Neisseria meningitidis expresses a single 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase that is inhibited primarily by phenylalanine

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Abstract: *Neisseria meningitidis* is the causative agent of meningitis and meningococcal septicemia is a major cause of disease worldwide, resulting in brain damage and hearing loss, and can be fatal in a large proportion of cases. The enzyme 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAH7PS) catalyzes the first reaction in the shikimate pathway leading to the biosynthesis of aromatic metabolites including the aromatic acids L-Trp, L-Phe, and L-Tyr. This pathway is absent in humans, meaning that enzymes of the pathway are considered as potential candidates for therapeutic intervention. As the entry point, feedback inhibition of DAH7PS by pathway end products is a key mechanism for the control of pathway flux. The structure of the single DAH7PS expressed by *N. meningitidis* was determined at 2.0 Å resolution. In contrast to the other DAH7PS enzymes, which are inhibited only by a single aromatic amino acid, the *N. meningitidis* DAH7PS was inhibited by all three aromatic amino acids, showing greatest sensitivity to L-Phe. An *N. meningitidis* enzyme variant, in which a single Ser residue at the bottom of the inhibitor-binding cavity was substituted to Gly, altered inhibitor specificity from L-Phe to L-Tyr. Comparison of the crystal structures of both unbound and Tyr-bound forms and the small angle X-ray scattering profiles reveal that *N. meningitidis* DAH7PS undergoes no significant conformational change on inhibitor binding. These observations are consistent with an allosteric response arising from changes in protein motion rather than conformation, and suggest ligands that modulate protein dynamics may be effective inhibitors of this enzyme.

Keywords: allostery; shikimate; aromatic amino acids; DAHPS

Abbreviations: BSA, bovine serum albumin; BTP, 1,3-bis[tris(hydroxymethyl)methyl]aminopropane; DAH7P, 3-deoxy-D-arabino-heptulosonate 7-phosphate; DAH7PS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; E4P, D-erythrose 4-phosphate; EDTA, ethylenediaminetetraacetic acid; ITC, isothermal titration calorimetry; PDB, protein data bank; PEG, polyethylene glycol; PEP, phosphoenolpyruvate; RMSD, root mean square difference; SAXS, small angle X-ray scattering; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; TMAO, trimethylamine N-oxide

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Introduction

The shikimate pathway leads to the formation of chorismic acid, which is the precursor for a range of aromatic metabolites including the aromatic amino acids L-Tyr, L-Phe, and L-Trp (Fig. 1).¹ As the pathway is present only in plants, microorganisms, and apicomplexan parasites, yet is absent from mammals, the enzymes of this pathway have attracted considerable attention as targets for inhibitor action.^{2–5}

The enzyme 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAH7PS) catalyzes the first step in the shikimate pathway, which is an aldol condensation reaction between phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P). Entry into the shikimate pathway is controlled at the protein level by allosteric regulation of DAH7PS. The activities of DAH7PS enzymes are usually regulated by inhibition with end-product amino acids L-Tyr, L-Phe, or L-Trp, or other intermediates of the pathway.⁶

Despite significant variation in amino acid sequences, all structurally characterized DAH7PS enzymes share a $(\beta/\alpha)_8$ TIM-barrel fold and possess similar active-site architecture.^{7–13} Originally DAH7PS enzymes were classified into different types (types I α , I β , and II) based on amino acid

sequence differences.^{14–17} As more structural information has become available it has become apparent that these sequence variations are related to different structural additions to the core catalytic barrel.^{6,8,13,18–22}

The nature of the extra barrel extensions is directly implicated in the sensitivity of the DAH7PS enzymes to allosteric control. Type I β DAH7PS enzymes can be devoid of decoration (associated with the absence of feedback inhibition) or possess an N-terminal or C-terminal extension of either a regulatory ACT domain or of chorismate mutase activity.^{8,18,21} Type I α DAH7PS enzymes have an N-terminal extension (a β -strand and two α -helices) and a two-stranded antiparallel β -sheet insertion in the $\alpha 5\beta 6$ loop.¹² The β -sheet decoration pairs with the β -strand portion of the N-terminal extension of an adjacent monomer at the dimer interface to create the binding sites for the feedback inhibitors.^{13,20} Lastly, the type II DAH7PS enzymes have an N-terminal extension (composed of a β -strand and two α -helices), which is usually coupled with an $\alpha 2\beta 3$ loop extension (providing a pair of α -helices).⁷ These decorations again form the binding sites for the aromatic amino acids that act as feedback inhibitors.^{19,23,24} The inhibition of the type II DAH7PS from *Mycobacterium tuberculosis* is synergistically inhibited by combinations of the aromatic amino acids. It is notable that the mechanisms of allosteric regulation for the DAH7PS enzymes of different types are remarkably different, although they have a common effect on catalytic activity.⁶ For instance, we have recently demonstrated that feedback inhibition of the type I β DAH7PS from *Thermotoga maritima* is achieved by domain re-organization and large structural changes, whereas inhibition of the type II DAH7PS from *M. tuberculosis* is achieved by more subtle perturbations in the molecular dynamics of the enzyme.^{21,24}

A number of species express multiple type I α DAH7PS isozymes, which are characterized by their differing sensitivity to inhibition by each aromatic amino acid.^{25–27} *Escherichia coli* expresses three isozymes sensitive either to L-Phe, L-Trp, or L-Tyr.²⁶ The L-Phe-sensitive isozyme accounts for around 80% of the DAH7PS activity for *E. coli*.²⁸ *Saccharomyces cerevisiae* expresses both an L-Tyr-sensitive and L-Phe-sensitive isozyme. These isozymes show high degrees of similarity and it has been shown that a single residue exchange in the allosteric binding site of *S. cerevisiae* DAH7PS (SceDAH7PS) allows the interconversion of L-Tyr and L-Phe sensitivities.¹³

