering of type 1 DAH7PS sequences. Each dot represents a protein sequence. A, two main clusters were identified by CLANS as indicated: type 1α subgroup and type 1β (also containing the related KDO8PS proteins). B, the CLANS analysis for the type 1β DAH7PS reveals 3 distinct clusters, the type 1β C-terminal CM-fused subgroup (red) including PniDAH7PS, the main type 1β subgroup (green) including GspDAH7PS, TmaDAH7PS, PuDAH7PS, and the KDO8PS group (blue).

tic divalent ion dependence of DAH7PS activity was observed, with Mn$^{2+}$ resulting in the fastest reaction rates (Fig. 3). Type Iβ DAH7PS enzymes are reported to show relatively diversified preferences for divalent metal ions, compared with the type Iα enzymes, which are largely reported to be Mn$^{2+}$ dependent (12, 26, 27). For type Iβ enzymes PgiDAH7PS, GspDAH7PS, and TmaDAH7PS show their greatest activities in the presence of Zn$^{2+}$, Cd$^{2+}$, and Mn$^{2+}$, respectively (2, 3, 11). Whereas PuDAH7PS and the DAH7PS from Aeropyrum pernix (ApeDAH7PS), two unregulated enzymes, have the best catalytic performances with Mn$^{2+}$ and Zn$^{2+}$, respectively (10, 28). The physiological relevance of the different divalent metal dependence of DAH7PS enzymes is still unclear and is likely to relate to environmental availability.

Consistent with the sequence-predicted compartmentalization of the DAH7PS and CM functions, the two individual domain variants, PniDAH7PS$^D$ and PniDAH7PS$^C_M$ exhibited only DAH7PS or CM activities, respectively. However, both separated domains were found to have significantly attenuated catalytic activities relative to that observed for the full-length enzyme. The separation of the two domains resulted in a dramatic increase in the $K_m$ values for PEP, E4P, and chorismate ($K_m^{PEP}$, $K_m^{E4P}$, and $K_m^{ch}$) and a decreased turnover number ($k_{cat}$) for the DAH7PS reaction (Table 2). A loss of catalytic efficiency for both domains on separation was also reported for PgiDAH7PS (2), but this has not been observed for other type Iβ DAH7PS where the CM domain is located at the N terminus, such as BsuDAH7PS (from Bacillus subtilis) and GspDAH7PS (2, 3).

Prephenate inhibits both DAH7PS and CM activities

The role of CM as a regulatory domain mediating the inhibition by prephenate, the product of the CM reaction, on DAH7PS has been clearly described for GspDAH7PS (3, 29). To determine whether prephenate has a similar inhibitory effect on PniDAH7PS, the DAH7PS catalytic activity of PniDAH7PS was examined in the presence of prephenate. PniDAH7PS catalytic activity was found to be extremely sensitive to prephenate; the relative half-maximal inhibition concentration (IC$_{50}$) of prephenate was calculated as 4.2 $\mu$M. However, under these conditions prephenate was not able to completely suppress the activity of PniDAH7PS, with ~25% of activity remaining even at the highest prephenate concentrations tested (Fig. 4A). Consistent with the predicted binding site for prephenate residing in the CM domain, the inhibition by prephenate of DAH7PS activity. To confirm that prephenate directly binds to the active site of the CM domain, the inhibition by prephenate of the CM activity of the PniDAH7PS was also assessed. As expected, prephenate displayed typical competitive inhibition of CM activity with a $K_i$ value of 1.2 $\mu$M (Fig. 4B).

PniDAH7PS is homodimeric

All characterized type 1β DAH7PS proteins adopt homotetrameric assemblies (3, 10, 30–32). This tetrameric assembly has been shown to be important for maintaining the normal function of DAH7PS, including catalysis and providing thermal stability and allosteric regulation (31, 33). The quaternary structure of full-length PniDAH7PS was investigated using analytical ultracentrifugation (AUC) and the absorbance versus radial position data were collected. Fitting the data into a continuous size distribution model, the transformed sedimentation coefficient distributions ($c(S)$) plots indicated a major species with a calculated mass of ~75 kDa, consistent with a homodimeric assembly (the theoretical molecular mass of the
The monomeric unit of PniDAH7PS is 40 kDa (Fig. 5A). The presence of 200 μM prephenate did not alter the sedimentation coefficient, suggesting that the allosteric inhibition by prephenate does not alter the quaternary assembly of PniDAH7PS.

The quaternary structures of the two truncated variants were also investigated. Intriguingly, the sedimentation velocity experiments for PniDAH7PS revealed a molecular mass of 29 kDa corresponding to a monomer (Fig. 5A), whereas PniDAH7PS<sub>CM</sub>, which was examined using analytical size exclusion chromatography (SEC) due to its low extinction coefficient, gave a clear single symmetrical peak corresponding to a mass of 23 kDa, consistent with a homodimeric species (Fig. 5C). These observations imply that the PniDAH7PS homodimer is assembled via dimerization of its CM domains, and that there may be limited interaction between DAH7PS domains in the homodimeric WT protein.

A large conformational change for PniDAH7PS in response to prephenate is observed by small angle X-ray scattering (SEC-SAXS) analysis was performed to obtain the low resolution profiles of PniDAH7PS<sup>D</sup>, PniDAH7PS<sup>CM</sup>, and PniDAH7PS, and further determine the effect of prephenate on the WT protein conformation in solution. The scattering data for PniDAH7PS<sup>D</sup> and PniDAH7PS<sup>CM</sup> were consistent with a compact globular and an elongated flat shape, respectively (Fig. 6, A and B, and Table 3). The Porod volumes for two variants, 49,700 Å<sup>3</sup> of PniDAH7PS<sup>D</sup> and 35,800 Å<sup>3</sup> of PniDAH7PS<sup>CM</sup>, combined with the pairwise distance distribution (P(r)) analysis corresponding to a monomer (Fig. 5), whereas PniDAH7PS<sup>CM</sup>, which was examined using analytical size exclusion chromatography (SEC) due to its low extinction coefficient, gave a clear single symmetrical peak corresponding to a mass of 23 kDa, consistent with a homodimeric species (Fig. 5C). These observations imply that the PniDAH7PS homodimer is assembled via dimerization of its CM domains, and that there may be limited interaction between DAH7PS domains in the homodimeric WT protein.

