The Crystal Structure of *Aquifex aeolicus* Prephenate Dehydrogenase Reveals the Mode of Tyrosine Inhibition*

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TyrA proteins belong to a family of dehydrogenases that are dedicated to *L*-tyrosine biosynthesis. The three TyrA subclasses are distinguished by their substrate specificities, namely the prephenate dehydrogenases, the arogenate dehydrogenases, and the cyclohexadienyl dehydrogenases, which utilize prephenate, *L*-arogenate, or both substrates, respectively. The molecular mechanism responsible for TyrA substrate selectivity and regulation is unknown. To further our understanding of TyrA-catalyzed reactions, we have determined the crystal structures of *Aquifex aeolicus* prephenate dehydrogenase bound with NAD⁺ plus either 4-hydroxyphenylpyruvate, 4-hydroxyphenylpropionate, or *L*-tyrosine and have used these structures as guides to target active site residues for site-directed mutagenesis. From a combination of mutational and structural analyses, we have demonstrated that His-147 and Arg-250 are key catalytic and binding groups, respectively, and Ser-126 participates in both catalysis and substrate binding through the ligand 4-hydroxyl group. The crystal structure revealed that tyrosine, a known inhibitor, binds directly to the active site of the enzyme and not to an allosteric site. The most interesting finding though, is that mutating His-217 relieved the inhibitory effect of tyrosine on *A. aeolicus* prephenate dehydrogenase. The identification of a tyrosine-insensitive mutant provides a novel avenue for designing an unregulated enzyme for application in metabolic engineering.

Tyrosine serves as a precursor for the synthesis of proteins and secondary metabolites such as quinones (1–3), alkaloids (4), flavonoids (5), and phenolic compounds (5, 6). In prokaryotes and plants, these compounds are important for viability and normal development (7).

The TyrA protein family consists of dehydrogenase homologues that are dedicated to the biosynthesis of *L*-tyrosine. These enzymes participate in two independent metabolic branches that result in the conversion of prephenate to *L*-tyrosine, namely the arogenate route and the 4-hydroxyphenylpyruvate (HPP)³ routes. Although both of these pathways utilize a common precursor and converge to produce a common end-product, they differ in the sequential order of enzymatic steps. Through the HPP route, prephenate is first decarboxylated by prephenate dehydrogenase (PD) to yield HPP, which is subsequently transaminated to *L*-tyrosine via a TyrB homologue (8). Alternatively, through the arogenate route, prephenate is first transaminated to *L*-arogenate by prephenate aminotransferase and then decarboxylated by arogenate dehydrogenase (AD) to yield *L*-tyrosine (9–11) (see Fig. 1A).

There are three classes of TyrA enzymes that catalyze the oxidative decarboxylation reactions in these two pathways. The enzymes are distinguished by the affinity for cyclohexadienyl substrates. PD and AD accept prephenate or *L*-arogenate, respectively, whereas the cyclohexadienyl dehydrogenases can catalyze the reaction using either substrate (12).

To ensure efficient metabolite distribution of the pathway intermediates, TyrA enzymes are highly regulated by various control mechanisms, including feedback inhibition, and genetic regulation by the Tyr operon (13–16). In some cases, *L*-tyrosine competes directly with substrate, be it prephenate or *L*-arogenate for the active site of arogenate or cyclohexadienyl dehydrogenases (14, 17–19). The product HPP can also serve as an efficient competitive inhibitor with respect to prephenate (20). Additionally, at the protein level PDs are only shown to be regulated at distinct allosteric sites or domains to modulate their activity. For example, the results of kinetic studies on the bifunctional *Escherichia coli* chorismate mutase-prephenate dehydrogenase (CM-PD) have indicated that this enzyme likely possesses a distinct allosteric site for binding tyrosine (21). In contrast, the *Bacillus subtilis* PD is the only enzyme reported to be competitively inhibited by HPP and *L*-tyrosine but is also noncompetitively inhibited by *L*-phenylalanine and *L*-tryptophan (12, 22). Additional regulatory control is thought to originate through a C-terminal aspartate kinase-CM-TyrA domain of the *B. subtilis* PD (23).