The Gram-negative bacterium *Neisseria meningitidis* is the causative agent of pyogenic meningitis and meningococcal septicemia. It is a major cause of disease worldwide, and can cause brain damage, hearing loss, and is fatal in 4–10% of sufferers.^{29,30}

Figure 1. The shikimate pathway for aromatic amino acid biosynthesis. The first step of the pathway is catalyzed by the enzyme DAH7PS. The pathway branches at chorismate for the biosynthesis of other important aromatic compounds including folate, quinone, and vitamins E and K.

Table I. Comparison of Kinetic Parameters for NmeDAH7PS Enzymes

DAH7PS	K_m^{PEP} (μM)	K_m^{E4P} (μM)	k_{cat} (s^{-1})	$k_{\text{cat}}/K_m^{\text{PEP}}$ ($\text{s}^{-1}\mu\text{M}^{-1}$)	$k_{\text{cat}}/K_m^{\text{E4P}}$ ($\text{s}^{-1}\mu\text{M}^{-1}$)
NmeDAH7PS	11 ± 1	43 ± 4	25.5 ± 0.5	2.3	0.59
NmeDAH7PS-S213G	8.7 ± 0.7	40 ± 4	14.8 ± 0.1	1.7	0.37
NmeDAH7PS (with L-Phe)	21 ± 1	92 ± 10	6.9 ± 0.4	0.33	0.075
NmeDAH7PS-S213G (with L-Tyr)	5.4 ± 0.5	21 ± 1	6.0 ± 0.1	1.11	0.29

L-Phe or L-Tyr concentrations were 300 μM .

The genome of *N. meningitidis* encodes a single DAH7PS (NmeDAH7PS) and sequence comparisons indicate this enzyme is a type I α DAH7PS (Fig. S1, Supporting Information). Herein, we report the structure and functional characterization of NmeDAH7PS. These studies provide insight into the regulation of aromatic amino acid biosynthesis in *N. meningitidis* mediated by NmeDAH7PS.

Results

Expression, purification, stability, and activity

The open-reading frame of the sole DAH7PS gene from *N. meningitidis* was cloned, over-expressed in *E. coli*, and the resulting protein was purified to homogeneity. NmeDAH7PS was catalytically active, with maximal activity at $\sim 40^\circ\text{C}$ (Fig. S2, Supporting Information). The kinetic parameters for NmeDAH7PS were determined (Table I) and were found to be most similar to the values reported for the L-Phe-sensitive *E. coli* DAH7PS (EcoDAH7PS) isozyme (Table S1, Supporting Information).³¹

NmeDAH7PS was activated, to varying extents, by different divalent metal ions, and no activity was apparent in the presence of EDTA (Table II). Mn^{2+} was found to be the most activating divalent metal ion for the enzyme, and Cd^{2+} was moderately activating. Co^{2+} , Zn^{2+} , Fe^{2+} , and Cu^{2+} also activated the enzyme, but to a lesser extent. The activation by these divalent metal ions is similar to that observed for other type I α DAH7PS enzymes. The three

DAH7PS isozymes expressed by *E. coli* and the L-Phe-sensitive *Sce*DAH7PS show a similar hierarchy of activity with the different metal ions, with Mn^{2+} also providing the highest levels of activity.^{32,33} In contrast, the L-Tyr-sensitive *Sce*DAH7PS isozyme was reported to have a slightly different response to metal ions, with the greatest activity observed in the presence of Cu^{2+} and Fe^{2+} , with only weak activation by Mn^{2+} or Mg^{2+} .³⁴

The active-site metal ion is thought to both organize the active site and directly activate the E4P carbonyl for reaction,^{10,35,36} but the precise reasons for the variation in the activation of the enzymes by different metal ions are unclear. It is likely that the metal selection relates to the bioavailability of the metal ions for the particular organism. However, it is noted, that as for NmeDAH7PS, most DAH7PS enzymes are activated by a range of metal ions.

The thermal stability of NmeDAH7PS in a range of different conditions was assessed by differential scanning fluorimetry (Table S2, Supporting Information). Additives, including various divalent metal ions and increasing concentrations of L-Tyr or L-Phe, made little difference to the stability of NmeDAH7PS, with the protein exhibiting a consistent T_m value of $47\text{--}49^\circ\text{C}$ in all conditions. The only exception was in the presence of Cd^{2+} , for which the T_m value decreased by 4°C . There have been limited previous studies on the thermal stability of DAH7PS from other organisms. However, PEP has been noted to stabilize the *Aeropyrum pernix*¹¹ and the L-Phe sensitive *E. coli* enzymes,³⁷ and some increase in thermal stability was noted for the *P. furiosus* DAH7PS in the presence of Mn^{2+} .³⁸

Table II. Activation of NmeDAH7PS by Different Divalent Metal Ions

Divalent metal	% Activity ^a
Mn	100
Cd	69
Co	34
Zn	24
Fe	15
Cu	8
Mg	1
Ni	0
Ca	0
Ba	0
Sr	0

^aAll solutions contained 10 μM EDTA and (except the metal solutions) were treated with Chelex resin before use. The assay mixture contained PEP (180 μM), divalent metal salt (100 μM), and NmeDAH7PS (5 μL , 1.7 mg/mL). Reactions were initiated with the addition of E4P (150 μM).