A large conformational change for PniDAH7PS in response to prephenate is observed by small angle X-ray scattering

Size exclusion chromatography in line with small angle X-ray scattering (SEC-SAXS) analysis was performed to obtain the low resolution profiles of PniDAH7PS<sup>D</sup>, PniDAH7PS<sup>CM</sup>, and PniDAH7PS, and further determine the effect of prephenate on the WT protein conformation in solution. The scattering data for PniDAH7PS<sup>D</sup> and PniDAH7PS<sup>CM</sup> were consistent with a compact globular and an elongated flat shape, respectively (Fig. 6, A and B, and Table 3). The Porod volumes for two variants, 49,700 Å<sup>3</sup> of PniDAH7PS<sup>D</sup> and 35,800 Å<sup>3</sup> of PniDAH7PS<sup>CM</sup>, combined with the pairwise distance distribution (P(r)) analysis corresponding to a monomer (Fig. 5), whereas PniDAH7PS<sup>CM</sup>, which was examined using analytical size exclusion chromatography (SEC) due to its low extinction coefficient, gave a clear single symmetrical peak corresponding to a mass of 23 kDa, consistent with a homodimeric species (Fig. 5C). These observations imply that the PniDAH7PS homodimer is assembled via dimerization of its CM domains, and that there may be limited interaction between DAH7PS domains in the homodimeric WT protein.
ysis, further supports a monomeric and dimeric assembly for PnIDAH7PS and PnIDAH7PSCM, respectively (Table 3). To verify these characterizations of PnIDAH7PS and PnIDAH7PSCM, homology modeling of monomeric PnIDAH7PS and homodimeric PnIDAH7PSCM was performed with Modeler (34) using PfuDAH7PS (PDB ID code 4C1L) and PfuCM (PDB ID code 1YBZ) as template structures, respectively (Fig. 6D). Both models are in good agreement with the corresponding scattering data ($\chi^2 = 1.1$ and 0.5 for PnIDAH7PS and PnIDAH7PSCM, respectively) (Fig. 6C).

The WT full-length enzyme was assessed following the investigation of the separated domains. The experimental profile of the prephenate-free PnIDAH7PS suggested an elongated protein with a Porod volume of 131,338 Å3, corresponding to an estimated molecular mass of ~77 kDa for PnIDAH7PS, a value consistent with the proposed homodimeric state of PnIDAH7PS in solution. Remarkably, when prephenate is included, a striking change in scattering profile was observed (Fig. 7). The significant decrease of the radius of gyration ($R_g$), maximum particle diameter ($D_{max}$), and Porod volume (Table 2) kinetic parameters for PnIDAH7PS, PnIDAH7PSD, and PnIDAH7PSCM, and kinetic constants of PgiDAH7PS and its DAH7PS- and CM-truncated variants (PgiDAH7PSD and PgiDAH7PSCM) for comparison

| Enzyme                  | DAH7PS activity | CM activity |
|-------------------------|-----------------|-------------|
|                         | $K_{M}^{PEP}$   | $K_{M}^{E4P}$| $k_{cat}$ | $k_{cat}/K_{M}^{PEP}$ | $k_{cat}/K_{M}^{E4P}$ | $k_{cat}/K_{M}^{	ext{chorismate}}$ |
| PnIDAH7PS               | 41 ± 4          | 58 ± 6      | 16.8 ± 0.5 | 0.41                  | 2.92 ± 0.03             | 7.6 ± 0.6          |
| PnIDAH7PSD              | 253 ± 61        | 803 ± 105   | 1.6 ± 0.2  | 6.3 × 10^{-3}         | 2.0 × 10^{-3}           | 1.68 ± 0.06        |
| PnIDAH7PSCM             | NA              | NA          | NA        | NA                    | NA                    | NA                  |
| PgiDAH7PS(2)            | 421 ± 43        | 1238 ± 141  | 1.6 ± 0.1  | 3.8 × 10^{-3}         | 1.3 × 10^{-3}           | 84 ± 5              |
| PgiDAH7PSD(2)           | 2724 ± 256      | 3638 ± 375  | 0.8 ± 0.1  | 2.9 × 10^{-4}         | 2.2 × 10^{-4}           | 337 ± 15            |
| PgiDAH7PSCM(2)          | NA              | NA          | NA        | NA                    | NA                    | NA                  |

*NA, not applicable.

Figure 3. Activation of the DAH7PS activities of PnIDAH7PS (blue) and PnIDAH7PSD (red) by different divalent metal ions (100 μM) relative to the activity with Mn$^{2+}$. The activities of both enzymes in the presence of the metal chelator EDTA are also shown as the controls. Error bars represent the S.D. from triplicate measurements.

Figure 4. A, response of PnIDAH7PS (black) and PnIDAH7PSD (red) to prephenate. B, inhibition of prephenate on the CM activity of PnIDAH7PS. Error bars represent the S.D. from triplicate measurements.

Table 2 Kinetic parameters for PnIDAH7PS, PnIDAH7PSD, and PnIDAH7PSCM, and kinetic constants of PgiDAH7PS and its DAH7PS- and CM-truncated variants (PgiDAH7PSD and PgiDAH7PSCM) for comparison

| Enzyme                  | DAH7PS activity | CM activity |
|-------------------------|-----------------|-------------|
|                         | $K_{M}^{PEP}$   | $K_{M}^{E4P}$| $k_{cat}$ | $k_{cat}/K_{M}^{PEP}$ | $k_{cat}/K_{M}^{E4P}$ | $k_{cat}/K_{M}^{	ext{chorismate}}$ |
| PnIDAH7PS               | 41 ± 4          | 58 ± 6      | 16.8 ± 0.5 | 0.41                  | 2.92 ± 0.03             | 7.6 ± 0.6          |
| PnIDAH7PSD              | 253 ± 61        | 803 ± 105   | 1.6 ± 0.2  | 6.3 × 10^{-3}         | 2.0 × 10^{-3}           | 1.68 ± 0.06        |
| PnIDAH7PSCM             | NA              | NA          | NA        | NA                    | NA                    | NA                  |
| PgiDAH7PS(2)            | 421 ± 43        | 1238 ± 141  | 1.6 ± 0.1  | 3.8 × 10^{-3}         | 1.3 × 10^{-3}           | 84 ± 5              |
| PgiDAH7PSD(2)           | 2724 ± 256      | 3638 ± 375  | 0.8 ± 0.1  | 2.9 × 10^{-4}         | 2.2 × 10^{-4}           | 337 ± 15            |
| PgiDAH7PSCM(2)          | NA              | NA          | NA        | NA                    | NA                    | NA                  |

*NA, not applicable.
3) clearly imply a transformation of the PniDAH7PS structure from an elongated shape to a smaller and more compact conformation is associated with prephenate binding.