Biochemical analyses of PD from *E. coli* CM-PD have provided a framework for understanding the molecular mecha-

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§ This abbreviation used is: HPP, 4-hydroxyphenylpyruvate; PD, prephenate dehydrogenase; AD, arogenate dehydrogenase; HPpropionate, hydroxyphenylpropionate; CM-PD, chorismate mutase-prephenate dehydrogenase.
nism of the TyrA enzymes. The E. coli PD-catalyzed reaction proceeds though a rapid equilibrium, random mechanism with catalysis as the rate-limiting step (24). Additionally, studies of the pH dependence of the kinetic parameters $V$ and $V/K$ indicate that a deprotonated group facilitates hydride transfer from prephenate to NAD$^+$ by polarizing the 4-hydroxy group of prephenate, whereas a protonated residue is required for binding prephenate to the enzyme-NAD$^+$ complex (25). The conserved residues His-197 and Arg-294 have been identified through extensive mutagenesis studies to fulfill these two roles (26, 27). Further analyses of the activities of wild-type protein and site-directed variants in the presence of a series of inhibitory substrate analogues support the idea that Arg-294 binds prephenate through the ring carboxylate (26).

The structures of AD from Synechocystis sp. and PD from *Aquilax aeolicus* (both in complex with NAD$^+$) have been reported by Legrand et al. (28) and by our group (29), respectively. Analyses of these structures have provided structural information on the conserved histidine and arginine residues. The structure *A. aeolicus* PD has also led to the identification of other active site residues that may play a role in enzyme catalysis, most notably Ser-126, which we propose facilitates catalysis by orienting the catalytic histidine and the nicotinamide moiety of NAD$^+$ into their catalytically efficient conformations. Ambiguities can arise from examination of the binary complexes, because prephenate has only been modeled in the active site. For example, analysis of the AD structure by Legrand et al. (28) places Arg-217 (equivalent to Arg-294 in *E. coli* and Arg-250 in *A. aeolicus*) too far from the active site to play a role in prephenate binding. Thus, the full complement of interactions between prephenate and TyrA proteins are still largely unknown, as are the interactions of the enzymes with L-tyrosine.

To further investigate the importance of residues involved in ligand binding, specificity, and catalysis, we have carried out co-crystallization studies of *A. aeolicus* PD with NAD$^+$ and prephenate, with NAD$^+$ and 4-hydroxyphenylpropionate (HPpropionate), a product analogue, and with NAD$^+$ and L-tyrosine. Accordingly, this study provides the first direct evidence that L-tyrosine binds to the active site of a prephenate dehydrogenase. We have investigated the role of Ser-126, His-147, His-217, and Arg-250 through the kinetic analysis of site-directed mutants and structural analysis of the co-crystal complexes. To understand the role of active site residues in substrate selectivity, comparative structural analysis of AD and PD was also conducted. The current study provides a basis for understanding the mechanism of substrate selectivity between the different classes of TyrA enzymes and details how *A. aeolicus* PD can accept prephenate as substrate and L-tyrosine as a competitive inhibitor.

**MATERIALS AND METHODS**

*Chemicals and Reagents—*Prephenate was prepared as described previously (30), while NAD$^+$ (free acid) was obtained from Roche Applied Science. L-Tyrosine was from ICN, whereas HPP and HPpropionate were from Aldrich. The keto form of HPP was prepared as outlined in Lindblad et al. (31). All other reagents were of molecular biology grade and were purchased from Sigma, Bioshop, or BDH. Oligonucleotides used for site-directed mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA).

**Table 1**

| Variant | Forward primer (5' → 3') |
|---------|-------------------------|
| S126A   | GGAATTCGAAACCTTAAAGGA  |
| H147N   | GTTGGAGGTGCCACGCTAGCAG |
| H217A   | GTTTCGACCTCCCCGCCCCTTTC |
| H217N   | GTTTCGACCTCCCCGCCCCTTTC |
| R250Q   | GTTTTTGAATCTTACGCAGTTGCA |

* Also contains the silent restriction site (CCGCGG) for Cfr421 to assist in screening mutant DNA.