NmeDAH7PS is inhibited by L-Phe, and to a lesser extent by L-Trp and L-Tyr

NmeDAH7PS was inhibited to the largest degree by the aromatic amino acid L-Phe, and to a lesser extent by L-Trp and L-Tyr [Fig. 2(a)]. This inhibition by all three aromatic amino acids has not been reported for the characterized L-Phe-sensitive type I α DAH7PSs from *E. coli* and *S. cerevisiae*. These DAH7PS enzymes exhibit sensitivity exclusively to only one of the aromatic amino acids.^{33,39}

The kinetic parameters of NmeDAH7PS determined in the presence of L-Phe indicate that the enzyme has a reduced affinity for both substrates, with the K_m values approximately doubling as well

Figure 2. Inhibition of (a) *NmeDAH7PS* by L-Phe (■), L-Trp (▲), and L-Tyr (●) in comparison to the (b) S213G variant (shown in corresponding open shapes). Error bars represent the standard deviation. Reaction mixtures contained 264 μM E4P, 100 μM PEP, 100 μM MnSO_4 in 50 mM BTP, pH 6.8, and 50 to 1000 μM of the amino acid. The reactions were initiated by the addition of either wild-type *NmeDAH7PS* (2 μL , 0.7 mg mL^{-1}) or the S213G variant (2 μL , 0.6 mg mL^{-1}) and carried out at 30°C. Triplicate measurements were made and averaged. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

as a lower k_{cat} (Table I). The specificity constants, $k_{\text{cat}}/K_{\text{m}}^{\text{PEP}}$ and $k_{\text{cat}}/K_{\text{m}}^{\text{E4P}}$, are 7- and 8-fold lower respectively than their corresponding values in the absence of L-Phe.

***NmeDAH7PS* crystallizes as a homotetramer**

NmeDAH7PS was crystallized in the presence of PEP and Mn^{2+} , in the absence of L-Phe.

NmeDAH7PS crystallizes as a homotetramer, similar to the tetramer architecture observed for the other structurally characterized type I α DAH7PS enzymes (Fig. S4, Supporting Information).¹² In common with all DAH7PS enzymes that have been structurally characterized, the monomer of *NmeDAH7PS* is a $(\beta/\alpha)_8$ TIM-barrel (Fig. 3), which houses the enzyme active site. Both PEP and Mn^{2+}

Figure 3. The crystal structure of *NmeDAH7PS* showing (a) the monomeric subunit and (b) the arrangement of the subunits in the homotetramer. The $(\beta/\alpha)_8$ TIM-barrel is colored blue and the N-terminal and internal extensions to the barrel are colored red and yellow, respectively. The active site is denoted by the position of PEP (cyan sticks) and Mn^{2+} (green sphere), and the position of the allosteric inhibitor binding site is marked by an asterisk. The salt bridge interaction (black dashes) between Arg126 and Glu27 is shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

are bound in the active site of each of the four subunits. The metal ion is coordinated by four protein ligands Cys63, His 270, Glu304, and Asp324 (Fig. S3, Supporting Information). These residues are completely conserved in known DAH7PSs. The metal coordination is bipyramidal, as described for the *S. cerevisiae* and *E. coli* DAH7PS structures, with the third coordination site occupied by water.^{12,35} This water ligand is thought to be displaced by the carbonyl oxygen of E4P, allowing the metal ion to position the aldehydic cosubstrate and facilitate carbon-carbon bond formation.³⁵ PEP interacts with conserved residues Arg167, Arg236, Lys188, Arg94, and Lys99 (Fig. S3, Supporting Information). In addition to the core catalytic component, the barrel is decorated with an N-terminal extension comprising a β -strand and two helices (β 0, α 0, and α 00), and a two β -strand extension (β 6a and β 6b) inserted internally into a barrel loop (between α 5 and β 6).

The four monomer subunits comprising the tetramer show close structural similarity (RMSD of pairwise alignment between the monomers of 0.12–0.15 Å) and associate as a dimer of tight dimers [Fig. 3(b)]. The tight-dimer interface is assisted by the formation of an extended antiparallel β -sheet where the β 0 strand from one monomer interdigitates across the interface with the internal extension (β 6a and β 6b) of the adjacent monomer. On average, the percentage of each monomer buried in the tight-dimer interfaces is ~18%. In contrast, the pair of tight dimers associates through a relatively weak interface that buries only a small portion of the surface area of each monomer (~3.8%). Although for the Tyr-sensitive *Sce*DAH7PS this interaction is purely hydrophobic,³⁵ in *Nme*DAH7PS salt bridges between Arg126 and Glu27 of adjacent subunits (subunits A and C or B and D) appear to be instrumental for maintaining this assembly of the tight dimers [Fig. 3(b)].

In the crystal structures of *Nme*DAH7PS, *Eco*DAH7PS and *Sce*DAH7PS the monomeric subunits share close structural similarity (RMSD of the pairwise comparisons between equivalent C α atoms of all monomers is 0.34–0.55 Å) and the tight dimer of *Nme*DAH7PS aligns more closely with that of *Eco*DAH7PS than *Sce*DAH7PS (RMSD of pairwise alignment of 0.47–0.52 Å and 0.60–0.69 Å, respectively). Notably, the twisted arrangement of the two dimers adopted in the structure of *Nme*DAH7PS is intermediate to that observed for *Eco*DAH7PS and *Sce*DAH7PS. The angle between the two tight dimers, as defined by the angle between the vectors connecting residue Met302 of each pair of monomers, is ~47°, which lies between that of *Sce*DAH7PS (~67°) and the relatively small twist observed for *Eco*DAH7PS (~27°, Fig. S4, Supporting Information).