The Kratky plot for the experimental data of prephenate-free PniDAH7PS exhibits a collapsed bell-shaped curve at the low q range, indicating a less compact structure containing some highly dynamical or poorly folded local regions. However, this relatively irregular structure becomes far more compact and organized in the presence of prephenate, as demonstrated by the more regular bell-shaped plot (Fig. 7). Moreover, the Porod-Debye plot reveals a curve with a plateau with prephenate, whereas a distinct ascending curve is observed in the absence of prephenate at low q values (Fig. 7D). The large difference in Porod-Debye plots clearly illustrates that the intrinsic flexibility of ligand-free PniDAH7PS is notably attenuated by the binding of prephenate (35, 36), which is consistent with the conformational change indicated by the Kratky plots.

To visualize the structural information available from the scattering profiles, rigid body modeling was carried out using BUNCH (37) to dock homology models for PniDAH7PSD and PniDAH7PSCM into the molecular envelopes derived from the SAXS data of PniDAH7PS. Both rigid body models for PniDAH7PS in the absence and presence of prephenate are in good agreement (χ² = 0.3 and 0.4, respectively) to their corresponding experimental SAXS data (Fig. 8A). As expected, the predicted rigid body model for the prephenate-free PniDAH7PS is elongated, and exhibits symmetry with a dumbbell shape, consisting of a central CM dimer with two flanking DAH7PS barrel domains (Fig. 8B). In this model, the loop α2–α3 of the CM domain is predicted to be in close proximity to loop β6–α6, which is involved in the active site of DAH7PS (Fig. 8B). This spatial arrangement is substantially changed when prephenate is present; the model predicts a structure for which the two barrel domains are found closer to the CM dimer, consistent with a more intimate association between two DAH7PS domains and CM dimeric moiety (Fig. 8B).

Discussion
Multifunctional enzymes play diverse roles in improving the performance of metabolic pathways. Here we have characterized a bifunctional enzymatic system from P. nigrescens where the catalytic efficiencies of a DAH7PS moiety and its C-terminal CM domain, catalyzing nonconsecutive reactions in aromatic amino acid biosynthesis, are enhanced by their association. This
Figure 6. SAXS profiles and homology models of PniDAH7PSD and PniDAH7PSCM. A, SAXS profiles of PniDAH7PSD (blue) and PniDAH7PSCM (red) (log I(q) versus q). B, Guinier plots for the scattering data of PniDAH7PSD (blue) and PniDAH7PSCM (red) (ln I(q) versus q²). C, the calculated scattering profiles (red line) of homology models for monomeric PniDAH7PSD (left) and PniDAH7PSCM homodimer (right) fitted to their corresponding scattering profiles (black circles), presenting χ² = 1.1 and 0.5, respectively. Note: the scattering profiles (black circles) used for these comparisons are identical data to those presented in panel A. D, the homology models for the monomeric PniDAH7PSD (upper) and PniDAH7PSCM homodimer (lower).

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association is further manipulated to provide allosteric regulation on the DAH7PS function.

Our findings show that this C-terminal CM-linked DAH7PS system is quite distinct from the N-terminal CM-linked DAH7PS enzymes, such as GspDAH7PS, for which detailed structural studies have been carried out (3, 32). First, it should be noted that the CM domain of PniDAH7PS is the sole source of CM activity within the cell, whereas organisms with the N-terminal CM fusions to DAH7PS have another efficient CM, present as a monofunctional unregulated enzyme (38). This difference may account for the observation that the N-terminal CM domains can be removed, with minimal catalytic penalty on either the DAH7PS or CM function (2). In contrast, for PniDAH7PS there is strong hetero-domain interplay between DAH7PS and CM moieties, such that their separation significantly attenuates both catalytic activities. Thus, removal of the CM domain does not just deny DAH7PS of allosteric control, suggesting that the grafted CM domain plays a dual role in delivering pathway regulation and efficient catalysis by both DAH7PS and CM.

The functional interdependency of the two enzymatic moieties appears to be related to a unique dimeric quaternary assembly for PniDAH7PS. This protein has an overall conformation in which DAH7PS catalytic barrels have no direct inter-

### Table 3

|                          | PniDAH7PSD | PniDAH7PS CM | PniDAH7PS prephenate free | PniDAH7PS + prephenate |
|--------------------------|------------|--------------|---------------------------|------------------------|
| $R_g$ (Å) (from $P(r)$)  | 19.3 ± 0.5 | 23.2 ± 0.6   | 44.1 ± 0.5                | 31.8 ± 0.2             |
| $I(0)$ (cm$^{-1}$) (from $P(r)$) | 0.06 ± 0.00 | 0.07 ± 0.02 | 0.11 ± 0.00               | 0.09 ± 0.00            |
| $B_r$ (Å) (from Guinier analysis) | 19.8 ± 1.1  | 22.2 ± 0.8   | 43.4 ± 0.7                | 31.8 ± 0.6             |
| $I(0)(cm^{-1})$ (from Guinier analysis) | 0.06 ± 0.00 | 0.07 ± 0.00 | 0.11 ± 0.00               | 0.09 ± 0.00            |
| $D_{max}$ (Å)            | 57.0       | 74.0         | 161.3                     | 102.3                  |
| $V_p$ (Å$^3$)            | 49,693     | 35,821       | 130,910                   | 108,708                |
| $M_{W_{Porod}}$ (Da)     | 31,058     | 22,388       | 81,819                    | 67,943                 |
| $M_{W_{Prot}}$ (Da)      | 29,464     | 11,191       | 40,364                    | 40,364                 |
| Number of subunits       | 1          | 2            | 2                         | 2                      |

* $V_p$, Porod volume.
* $M_{W_{Porod}}$, molecular weight estimated from Porod volume.
* $M_{W_{Prot}}$, molecular weight calculated based on protein sequence. The uncertainties represent S.D. estimated by the error propagation from the experimental data.