**Structure of Prephenate Dehydrogenase**

Site-directed Mutagenesis—The expression plasmid (Δ19PD) encoding residues 20–311 of the *A. aeolicus* VF5 PD protein (AAC07589 from GenbankTM) has been previously described (19). Site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene), whereby complimentary oligonucleotides containing the desired mutations for Δ19PD were used. Table 1 summarizes the mutants generated and the respective oligonucleotides used for mutagenesis. All Δ19PD mutants were verified by DNA sequencing.

Protein Expression and Purification—All constructs of *A. aeolicus* VF5 PD were expressed and purified as previously described (29) with the following modifications. Cells harboring recombinant mutant Δ19PD were disrupted by French press followed by sonication, and the heat treatment step was omitted. Following chromatography of the thrombin-treated enzyme on nickel-nitrilotriacetic acid resin, PDs were subjected directly to size exclusion chromatography on a Superdex 200 column.

Determination of Enzyme Activity and Dissociation Constants for Ligand Binding—Enzyme activity of *A. aeolicus* PD in the presence of NAD$^+$ and prephenate was monitored in a 1-ml reaction cuvette containing 50 mM HEPES and 150 mM NaCl at pH 7.5 as previously described (19). Briefly, the reaction mixture was incubated at 55 °C for 2 min, and then the reaction was initiated by the addition of enzyme. The production of NADH was followed at 340 nm spectrophotometrically. The turnover numbers and Michaelis constants for substrates were obtained by fitting initial velocity data to the Michaelis-Menten equation. Inhibition constants ($K_i$) for the dissociation of HPP, HPpropionate, or L-tyrosine from the enzyme-NAD$^+$ complex were obtained by fitting initial velocity data to the equation for linear competitive inhibition. These data were obtained by varying prephenate at four concentrations ranging from below to above the $K_m$ (in the presence of 2 mM NAD$^+$) and HPP from 0.2 to 1.0 mM and L-tyrosine ranging from 0 to 5 mM. Percent residual activity as a function of L-tyrosine concentration was monitored as described by Bonvin et al. (19). All kinetic data were fitted to the appropriate rate equations by using the computer programs of Cleland (32) or GraFit (Version 5.0, Leatherbarrow). Changes in fluorescence emission as a function of prephenate concentration were used to determine dissociation constants of the prephenate from the binary complex as described by Bonvin et al. (19). Protein concentration was...
estimated using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) with bovine serum albumin (Sigma) as a standard.

**Crystallization of PD**—The initial crystallization condition was determined with a sparse matrix crystallization (Hampton Research Crystal Screen™) screen at room temperature using the hanging drop, vapor-diffusion technique. The optimized co-crystallization condition consists of 48% 2-methyl-1,3-propanediol and 100 mM HEPES at pH 7.8, and co-crystals were obtained by supplementing the protein solution (at a concentration of 12 mg/ml) with 5 mM NAD⁺ and either prephenate, HPpropionate, or L-tyrosine. Site-directed mutagenesis studies targeted residues Ser-126, His-147, His-217, and Arg-250, which are considered important for either the enzyme-catalyzed reaction or for conferring ligand selectivity.

**Crystallographic Data Collection**—X-ray diffraction data were collected from single crystals at a temperature of 100 K in a nitrogen stream on beamline SBC19 at a wavelength 0.9794 Å at the Argonne National Laboratory, Advanced Photon Source. The diffraction data were processed and scaled with the HKL3000 suite of programs (DENZO/SCALEPACK) (33). The enzyme-ligand structures were sufficiently isomorphous with the previously described Δ19PD-NAD⁺-complexed structure (29) to allow the immediate use of the RIGID routine of CNS (Crystallography and NMR system) (34), to correctly place the search model. Model visualization and rebuilding were done with the program O (35).