Substitution of Ser213 to Gly reduces the inhibition by L-Phe and enhances inhibition by Tyr

Comparison of the *Nme*DAH7PS structure with the L-Phe- and L-Tyr-bound structures of *Eco*DAH7PS and *Sce*DAH7PS allows the inhibitor-binding site to be deduced. This also allows the identification of the residue that may, at least partially, determine the selectivity of *Nme*DAH7PS for inhibition by a particular amino acid [Fig. 3(a)]. *Nme*DAH7PS bears a Ser residue at position 213 and for other type Ia DAH7PS enzymes, the identity of the residue at this position has been shown to correlate with sensitivity to either L-Phe (Ser) or L-Tyr (Gly).¹³ A Ser in this position is consistent with the inhibition pattern observed for *Nme*DAH7PS where L-Phe is the most effective inhibitor (Fig. 2).

The S213G replacement was created in *Nme*DAH7PS to modify inhibitor selectivity from L-Phe to L-Tyr. This variant was catalytically active, with similar thermal stability and kinetic parameters to wild-type *Nme*DAH7PS (Table I). *Nme*DAH7PS S213G was crystallized and the structure was solved to a resolution of 2.4 Å. The tetrameric form of this protein shows very close overall structural resemblance to the wild-type enzyme (RMSD for C α atoms of 0.33 Å).

The S213G variant of *Nme*DAH7PS was more sensitive to inhibition by L-Tyr than L-Phe, and less sensitive to inhibition by L-Phe than the wild-type enzyme (Fig. 2b), confirming the identity of the residue at this position is important for the inhibitor selectivity of *Nme*DAH7PS. The main effect of the presence of L-Tyr on the *Nme*DAH7PS S213G variant is a reduction in the k_{cat} value (Table I).

The structure of the S213G variant of *Nme*DAH7PS in complex with L-Tyr was determined. This protein displays close structural similarity with the unliganded S213G variant (RMSD between equivalent C α atoms of 0.31 Å). The interactions between the interfaces are identical to those observed for the wild-type enzyme. However, additional hydrogen-bonding interactions, fabricated by supervenient residues resulting from the improved ordering of the N terminus upon L-Tyr binding, cause a small increase in the buried surface area of the S213G variant tight-dimer interface (now burying, on average, ~21% of the surface area of each monomer).

Tyr is observed bound to all four potential binding sites in the tetramer structure of the S213G variant of *Nme*DAH7PS [Fig. 4(a)]. A hydrogen bond is observed between the hydroxyl group of L-Tyr with the main chain amide of Val214, which is located on the β 6a strand of the internal extension. The side-chain hydroxyl and main-chain amide group of Ser182 form hydrogen bonds with the carboxyl functionality of the L-Tyr ligand. Hydrogen bonds are also observed between the amino group of Tyr and

Figure 4. (a) The Tyr binding site in the S213G variant of *Nme*DAH7PS (unbound form colored green, Tyr-bound form colored magenta). The electron density ($2F_o - F_c$) of L-Tyr (yellow sticks) is contoured at 2σ and the residues contributed from the adjacent monomer are colored cyan for the bound form. (b) The Phe binding site of Phe-regulated *Eco*DAH7PS (unbound form colored green, PDB code 1N8F, Phe-bound form colored magenta, PDB code 1KFL). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the side chain of Gln153 and with Asp9 and for some subunits, Asp8, contributed by the N-terminal extension of the adjacent monomer of the tight dimer.

These interactions, the location of the allosteric inhibitor binding site and the ordering of the $\beta 0$ strand at the N-terminus are consistent with those observed for the ligand-bound *Eco*DAH7PS and *Sce*DAH7PS enzymes.^{13,20} The structural shift involving residues located on the $\beta 0$ (from the adjacent monomer of the tight dimer) and $\beta 6a/\beta 6b$ strands and the $\beta 3-\alpha 3$ loop observed for *Eco*DAH7PS²⁰ [Fig. 4(b)] is noticeably absent for *Nme*DAH7PS S213G. However, the narrowing of the ligand binding cavity that occurs on ligand binding for *Eco*DAH7PS, is much smaller for the S213G variant of *Nme*DAH7PS.

Isothermal titration calorimetry

The binding affinity of L-Phe to *Nme*DAH7PS was measured by isothermal titration calorimetry (ITC) with cooperativity observed in the binding isotherm ([Fig. 5(a)]). The data were fitted with a two-site sequential model giving K_d values of $1.56 \pm 0.05 \mu M$ and $21.1 \pm 0.4 \mu M$. The absence of observable heat change on titration with Tyr to *Nme*DAH7PS is consistent with the relative ineffectiveness of Tyr as an inhibitor of the enzyme.

Unlike the wild-type enzyme there was no observable change in enthalpy on titration with Phe for the S213G variant; however, an enthalpy of binding was observed and fitted with a one-site model

for Tyr giving a K_d value of $22 \pm 2 \mu M$ [Fig. 5(b)]. This mirrors the switch in inhibitor sensitivity measured in the enzyme activity assay and the ability of the single S213G replacement to tune ligand specificity. Compared with the wild-type enzyme titration with L-Phe, no evidence of cooperativity was observed in the binding isotherm of S213G with L-Tyr. This may be a consequence of the assay conditions and the relatively poor binding of this ligand in the variant protein, rather than necessarily reflecting a change in binding.