Figure 7. Analysis of the SAXS profiles of PniDAH7PS in the absence (blue) and presence (red) of prephenate. A, SAXS profiles ($\log I(q)$ versus $q$). B, Guinier plots ($\ln I(q)$ versus $q^2$). C, Kratky plot ($q^2 I(q)$ versus $q$). D, Porod-Debye plot ($q^4 I(q)$ versus $q^4$) limited to the range of the SAXS data for which the Guinier linearity is observed.
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Figure 8. Rigid body modeling for PniDAH7PS in the absence and presence of prephenate against SAXS profiles. A, the calculated scattering profiles (red line) of rigid body models for the prephenate-free (left) and bound (right) PniDAH7PS fit to their corresponding experimental data (black scatter), presenting $\chi^2 = 0.3$ and 0.4, respectively. The bottom panels show residual plots. Note: the scattering profiles of the prephenate-free and bound PniDAH7PS for the model fitting is the same data presented in Fig. 7A. B, the rigid body models for the prephenate-free (upper) and bound (lower) PniDAH7PS. The blue and red dummy models are the predicted DAH7PS and CM domains, respectively.

action, distinguishing it from the homotetrameric or homodimeric assemblies found in all other characterized DAH7PS proteins (3, 10, 13, 14, 31, 39).

Homomeric domain interfaces play important roles for DAH7PS, particularly for the type I DAH7PS proteins where one of the interfaces in the homodimeric protein is located proximally to the active site, and therefore likely plays a role in the integrity of the active site and hence supports catalysis (31, 39). With no direct interaction between the DAH7PS barrels for dimeric full-length PniDAH7PS, it appears likely that the interface to support catalysis is provided by a hetero-domain interaction between the DAH7PS barrel and the CM moiety.
This conclusion is supported by the X-ray scattering data, which supports a dimeric structure for full-length DAH7PS where the DAH7PS barrels are only able to form interdomain interfaces with the CM domains.

If this hetero-domain interaction provides the structural basis to support efficient enzymatic functions for both DAH7PS and CM, interrupting this interaction would be expected to cause the same outcome as physically separating the two domains, that is to result in comparable attenuation of both catalytic activities. Prephenate binding does just this, as evidenced by the dramatic conformational change of PniDAHPS on the addition of this allosteric inhibitor. Thus, catalytic enhancement and regulation appear to share a common mechanism.

The inhibition of the DAH7PS activity by prephenate is incomplete. This observation supports a model for which hetero-domain disruption confers allosteric response, as the isolated monomeric PniDAHPS also displays residual activity. It is also worth noting that the concentration of prephenate reports only on the Tyr/Phe branch of aromatic amino acid biosynthesis and thus may not be an indicator of the requirement for Trp. Residual DAH7PS activity thus prevents a complete shutdown of Trp biosynthesis.

The domain interactions and functional interplay between efficient catalysis and regulation for the PniDAH7PS system substantially expand the space for reconsidering the evolutionary trajectory of DAH7PS. Given that the homomeric association is not a necessary condition for yielding a fully functional type Iβ DAH7PS, an evolutionary trajectory where the ancestor of contemporary type Iβ DAH7PS enzymes was monomeric, and was subsequently adapted to self-associate or by fusion with a C-terminal CM is conceivable. Given the common structural solution for supporting regulation and efficient catalysis, it appears likely that these benefits of fusion co-evolved. Unraveling the evolutionary details for this heterogenic DAH7PS proteins await a deeper phylogenetic investigation and more experimental investigation.

The linkage between CM and DAH7PS is a repeated observation in contemporary systems. As well as the N-terminal–fused CM found for type Iβ DAH7PS protein, type II DAH7PS proteins also demonstrate a direct CM-DAH7PS interaction (40–42). Albeit that this is an interchain, rather than intrachain association, this type II system bears significant resemblance to the type II system. For instance, the DAH7PS and the intracellular CM from Mycobacterium tuberculosis (MtuDAH7PS and MtuCM) form an octameric complex, where the hetero-interactions between homotetrameric MtuDAH7PS and MtuCM homodimer are required for full activity of the CM subunit. Allostery for the CM activity is delivered by disruption of heteromeric interface (42, 43). These common properties of hetero-functional dependence and allostery shared by PniDAH7PS and MtuDAH7PS-CM complexes reveal a sophisticated functionality that arises from the organization as multi-functional enzyme or enzymatic complex. Multifunctionality thus appears to be an efficient evolutionary strategy to support both enzymatic catalysis and regulation in the shikimate pathway.

### Experimental procedures

#### Amino acid sequence alignments and CLANS

Seed sequences of DAHP synthetase I (PF00793), representing type Iβ and type Iα DAH7PS and KDO8PS, was extracted from the Pfam database (44) and aligned using Clustal Ω (45). Aligned seeds sequence file was then submitted to jackhammer, a tool of the HMMER web server (46) to generate a hidden Markov model for each multiple sequence alignment, which is in turn used to retrieve type I DAH7PS and KDO8PS sequences from four databases: UniProt (47), RefSeq (48), Pfamseq (44), and NR (48). After removing redundancy with a 90% sequence identity threshold by Jalview (49), all candidate sequences were processed with the clustering method implemented in CLANS (50). Following an all-against-all BLAST search of the sequences, the force-directed pairwise similarities clustering algorithm was run for more than 5000 iteration cycles at a p value of $10^{-40}$, yielding three clusters that were identified as type Iβ, type Iα, KDO8PS, and rendered as 3D graphs. After removal of the outliers, the sequences of type Iβ and KDO8PS were extracted and put into CLANS again to obtain more distinct clusters. Sequence alignments were generated with T-Coffee (51) and rendered with ESPript 3.

#### Construction of expression vectors

The gene encoding PniDAH7PS was codon-optimized for the expression in engineered Escherichia coli, and synthesized by Geneart (Life Technologies, Inc.). Using In-Fusion HD Cloning technology (Clontech, Inc.), the synthetic PniDAH7PS gene was amplified using specific primers (Table 4) and inserted into the expression vector pET28a between NdeI and XhoI restriction sites for recombinant expression of the PniDAH7PS with an N-terminal cleavable His$_6$ tag and a cleavage site for tobacco etch virus (TEV) protease.