The remainder of the model was manually built with O, and simulated annealing refinement was subsequently conducted with CNS after every round of model building. In the later stages of model rebuilding and structure refinements, Coot and Refmac with TLS refinement were, respectively, used for the Δ19PD-NADH·HPpropionate, and Δ19PD-NAD⁺-l-tyrosine structures, until each of the model converged (36–38). All molecules of PD were rebuilt prior to every round of refinement, and non-crystallographic symmetry constraints were not applied during refinement. NAD⁺ molecules and ligands were fitted to the unaccounted electron density in each molecule in the tetramer after the second round of refinement. PDB, topology, and parameter files for NAD⁺, tyrosine, HPpropionate, and HPP were obtained from the HIC-up server (41). Representative figures from the crystal structure were produced with PyMOL.

**RESULTS AND DISCUSSION**

The primary objectives of this study were to identify active site residues that are directly involved in the catalytic and regulatory mechanisms of PD and to determine the mechanism that contributes to substrate selectivity among enzymes in the TyrA family. Co-crystallization was conducted in the presence of NAD⁺ and either prephenate, HPpropionate, or l-tyrosine. Site-directed mutagenesis studies targeted residues Ser-126, His-147, His-217, and Arg-250, which are considered important for either the enzyme-catalyzed reaction or for conferring ligand selectivity.

**Crystallographic and Structural Summaries of Δ19PD-NADH·HPP, Δ19PD-NAD⁺·HPpropionate, and Δ19PD-NAD⁺·l-Tyrosine**—Crystals of Δ19PD-NAD⁺·HPP, Δ19PD-NAD⁺·HPpropionate, and Δ19PD-NAD⁺·l-tyrosine grew under identical precipitant and pH crystallization conditions. These crystals belong to space group P2₁2₁2₁, and their respective X-ray data collection statistics are given in Table 2.

| X-ray data | HPP | Δ19PDH-NAD⁺ in complex with | Tyrosine |
|------------|-----|-----------------------------|---------|
| Space group | P2₁₂₁₂₁ | P2₁₂₁₂₁ | P2₁₂₁₂₁ |
| Unit cell (Å³) | 82.50 × 92.48 × 164.08 | 82.02 × 92.36 × 163.83 | 79.34 × 93.74 × 163.81 |
| Molecules in asymmetric unit | 4 | 4 | 4 |
| Resolution (Å) | 2.15 | 2.25 | 2.15 |
| Wavelength (Å) | 0.9794 | 0.9794 | 0.9794 |
| Total observations (no.) | 605,225 | 509,943 | 645,457 |
| Unique reflections (no.) | 66,487 | 58,247 | 77,652 |
| Completeness (%) | 96.4 | 97.4 | 93.8 |
| R₁ (I/σ(I)) | 47.3 (1.3)* | 38.2 (1.3) | 40.0 (2.5) |
| Rₚ (I) | 4.8 (52.0) | 4.3 (55.3) | 6.7 (42.2) |

*Numbers in parentheses refer to the highest resolution shell.

Although continuous, interpretable electron density was observed for the vast majority of these structures, the following residues were excluded in the model due to poor or absent densities: for the Δ19PD-NADH·HPP structure, residues 20–25 and 309–311 for chain D, residues 20–24 and 310–311 in Chain B, residues 20–24 and 306–311 in Chain C, residues 20–29 and 306–311 in Chain B, 20–25 and 311 in Chain A, residues 20–25 and 311 for chain D, residues 20–29 and 20–26 and 306–311 in Chain B, 20–24 and 311 in Chain C, and 20–26 and 311 in Chain D. Poor quality of electron density in these regions of the maps contributed to the higher than normal refinement factors, R_free and R_work (Table 3).