Small angle X-ray scattering indicates allosteric inhibitor binding is not accompanied by conformational change

Small angle X-ray scattering (SAXS) profiles were determined to assess the solution structure of *Nme*DAH7PS. The scattering profile for *Nme*DAH7PS was consistent with the enzyme adopting a tetrameric assembly similar to that observed in the crystal structure [Fig. 6(a)].

The scattering profiles for wild-type and S213G *Nme*DAH7PS determined in either the absence or presence of inhibitors, L-Phe or L-Tyr, were found to be virtually superimposable, indicating that inhibitor binding does not induce substantial conformational change (Fig. 6). This observation is consistent with the comparison between the unbound and L-Tyr-bound crystal structures of the S213G variant of *Nme*DAH7PS, which show minimal structural changes occur on ligand binding, thus suggesting that the allosteric mechanism operates with relatively subtle changes in protein conformation.

Figure 5. Enthalpy changes measured by ITC of (a) L-Phe binding to *NmeDAH7PS* and (b) L-Tyr binding to *NmeDAH7PS* S213G.

Discussion

N. meningitidis expresses a single DAH7PS enzyme, principally inhibited by L-Phe, and to lesser extent by L-Tyr and L-Trp. In contrast, *E. coli* expresses three isozymes, and each of these enzymes is solely sensitive to one of the aromatic amino acids, L-Phe, L-Tyr, or L-Trp. This expression of differentially sensitive isozymes is a strategy for controlled entry into this branched biosynthetic pathway for aromatic metabolite biosynthesis. The L-Phe-, L-Tyr-, and L-Trp-sensitive isozymes of *E. coli* account for ~80, 20, and <1%, respectively, of the total cellular DAH7PS activity, reflecting the relative cellular demands of these aromatic amino acids.²⁸ Intriguingly, the expression of a single DAH7PS in *N. meningitidis* with some sensitivity to all three amino acids, yet significantly more sensitive to L-Phe, correlates well with the relative contributions that the *E. coli* isozymes make to the overall DAH7PS activity. This feature of the *N. meningitidis* DAH7PS may circumvent the need for expression of multiple isozymes and thereby limit unnecessary resource expenditure.

In the absence of inhibitors, the sizes of the inhibitor binding cavities of *NmeDAH7PS*, *EcoDAH7PS*, and *SceDAH7PS* are comparable. However, whereas a large contraction of the cavity is observed for both *EcoDAH7PS* and *SceDAH7PS* on inhibitor binding, this change is markedly less discernible for *NmeDAH7PS* S213G. This difference in binding site properties may account for the observed inhibitor promiscuity of *NmeDAH7PS*. For

NmeDAH7PS, as for both isozymes of *SceDAH7PS*, selectivity for L-Phe or L-Tyr is interconverted by this single amino acid exchange,¹³ strengthening the claim that sensitivity to L-Tyr as opposed to L-Phe or L-Trp can be identified readily by an examination of the primary sequence of type Iα DAH7PS enzymes. While more sensitive to L-Tyr than L-Phe, the S213G variant of *NmeDAH7PS* does not show the degree of inhibition in the presence of L-Tyr as the wild-type enzyme does to L-Phe, suggesting that fine tuning may be required to optimize the modified inhibitor response.

The inhibition of the wild-type *NmeDAH7PS* by L-Trp contrasts to the inhibitor selectivity reported for other L-Phe- or L-Tyr-sensitive type Iα DAH7PS enzymes. Unlike L-Phe- and L-Tyr-sensitive isozymes, there has been only limited characterization of L-Trp-sensitive enzymes.^{40,41} In contrast to the *E. coli* L-Trp-sensitive isozyme, which is reduced to half its catalytic activity by ~2 μM of L-Trp,⁴⁰ *NmeDAH7PS* was found to be significantly less sensitive to L-Trp. Therefore, the physiological role of the response to L-Trp is not clear, and L-Phe and L-Tyr cellular levels may be sufficient to report on the requirements for DAH7PS activity in relation to L-Trp biosynthesis. Sequence alignments reveal that the *E. coli* L-Trp-sensitive isozyme also bears a Ser at the equivalent position to Ser213 in *NmeDAH7PS*. The presence of this Ser correlates with the observation that the S213G mutation of *NmeDAH7PS* attenuates the sensitivity of the enzyme to inhibition by L-Trp.

Figure 6. Comparison of the SAXS profiles of *NmeDAH7PS* (closed circles) and S213G variant (open circles) collected in the absence of ligand (a and b), presence of L-Tyr (c and d) or L-Phe (e and f) in comparison to the theoretical scattering generated from crystallographic coordinates of both the wild-type (PDB code 4HSN, black solid line) and S213G variant (PDB code 4IXX, black dashed line) crystal structures using CRY SOL. Discrepancies from the fit of the theoretical scattering to the experimentally determined data (χ) of *NmeDAH7PS* and the S213G variant are 1.58 and 1.42, respectively. Scattering data were collected from *NmeDAH7PS* and the S213G variant using protein at 2.5 mg mL⁻¹ in buffer [10 mM BTP (pH 7.5), 100 mM KCl, 200 μ M PEP], with and without 1 mM L-Tyr or L-Phe. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

While allostery has often been attributed to structural changes mediated by ligand binding, it is now recognized that subtle changes in protein motion can be associated with the allosteric response.^{42–45} We observed no large changes in conformation associated with ligand binding for *NmeDAH7PS*, either in the crystalline form or in solution. The small conformational changes noted for the L-Phe-sensitive *E. coli* enzyme and observed in the L-Tyr-sensitive *S.*

cerevisiae DAH7PS that were suggested to delineate a signal transduction pathway from allosteric site to active site, were not evident in our structures. Different types of DAH7PS enzymes display a variety of allosteric mechanisms, and in some cases inhibitor binding appears to be associated with significant conformational changes.²¹ From the analysis of structural data determined for *NmeDAH7PS*, it appears that modest changes in protein structure or dynamics

may be responsible for mediating the regulatory signal of *Nme*DAH7PS, highlighting the variation in allosteric mechanisms and architecture that are associated with the DAH7PS enzyme family.