To generate constructs for the expression of the separate DAH7PS and CM moieties, PniDAH7PS$^{\text{D}}$ and PniDAH7PS$^{\text{CM}}$, the sequences encoding the DAH7PS (Met$^1$–Lys$^{261}$) and CM (His$^{262}$–Asn$^{354}$) domains of PniDAH7PS were amplified with two pairs of specific primers (Table 4). To the products of the first round PCR were added attB1 and attB2 recombiant sites at 5′ and 3′ ends, respectively, via a second PCR using a pair of generic gateway primers (Table 4). The purified PCR products were incorporated into the donor vector pDONR-221 using BP clonease enzyme mix (Life Technologies, Inc.) (52). Subsequently PniDAH7PS$^{\text{D}}$ and PniDAH7PS$^{\text{CM}}$ genes within the recombinant pDONR-221 plasmids were transferred into the destination vector pDEST17 (pDEST17-PniDAH7PS$^{\text{D}}$) and pDEST15 (pDEST17-PniDAH7PS) using LR clonase enzyme mix (Life Technologies, Inc.) (53). The pDEST17-PniDAH7PS$^{\text{D}}$ and pDEST17-PniDAH7PS expression vectors, respectively, produce the truncated variants of the PniDAH7PS$^{\text{D}}$ with an N-terminal cleavable His$_6$ tag and the PniDAH7PS$^{\text{CM}}$ with an N-terminal cleavable GST tag, respectively. The removal of either the His$_6$ tag or the GST tag by TEV protease cleavage also leaves four non-native residues (Gly-Ser-Gly-Ala) in front of the N terminus of either variant.
Protein expression and purification

The expression vectors pET28a-PniDAH7PS and pDEST17-PniDAH7PS were transformed into E. coli BL21 (DE3) pBB540/pBB542 cells, and pDEST15-PniDAH7PSCM was transformed into E. coli BL21 (DE3) pLysS cells. LB medium containing 50 g/ml of kanamycin, 25 g/ml of chloramphenicol, and 10 g/ml of spectinomycin was used for the expression of PniDAH7PS and PniDAH7PSD. LB medium containing 100 g/ml of ampicillin and 25 g/ml of chloramphenicol was used for the expression PniDAH7PSCM.

Culture growth were all at 37 °C. Induction of protein expression was initiated by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside at A600 0.4–0.8, followed by further overnight growth at 23 °C.

Cells were harvested using centrifugation (12,000 g, 4 °C, 15 min) and resuspended in lysis buffer (50 mM Bistris propane, 200 mM KCl, 200 μM PEP, and 10 μM of imidazole) for the expression of PniDAH7PS and PniDAH7PSD. LB medium containing 100 μg/ml of ampicillin and 25 μg/ml of chloramphenicol was used for the expression PniDAH7PSCM. Culture growth were all at 37 °C. Induction of protein expression was initiated by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside at A600 0.4–0.8, followed by further overnight growth at 23 °C.

Purification of PniDAH7PS and PniDAH7PSD—The clarified cell lysate of PniDAH7PS or PniDAH7PSD was filtered and loaded to a HiTrap TALON crude column (filled with a cobalt-based affinity resin, GE Healthcare) pre-equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH 7.4), then eluted with elution buffer (20 mM sodium phosphate, 500 mM NaCl, 250 mM imidazole, pH 7.4). The eluted fractions were pooled together and desalted. The His6 tag was removed by TEV protease cleavage overnight at 4 °C and the protein was reapplied to the TALON column again to remove the TEV protease. The His6 tag, and any uncleaved protein. The untagged proteins were further purified by SEC (Superdex S200 26/60 column, GE Healthcare) using a buffer of 10 mM Bistris propane, 150 mM NaCl, 200 μM PEP, pH 7.4.

Purification of PniDAH7PSCM—The crude cell extract of either PniDAH7PSCM was filtered and applied to a GSTrapTM HP column (GE Healthcare) pre-equilibrated with binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3) and then eluted with the elution buffer (50 mM Tris-HCl, 10 mM reduced GSH, pH 8.0). The eluted fractions were collected and desalted, and then were subjected to TEV cleavage overnight at 4 °C for the removal of the GST tag. Following the cleavage reaction, the protein mixture was reloaded onto the GSTrap column to remove the TEV protease, the cleaved GST tag, and uncleaved protein. The protein of interest was collected from the flow-through and further purified by SEC using HiLoad 16/60 Superdex 75 column (GE Healthcare) and SEC buffer containing 10 mM Bistris propane, pH 8.1, 150 mM NaCl.

Kinetic characteristics

A modified continuous assay for the spectrophotometric analysis was employed for determining the kinetic parameters of the WT enzyme and two truncated variants. Enzymatic reactions were carried out in 1-cm path length quartz cuvettes at 35 °C, and the reaction rates were monitored by the disappearance of PEP at 232 nm or chorismate at 274 nm for DAH7PS and CM activities, respectively.

The determination of metal dependence of PniDAH7PS and PniDAH7PSD was carried out with 200 mM PEP and 225 μM E4P in the presence of 100 μM MnSO4, CdCl2, CoCl2, CuCl2, CaCl2, MgSO4, NiCl2, SrCl2, PdCl2, or EDTA.

The reaction mixture for determining the kinetic parameters for the DAH7PS domain of PniDAH7PS contained 100 μM Mn2+, 7.3 × 10−2 μM enzyme in 50 mM Bistris propane buffer, pH 7.4. To determine kinetic parameters, the PEP concentra-
tion was fixed at 338 μM and the concentration of E4P was fixed at 329 μM, while conducting assays with varied concentrations of the other substrates.

The assays for determining the kinetic parameters for *Pn*DAH7PSD contained 100 μM Mn²⁺, 0.41 μM enzyme in 50 mM Bistris propane buffer, pH 7.4. Due to the high apparent *Kₐ* for E4P of this truncated variant and the dimerization of E4P at high concentrations (54), kinetic parameters were determined by measuring the rate of consumption of PEP at different fixed concentrations of PEP (121, 242, and 484 μM, respectively) as the E4P concentration was varied (75–445 μM). The data were fitted to the equation of Alberty (55),

\[
\nu_{\text{max}} = \frac{V_{\text{max}}[A][B]}{K_{i}[A] + K_{m} + [A][B] + K_{d}K_{m}} \quad (\text{Eq. 1})
\]

where *V*ₘₐₓ is the maximum possible *υυₜₜ*; [A] is the concentration of the variable substrate (E4P), and [B] is the concentration of the substrate held constant (PEP) for each round Michaelis-Menten kinetic measurement; *K*ₘₐₓ (Kₐ[A]) are the concentrations of substrate B (A), which gives one-half *V*ₘₐₓ when substrate A (B) is saturating; *K*ₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚportion of substrate B (A), which gives one-half *V*ₘₐₓ when substrate A (B) is saturating; *K*ₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚportion of substrate B (A), which gives one-half *V*ₘₐₓ when substrate A (B) is saturating.

The assay for determining the kinetic parameters for the CM domain of *Pn*DAH7PS contained 6.8 × 10⁻² μM enzyme in 50 mM Bistris propane buffer, pH 7.4, and initiated by the addition of chorismate with a series of concentrations.

**Feedback inhibition**

The inhibition of DAH7PS activity for *Pn*DAH7PS and *Pn*DAH7PSD was characterized by varying concentrations of prephenate (0–315 μM) in a reaction carried out at 35 °C, containing 100 μM Mn²⁺, 200 μM PEP, 225 μM E4P and either 7.3 × 10⁻² μM enzyme (*Pn*DAH7PS) or 0.41 μM enzyme (*Pn*DAH7PSD), in 50 mM Bistris propane buffer (pH 7.4). The calculation of IC₅₀ was obtained by fitting data to Equation 2.

\[
Y = \frac{\text{Max} + (\text{Max} - \text{Min})}{1 + 10^{X - \text{LogIC₅₀}}} \quad (\text{Eq. 2})
\]

The product inhibition of prephenate on the CM activity of *Pn*DAH7PS was examined also using the continuous spectrophotometric assay in 50 mM Bistris propane buffer, pH 7.4, at 35 °C. The *K*ᵢ value for prephenate was determined by varying the prephenate concentration from 0 to 20 μM, with a chorismate concentration range of 0–310 μM.

**Determination of quaternary structure**

**ALIC—Sedimentation velocities of *Pn*DAH7PS and *Pn*DAH7PSD** at varied concentrations were determined using a Beckman Coulter Model XL-1 analytical ultracentrifuge equipped with a UV-visible dual-beam UV-visible spectrophotometer with a monochromator. The reference buffer solution (10 mM Bistris propane, 150 mM NaCl, pH 7.4) and sample solutions were examined in 12-mm double sector aluminum center pieces with quartz windows that were mounted in a 4-hole An60Ti rotor. Samples (380 μl/cell) and buffer reference (400 μl/cell) were centrifuged at 50,000 rpm at 20 °C and absorbance data were collected in continuous mode at 275 nm without averaging. The partial specific volume (ι) of the samples (0.7445 ml/g for *Pn*DAH7PS and 0.7479 ml/g for *Pn*DAH7PSD), buffer density (1.0049 g/ml), and buffer viscosity (1.0214 cP) were calculated using SEDNTERP (56). The data were fitted to a continuous size distribution model using the program SEDFIT (57).

**Analytical SEC—**Analytical SEC was carried out using a Superdex 10/300 GL column (GE Healthcare) pre-equilibrated with the SEC buffer for *Pn*DAH7PSCM. 500 μl of 0.5 mg/ml of protein standards (GE Healthcare) in SEC buffer, RNase A (13.7 kDa), ovalbumin (43 kDa), conalbumin (75 kDa), aldolase (158 kDa), and blue dextran (2000 kDa), were applied at a flow rate of 0.2 ml/min. Following analysis of the standard proteins, 500 μl of a *Pn*DAH7PSCM sample at 1 mg/ml was injected onto the column followed by elution with the SEC buffer. The elution volumes of blue dextran and other standard proteins were, respectively, recorded as the void volume (*V*ₖ) and *V*ₑ. Using the equation *K*ₐᵥ = (*V*ₑ − *V*ₖ)/(*V*ₑ − *V*₀), *V*ₑ values were transformed to *K*ₐᵥ (a coefficient defining the proportion of the bead pores that can be occupied by molecule) values that were further plotted against log (mass of protein standard (Da)) to generate a calibration curve and a regression equation. With the aid of this correlation between the elution volume and protein mass, the calculated mass of sample was obtained based on its elution volume.

**SAXS**

SAXS experiments were conducted at the Australian Synchrotron, using the SAXS/WAXS equipped with a Pilatus detector (1 m, 170 × 170 mm, effective pixel size, 172 × 172 mm) (58). Samples were examined by X-ray with a wavelength of 1.0332 Å, and the subsequently scattered X-ray intensities were recorded using a sample detector at a distance of 1.6 m from the sample (providing a q-range of 0.0015–3.0 Å⁻¹). The *Pn*DAH7PS sample was eluted from a SEC column (Superdex 200 5/150) by SEC buffer (10 mM Bistris propane, pH 7.4, 150 mM NaCl, 200 mM PEP) with or without 400 μM prephenate. *Pn*DAH7PSD and *Pn*DAH7PSCM were eluted, respectively, by their SEC buffers as described above. The eluted sample passed into a 1.5-mm thin-walled glass capillary where the sample was examined by X-ray at 25 °C at 2-s intervals, and the scattering data were collected.

The reduction and buffer subtraction for raw scattering data of protein sample were performed using Scatterbrain developed at the Australian Synchrotron. Processed data were plotted (f(q) versus q) and analyzed using Primus (59) and Origin 8.

The SEC-SAXS data set for *Pn*DAH7PS, in either the absence or presence of prephenate, displays a single symmetric peak over ~120 frames. The maximum scattering was observed at frame 373 and frame 353, respectively, in the prephenate-free and prephenate-bound data sets. To confirm conformational homogeneity, three different groups of data were summed for each data set. The summed data covered the maximum scattering intensity and either side of the scattering maxima. The same scattering profile was observed across the scattering peak. Therefore, frames 371–375 and frames 351–355 were summed for the prephenate-free and prephenate-bound conditions, respectively.
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The data sets for structural analysis were recorded with 447 data points over the range 0.005 ≤ q ≤ 0.35 Å⁻¹. The radius gyration (R_g) of protein particle was evaluated from Guinier plots (ln I(q) versus q²) of scattering data. Also the linear Guinier plots in the q range of q less than 1.3/R_g is a crucial indicative of the monodispersity of samples. Porod volume and the maximum dimension of protein particle were evaluated, respectively, from the Porod plot and the pair-distance distribution (P(r)) function yielded via Fourier transformation. MMPorod were obtained by dividing the Porod volumes by 1.7.