Materials and methods

Bacterial strains, plasmids, media, and growth conditions

The open reading frame for *N. meningitidis* DAH7PS (*Nme*DAH7PS) was amplified from genomic DNA (*N. meningitidis*, serotype B strain MC58, ATCC BAA-355) using standard methodologies and the primers 5'-GGTGGTCATATGACACAC CATTACCCG and 5'-CGAGGATCCTTTATCACTTG CGCGTGC to introduce NdeI and BamHI restriction sites (underlined). The PCR product was eluted from the gel, digested with NdeI (front site), and BamHI (back site) cloned into a pT7-7 vector and the sequence verified after transformation in *E. coli* XL-1 competent cells. The recombinant pT7-7-*Nme*DAH7PS plasmid was transformed into *E. coli* BL21 (DE3) cells for protein expression. Cultures of the expression cell line (250 mL) were grown in lysogeny broth containing $100\text{ }\mu\text{g mL}^{-1}$ ampicillin at 37°C until mid-exponential phase [attenuance (OD_{600}) = 0.4–0.6] was reached, at which point protein expression was induced by addition of 1 mM isopropyl β -D-thiogalactopyranoside. Cells were harvested by centrifugation ($5,500g$ for 15 min at 4°C or $12,000g$ for 10 min at 4°C) 4 h after induction. Cell pellets were stored at -80°C until lysis.

Site-directed mutagenesis

The mutant variant S213G of *Nme*DAH7PS was created by site-directed mutagenesis using a QuikChange® II Site-Directed Mutagenesis Kit (Stratagene). The pT7-7-*Nme*DAH7PS plasmid was used as the template, and the S213G mutation created using the primers 5'-CGCACTTTCCTGGGTGTAA CCAAGGCCG and 5'-CGGCCTTGTTACACCCAGGA AATGATGCG (mutation underlined). PCR products were transformed into *E. coli* XL1-Blue cells and plasmids analyzed by agarose gel electrophoresis. Plasmids of the expected size were sequenced to identify those of successful mutagenesis, after which the plasmids were transformed into competent *E. coli* BL21 (DE3) cells.

Enzyme purification

Frozen cell pellets were thawed and re-suspended in lysis buffer [10 mM BTP buffer (pH 7.3), 1 mM EDTA, 200 mM KCl, and 200 μM PEP] and lysed either using a French Press followed by sonication on ice, or by sonication on ice alone. Cell debris was removed by centrifugation ($24,000g$ for 20 min at 4°C). The supernate was filtered and diluted with buffer A [10 mM BTP buffer (pH 7.3), 1 mM EDTA, and 200 μM PEP] before being loaded onto a pre-

equilibrated 8 mL Source™ 15Q (Amersham) anion-exchange column and washed with several column volumes of buffer A. *Nme*DAH7PS was eluted from the column using a gradient of increasing NaCl concentration (0 to 1 M) at 4°C , and typically eluted at a NaCl concentration of 0.1 M. Fractions containing *Nme*DAH7PS (as determined by SDS-PAGE and enzyme activity) from anion exchange were pooled and either ammonium sulfate added to a final concentration of 1 M, or NaCl to a final concentration of 3 M. The sample was refiltered and applied to a 8 mL Source™ 15Phe column (Amersham) pre-equilibrated with buffer B [buffer A (pH 7.3) plus 1 M ammonium sulfate or 3 M NaCl]. *Nme*DAH7PS eluted from the column at room temperature with either a linear gradient or immediate step from buffer B to buffer A. Fractions containing *Nme*DAH7PS from hydrophobic-interaction chromatography were pooled, filtered, and further purified by size-exclusion chromatography (SEC) using a HiLoad 26/60 Superdex™ 200 prep grade column (GE Healthcare). Fractions from SEC containing *Nme*DAH7PS were pooled, desalted, and concentrated by repeated dilution and concentration using a 10 kDa MWCO device (Vivascience). Aliquots of purified *Nme*DAH7PS were flash-frozen and stored at -80°C . Protein concentrations were determined by the method of Bradford, using BSA as a protein standard, or by absorption at 280 nm ($\epsilon = 31,650\text{ M}^{-1}\text{ cm}^{-1}$).

Enzyme assays

The assay system for *Nme*DAH7PS was a modified form of the assay used by Schoner and Herrmann as previously described.⁴⁶ The assays to determine the optimum temperature of *Nme*DAH7PS activity contained PEP (180 μM) and MnSO_4 (100 μM) in 50 mM BTP buffer containing 10 μM EDTA, which was pH 6.8 at the required temperature. After incubation at the required temperature for 6 min, E4P (150 μM) was added, and then 1 min later, the reaction was initiated by the addition of enzyme (5 μL , 1.7 mg mL^{-1}).