Homology and rigid body modeling

Homology modeling—Homology models of monomeric PniDAH7PS⁰ and, both monomeric and homodimeric PniDAH7PS⁴ were generated using MODELLER (37) utilizing the “automodel” routine to create 100 models, using the crystal structures of PfuDAH7PS (PDB ID code 4C1L) and PfuCM (PDB ID code 1YBZ) as templates (9, 60). The best model was selected based on models exhibiting the lowest MODELLER objective function value and the lowest global DOPE score, followed by visual inspection and a residue-by-residue DOPE score analysis.

Rigid body modeling—Rigid body modeling against SAXS data were carried out using BUNCH (37) and homology models of PniDAH7PS⁰ and PniDAH7PS⁴. Prior to running BUNCH modeling for the PniDAH7PS⁴ homodimer, an initial structural model of monomeric PniDAH7PS⁴ was preliminarily constructed by pre-bunch via linking the models of PniDAH7PS⁰ and PniDAH7PS⁴ together according to the sequence of PniDAH7PS⁴. Sequentially, the initial structure was inputted into BUNCH together with the experimental SAXS data and calculated scattering amplitudes for the PniDAH7PS⁰ and PniDAH7PS⁴ models. Then, 80–100 steps of simulated annealing, constrained by P2 symmetry and contact information for the PniDAH7PS⁴ dimer, were carried out, which gave rise to a rigid body model and a fit to the corresponding SAXS profile.

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References

1. Singh, H., Arentson, B. W., Becker, D. F., and Tanner, J. J. (2014) Structures of the PutA peripheral membrane flavoenzyme reveal a dynamic substrate-channeling tunnel and the quinone-binding site. Proc. Natl. Acad. Sci. U.S.A. 111, 3389–3394 CrossRef Medline
2. Wu, J., and Woddard, R. W. (2006) New insights into the evolutionary links relating to the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase subfamilies. J. Biol. Chem. 281, 4042–4048 CrossRef Medline
3. Nazmi, A. R., Lang, E. J. M., Bai, Y., Allison, T. M., Othman, M. H., Panjikar, S., Arcus, V. L., and Parker, E. I. (2016) Interdomain conformational changes provide allosteric regulation en route to chorismate. J. Biol. Chem. 291, 21836–21847 CrossRef Medline
4. Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., and Davies, D. R. (1988) Three-dimensional structure of the tryptophan synthase αβ2 multienzyme complex from Salmonella typhimurium. J. Biol. Chem. 263, 17857–17871 Medline
5. Bentley, R. (1990) The shikimate pathway: a metabolic tree with many branches. Crit. Rev. Biochem. Mol. Biol. 25, 307–384 CrossRef Medline
6. Light, S. H., and Anderson, W. F. (2013) The diversity of allosteric controls at the gateway to aromatic amino acid biosynthesis. Protein Sci. 22, 395–404 CrossRef Medline
7. Walker, G. E., Dunbar, B., Hunter, I. S., Nimmo, H. G., and Coggins, J. R. (1996) Evidence for a novel class of microbial 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase in Streptomyces coelicolor A3(2), Streptomyces rimosus and Neurospora crassa. Microbiology142, 1973–1982 Medline
8. Subramaniam, P. S., Xie, G., Xia, T., and Jensen, R. A. (1998) Substrate ambiguity of 3-deoxy-D-arabino-2-octulosonate 8-phosphate synthase from Neisseria gonorrhoeae in the context of its membership in a protein family containing a subset of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases. J. Bacteriol. 180, 119–127 Medline
9. Schofield, L. R., Anderson, B. F., Patchett, M. L., Norris, G. E., Jameson, G. B., and Parker, E. J. (2005) Substrate ambiguity and crystal structure of Pyrococcus furiosus 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase: an ancestral 3-deoxyald-2-ulosonate-phosphate synthase? Biochemistry 44, 11950–11962 CrossRef Medline
10. Zhou, L., Wu, J., Janakiramam, V., Shumilin, I. A., Bauere, R., Kretesing, R. H., and Woodard, R. W. (2012) Structure and characterization of the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from Aeropyrum pernix. Bioorg. Chem. 40, 79–86 Medline
11. Wu, J., Howe, D. L., and Woodard, R. W. (2003) Thermotoga maritima 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase: the ancestral eubacterial DAHP synthase? J. Biol. Chem. 278, 27525–27531 CrossRef Medline
12. Cross, P. J., Pietersma, A. L., Allison, T. M., Wilson-Coutts, S. M., Cochran, F. C., and Parker, E. J. (2011) Neisseria meningitidis expresses a single 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase that is inhibited primarily by phenylalanine. Protein Sci. 22, 1087–1099 CrossRef Medline
13. Webby, C. J., Baker, H. M., Lott, J. S., Baker, E. N., and Parker, E. J. (2005) The structure of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from Mycobacterium tuberculosis reveals a common catalytic scaffold and ancestry for type I and type II enzymes. J. Mol. Biol. 354, 927–939 CrossRef Medline
14. Cross, P. J., Dobson, R. C., Patchett, M. L., and Parker, E. J. (2011) Tyrosine latching of a regulatory gate affords allosteric control of aromatic amino acid biosynthesis. J. Biol. Chem. 286, 10216–10224 CrossRef Medline
15. Lang, E. J., Cross, P. J., Mittelstädt, G., Jameson, G. B., and Parker, E. J. (2014) Allosteric ACtion: the varied ACT domains regulating enzymes of amino-acid metabolism. Curr. Opin. Struct. Biol. 29, 102–111 CrossRef Medline
16. Grant, G. A. (2006) The ACT domain: a small molecule binding domain and its role as a common regulatory element. J. Biol. Chem. 281, 33825–33829 CrossRef Medline
17. Curien, G., Biou, V., Mas-Droux, C., Robert-Genthon, M., Ferrer, J. L., and Dumas, R. (2008) Amino acid biosynthesis: new architectures in allosteric chemistry. Crit. Rev. Biochem. Mol. Biol. 43, 325–339 CrossRef Medline
18. Aravind, L., and Koonin, E. V. (1999) Gleaning non-trivial structural, functional and evolutionary information about proteins by iterative database searches. J. Mol. Biol. 287, 1023–1040 CrossRef Medline
19. Cross, P. J., and Parker, E. J. (2013) Allosteric inhibitor specificity of Thermotoga maritima 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase. FEBS Lett. 587, 3063–3068 CrossRef Medline
20. Cross, P. J., Allison, T. M., Dobson, R. C., Jameson, G. B., and Parker, E. J. (2013) Engineering allosteric control to an unregulated enzyme by transfer of a regulatory domain. Proc. Natl. Acad. Sci. U.S.A. 110, 2111–2116 CrossRef Medline
21. Fan, Y., Cross, P. J., Jameson, G. B., and Parker, E. J. (2018) Exploring modular allosteria by interchangeable regulatory domains. Proc. Natl. Acad. Sci. U.S.A. 115, 3006–3011 CrossRef Medline