Michaelis-Menten kinetics

For wild-type *Nme*DAH7PS the reactions to determine K_m^{E4P} used 203 μM PEP and 44 μM to 448 μM E4P, and for K_m^{PEP} 224 μM E4P and 4 to 102 μM PEP. The reactions were initiated by the addition of *Nme*DAH7PS (2 μL , 0.8 mg mL^{-1}). For the *Nme*DAH7PS variant S213G, to determine K_m^{E4P} 270 μM PEP and 20 to 423 μM E4P was used. For K_m^{PEP} 211 μM E4P and 20 to 182 μM PEP was used. The reactions were initiated by the addition of *Nme*DAH7PS S213G (2 μL , 1.3 mg mL^{-1}).

To determine the K_m^{E4P} for wild-type *Nme*DAH7PS in the presence of inhibitor, the reactions used 130 μM PEP, and 60 to 600 μM E4P. For K_m^{PEP}

determination, 480 μM E4P and 5 to 218 μM PEP were used. All reactions contained 300 μM Phe and were initiated with enzyme (2 μL at 1.1 mg mL⁻¹). For the *Nme*DAH7PS S213G variant, the K_m^{E4P} and K_m^{PEP} were determined using 33 μM PEP and 48 to 480 μM E4P and 746 μM E4P and 5 to 110 μM PEP, respectively in the presence of 300 μM Tyr. Reactions were initiated by the addition of enzyme (2 μL , 0.6 mg mL⁻¹).

The values of K_m and k_{cat} were determined by fitting the initial-rate data to the Michaelis-Menten equation using Graft (Erithicus).

Metal activation

All solutions for metal activation experiments (except the metal solutions) contained 10 μM EDTA and were treated with Chelex (BioRad) resin before use. The assay mixture used 180 μM PEP, 100 μM of the divalent metal salt and *Nme*DAH7PS (5 μL , 1.7 mg mL⁻¹). The reactions were initiated by the addition of 150 μM E4P. The *Nme*DAH7PS used contained 10 mM EDTA. The metal salts used were MnSO₄, ZnSO₄, CdSO₄, CoSO₄, NiCl₂, CaCl₂, CuSO₄, FeCl₂, MgSO₄, SrCl₂, and BaCl₂.

Differential scanning fluorimetry

The melting temperature of wild-type and S213G *Nme*DAH7PS were determined by differential scanning fluorimetry using an iCycler iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad), based on the method of Nordlund *et al.*⁴⁷

Isothermal titration calorimetry

Binding of wild-type or S213G *Nme*DAH7PS to L-Phe and L-Tyr was measured by ITC using a VP-ITC unit operating at 298 K (Microcal, GE Healthcare). Before use, the protein was buffer exchanged against binding buffer [0.5 mM MnSO₄ in 50 mM BTP buffer (pH 7)] and all solutions were degassed in a vacuum. To improve the solubility of L-Tyr, 10 μL aliquots of 10 M NaOH were added stepwise to the solution. Protein concentration was measured by UV absorption immediately before titrations were started. The titrations were comprised of 28 injections, one 2 μL injection followed by 27 10 μL injections of the amino acid. The initial datum point was routinely deleted to allow for diffusion of ligand across the needle tip during the equilibration period. A heat of dilution experiment was measured independently and subtracted from the integrated data before curve fitting in Origin 7.0. For the binding of wild-type *Nme*DAH7PS to L-Phe, 16 μM of *Nme*DAH7PS was used and the syringe contained 0.6 mM L-Phe; and the data were fitted with the two-site sequential-binding model supplied by MicroCal. For the S213G variant the concentrations were 35 μM and 1.6 mM, respectively. For wild-type *Nme*DAH7PS with L-Tyr, 5 mM L-Tyr was titrated into 13

μM *Nme*DAH7PS and for the S213G variant the concentrations were 0.4 mM L-Tyr and 15 μM protein. A curve was fitted with a one-site model (MicroCal) keeping the stoichiometry fixed ($n = 1$) for the binding of the S213G variant with L-Tyr after the second datum point was deleted.

Crystallization

Crystals of *Nme*DAH7PS (both wild-type and S213G variant) were grown by hanging-drop vapor diffusion. A protein solution [11 mg mL⁻¹ in 10 mM BTP buffer (pH 7.3)] was mixed 1:1 (v/v) with a reservoir solution containing 0.2 M trimethylamine *N*-oxide (TMAO), 0.1 M Tris (pH 8.5), 15–20% (w/v) PEG 2000 mme, 0.4 mM MnSO₄, and 0.4 mM PEP. The drop sizes were 2 μL , and the volume of the reservoir solution was 500 μL . The crystallization trays were left at 20°C. Crystals typically began to form after 3 days and were fully formed within 5 days. For the S213G variant, crystals grew in a different form that did not diffract to a high resolution. Transport of crystallization trays from our laboratory in New Zealand to the Australian Synchrotron as hand luggage by airplane allowed the previously formed crystals to redissolve and new crystals to grow in the same form and morphology as those of wild-type *Nme*DAH7PS. Immediately before data collection, crystals were transferred briefly into a cryoprotectant composed of 20% glycerol in the respective reservoir solution. For the L-Tyr-bound S213G variant structure, the cryoprotectant solution contained 0.2 M TMAO, 0.1 M Tris (pH 8.5), 20% (w/v) PEG 2000 mme, 0.2 mM MnSO₄, 0.2 mM PEP, 0.2 mM Tyr, and 20% (w/v) PEG 400. The crystal was left to soak for 20 min.

Determination and refinement of structure

Data sets were collected at the Australian Synchrotron using the MX1 and MX2 beamlines and were processed using XDS⁴⁸ and SCALA (CCP4 suite).⁴⁹ The results are summarized in Table III, along with key structure refinement details. The wild-type and S213G variant structures of *Nme*DAH7PS crystallized in the monoclinic space group *P*12₁1 and diffracted to 2.0 Å (wild type), 2.4 Å (S213G variant), and 2.1 Å (L-Tyr-bound S213G variant), with the following unit cell dimensions: $a = 73$, $b = 137$, $c = 76$, $\alpha = 90^\circ$, $\beta = 96^\circ$, and $\gamma = 90^\circ$. The monomeric structure of *S. cerevisiae* DAH7PS (from PDB entry 1OF8), after modification by Chainsaw,⁵⁰ was used as the search molecule in Phaser⁵¹ to solve by molecular replacement the structure of wild-type *Nme*DAH7PS. The wild-type structure was used to determine the structure of the L-Tyr-bound S213G variant, which in turn was used to solve the structure of the unbound S213G variant structure, carrying through the same set of reflections for calculation of R_{free} . Refinements were conducted

Table III. Crystal Parameters, Data Collection, and Refinement Statistics

	<i>NmeDAH7PS</i>	<i>NmeS213G</i>	<i>NmeS213G</i> with Tyr
A. Data collection			
Crystal system; space group	Monoclinic, <i>P12₁1</i>	Monoclinic, <i>P12₁1</i>	Monoclinic, <i>P12₁1</i>
Unit cell parameters (Å)	73.46, 137.3, 76.36,	73.06, 132.43, 75.03, 90.00,	73.52, 136.99, 76.21, 90.00,
<i>a</i> , <i>b</i> , <i>c</i> , α , β , γ	90.00, 96.42, 90.00	95.72, 90.00	96.63, 90.00
Resolution range (Å)	19.79–2.00 (2.05–2.00)	19.59–2.40 (2.47–2.40)	19.57–2.10 (2.21–2.10)
Measurements	373,764	208,167	320,885
Unique reflections	100,515	55,141	86,154
Redundancy	3.7 (3.8)	3.8 (3.8)	3.7 (3.8)
Completeness (%)	99.3 (100)	99.4 (99.9)	98.8 (99.7)
<i>I</i> / σ (<i>I</i>)	7.9 (2.0)	11.0 (1.6)	14.0 (1.9)
<i>R</i> _{merge}	0.062 (0.369)	0.067 (0.486)	0.047 (0.393)
Wilson <i>B</i> -value (Å ²)	27.2	34.8	35.3
B. Refinement			
Resolution (Å)	19.79–2.00 (2.05–2.00)	19.59–2.40 (2.46–2.40)	19.53–2.10 (2.16–2.10)
<i>R</i> _{cryst}	0.1754	0.1914	0.2040
<i>R</i> _{free}	0.2085	0.2247	0.2424
Chain length	351	351	351
Observed number of residues	341 in all chains	340 (chain A), 339 (chain B), 340 (chain C), 342 (chain D)	345 (chain A), 343 (chain B), 347 (chains C and D)
Water molecules	561	100	257
Other	Mn ²⁺ , PEP, SO ₄ ²⁻	Mn ²⁺ , SO ₄ ²⁻	Mn ²⁺ , PEP, L-Tyr
Mean <i>B</i> (Å ²)			
Protein	36.87	49.04	39.58
Water	38.56	33.74	36.49
Other	54.10	58.01	50.06
r.m.s.d. from target values			
Bond lengths (Å)	0.019	0.013	0.015
Bond angles (°)	1.781	1.444	1.610
Dihedral angles (°)	5.529	4.962	5.210
Ramachandran			
Preferred (%)	95.9	96.3	96.4
Allowed (%)	3.4	2.5	2.6
Outliers (%)	0.7	1.2	1.0
PDB entry	4HSN	4IXX	4HSO

with Refmac5,⁵² and electron density maps were analyzed with COOT.⁵³ The validation tools of COOT and MolProbity⁵⁴ were used to check the structures. All diagrams were drawn with PyMOL (Schrödinger, LLC).

Small angle X-ray scattering

SAXS measurements – Measurements were performed at the Australian Synchrotron SAXS/WAXS beamline equipped with a Pilatus detector using methods previously described.²¹

SAXS data analysis

Scattered intensity (*I*) was plotted versus *q*. Extrapolation of the *NmeDAH7PS* *I*(*q*) profiles to zero angle [*I*(0)] indicated a molecular mass consistent with the *NmeDAH7PS* tetramer. All samples were devoid of an increase in intensity at low *q* (increase is indicative of aggregation). Radius of gyration (*R*_g) did not vary significantly over the concentrations measured, and all Guinier plots were linear for *q*·*R*_g < 1.3. The data sets for structural analyses were recorded with 294 data points over the range 0.013 ≤ *q* ≤ 0.400 Å^{−1}. 1D profiles were background subtracted and Guinier fits were made using

PRIMUS.⁵⁵ Indirect Fourier transform was performed using GNOM⁵⁶ to yield the function *P*(*r*), which gives both the relative probabilities of distances between scattering centers and the maximum dimension of the scattering particle *D*_{max}. The SAXS experimental parameters calculated for each sample are detailed in Table S3, Supporting Information. Theoretical scattering curves were generated from atomic coordinates and compared with experimental scattering curves using CRY SOL.⁵⁷

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