22. Fukui, K., Kato, N., Kato, H., Watanabe, K., and Tatematsu, N. (1999) Incidence of Prevotella intermedia and Prevotella nigrescens carriage among family members with subclinical periodontal disease. J. Clin. Microbiol. 37, 3141–3145 Medline

23. Stingu, C. S., Schauermann, R., Jentsch, H., Eschrich, K., Brosteau, O., and Rodloff, A. C. (2013) Association of periodontitis with increased colonization by Prevotella nigrescens. J. Investig. Clin. Dent. 4, 20–25 CrossRef

24. Yakob, M., Söder, B., Meurnan, J. H., Jogestrand, T., Nowak, J., and Söder, P. O. (2011) Prevotella nigrescens and Porphyromonas gingivalis are associated with signs of carotid atherosclerosis in subjects with and without periodontitis. J. Periodontal Res. 46, 749–755 CrossRef Medline

25. Jensen, R. A., Xie, G., Calhoun, D. H., and Bonner, C. A. (2002) The correct calculation of the catalytic mechanism and indicates a new mechanism of allosteric regulation of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. J. Mol. Evol. 54, 416–423 CrossRef Medline

26. Stephens, C. M., and Bauerle, R. (1991) Analysis of the metal requirement of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from Escherichia coli. J. Biol. Chem. 266, 20810–20817 Medline

27. Paravicini, G., Schmidhei, T., and Braus, G. (1989) Purification and properties of the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (phenylalanine-inhibitable) of Saccharomyces cerevisiae. Eur. J. Biochem. 186, 361–366 CrossRef Medline

28. Schofield, L. R., Patchett, M. L., and Parker, E. J. (2004) Expression, purification, and characterization of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from Pyrococcus furiosus. Protein Expr. Purif. 34, 17–27 CrossRef Medline

29. Pratap, S., Dev, A., Kumar, Y., Yadav, R., Narwal, M., Tomar, S., and Kumar, P. (2017) Structure of chorismate mutant-like domain of DAHPs from Bacillus subtilis complexed with novel inhibitor reveals conformational plasticity of active site. Sci. Rep. 7, 6364 CrossRef Medline

30. Shumilin, I. A., Bauerle, R., Wu, J., Woodard, R. W., and Kretsinger, R. H. (2012) Dynamic cross-talk among remote binding sites: the molecular mechanism of allosteric plasticity of active site. J. Mol. Biol. 415, 656–673 CrossRef Medline

31. Nazmi, A. R., Schofield, L. R., Dobson, R. C., Jameson, G. B., and Parker, E. J. (2014) Destabilization of the homotetrameric assembly of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from the hyperthermophile Pyrococcus furiosus enhances enzymatic activity. J. Mol. Biol. 426, 656–673 CrossRef Medline

32. Light, S. H., Halavaty, A. S., Minasov, G., Shuvalova, L., and Anderson, W. F. (2012) Structural analysis of a 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase with an N-terminal chorismate mutase-like regulatory domain. Protein Sci. 21, 887–895 CrossRef Medline

33. Jiao, W., Hutton, R. D., Cross, P. J., Jameson, G. B., and Parker, E. J. (2012) Dynamic cross-talk among remote binding sites: the molecular basis for unusual synergistic allosteroy. J. Mol. Biol. 415, 716–726 CrossRef Medline

34. Webb, B., and Sali, A. (2016) Comparative protein structure modeling using MODELLER. Curr. Protoc. Protein Sci. 86, 2.9.1–2.9.37 Medline

35. Rambo, R. P., and Tainer, J. A. (2011) Characterizing flexible and intrinsically unstructured biological macromolecules by SAS using the Porod-Debye law. Biopolymers 95, 559–571 CrossRef Medline

36. Hammel, M. (2012) Validation of macromolecular flexibility in solution by measuring the tyrosine-sensitive isoenzyme from Escherichia coli. J. Biol. Chem. 251, 5440–5447 Medline

37. Blackmore, N. J., Nazmi, A. R., Hutton, R. D., Webby, M. N., Baker, E. N., Jameson, G. B., and Parker, E. J. (2015) Complex formation between two biosynthetic enzymes modifies the allosteric regulatory properties of both: an example of molecularmosis. J. Biol. Chem. 290, 18187–18198 CrossRef Medline

38. Cross, P. J., Heyes, L. C., Zhang, S., Nazmi, A. R., and Parker, E. J. (2016) The functional unit of Neisseria meningitidis 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase is dimeric. PLoS One 11, e0145187 CrossRef Medline

39. Schuck, P. (2000) Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamn equation modeling. Biophys. J. 78, 1606–1619 CrossRef Medline
Hetero-domain interactions of a bifunctional enzyme

58. Kirby, N. M., Mudie, S. T., Hawley, A. M., Cookson, D. J., Mertens, H. D., Cowieson, N., and Samardzic-Boban, V. (2013) A low-background-intensity focusing small-angle X-ray scattering undulator beamline. *J. Appl. Crystallogr.* **46**, 1670–1680 CrossRef

59. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H. J., and Svergun, D. I. (2003) *PRIMUS*: a Windows PC-based system for small-angle scattering data analysis. *J. Appl. Crystallogr.* **36**, 1295–1296 CrossRef

60. Lee, D., Chen, L., Nguyen, D., Dillard, B. D., Tempel, W., Habel, J., Zhou, W., Chang, S.-H., Kelley, L.-L. C., Liu, Z.-J., Lin, D., Zhang, H., Praissman, J., Bridger, S., Enah, J. C., *et al.* (2005) PDB ID: 1YBZ Conserved hypothetical protein from *Pyrococcus furiosus* Pfis-1581948-001 CrossRef