Crystals of Δ19PD-NAD⁺·HPpropionate and Δ19PD-NAD⁺·l-tyrosine were obtained from co-crystallization studies with 5 mM NAD⁺ and 10 mM HPpropionate or 2.25 mM l-tyrosine, respectively. Interestingly, the Δ19PD-NADH·HPP crystals were obtained from co-crystallization experiment containing 5 mM NAD⁺ and 10 mM prephenate. The presence of NADH in the protein crystal was determined spectrophotometrically.
metrically at 340 nm. The presence of HPP and NADH instead of prephenate and NAD$^+$, in the structure, indicates that prephenate was enzymatically converted to HPP during the co-crystallization studies. Fig. 1B shows the chemical structure of each representative ligand used in structure determination of Δ19PD. All ligand types were located in the $F_o - F_c$ difference electron density map after the initial round of refinement. Accordingly, these molecules were built into the model prior to the second round of refinement (Fig. 2, A–C).

Only one molecule of the product or product analogue is consistently identified per dimer. In contrast, both subunits in the dimer contain a molecule of NAD$^+$ or NADH. For example, for each dimer of the ternary complex Δ19PD-NADH-HPP, one subunit contains a molecule of NADH and one molecule of product (we define this as a “paired” occupancy), whereas the other subunit contains only one molecule of NAD$^+$, but no product (we defined this as the “unpaired” occupancy). It is unlikely that interdimer interaction in the crystal packing prevents the binding of HPP to both binding sites simultaneously, because the structure was obtained from co-crystallization studies. The presence of NAD$^+$ in the active site suggests that NAD$^+$ binds first followed by prephenate; similarly, HPP needs to be released before NADH from the active site. This is highly speculative: this model is supported by the fact that we never identified that HPP, in the absence of NADH, occupied the active site. It is also likely that NAD$^+$ binding is required for crystallization. The observation of paired and unpaired occupancy of product analogue in the dimer is indicative that substrate binding and product release are ordered, which is consistent with kinetic data obtained for arogenate dehydrogenase from Synechocystis sp. in which arogenate preferentially binds first (14). However, this finding contrasts the random kinetic mechanism proposed from the analysis of initial velocity and dead-end and product inhibition studies of PD and AD from a number of organisms, including E. coli and Arabidopsis thaliana (17, 19, 24, 28).

**Conformational Shifting upon Substrate Binding**—Superimposition of the crystal structure of PD indicated that substrate binding induces a global conformational change on the dimer. The dimeric protein is in a closed conformation with only NAD$^+$ bound and the binding of HPP or a product analogue moves the two subunits away by about 5Å (Fig. 3, A and B, inset). This global conformational change is imparted mainly on the unpaired monomer. Therefore, the unpaired monomer is in the “open” conformation (Fig. 3A). We speculated that this global conformational change could negatively influence the binding of ligands, product

### TABLE 3 Refinement statistics

|                     | Δ19PDH-NAD$^+$ in complex with | HPP | HPpropionate | Tyrosine |
|---------------------|--------------------------------|-----|--------------|----------|
| $R_{\text{cryst}}$ | 21.1                           | 19.3| 19.6         |          |
| $R_{\text{free}}$  | 25.9                           | 23.0| 25.0         |          |
| $\alpha$ cutoff     | 0.0                            | 0.0 | 0.0          |          |
| Root mean square deviation bond length (Å) | 0.016 | 0.008 | 0.007 |          |
| Root mean square deviation dihedral angle (°) | 21.1 | 20.8 | 20.9 |          |
| Angle               | 1.3                            | 1.3 | 1.2          |          |
| Improper angle      | 0.84                           | 0.87| 0.85         |          |
| Mean $B$-factor (Å$^2$) | 38.95 | 56.0 | 47.7 |          |
| Percentage of residues in Favored regions | 87.6 | 86.4 | 90.2 |          |
| Additional allowed regions | 12.3 | 13.4 | 9.5 |          |
| Disallowed regions  | 0.1                            | 0.2 | 0.2          |          |
| Number of atoms or molecules |                  |     |              |          |
| Protein atoms       | 9380                           | 9408| 9453         |          |
| NAD$^+$ molecules   | 4                              | 4   | 4            |          |
| Tyrosine molecules  | 1                              | 1   | 2            |          |
| HPpropionate molecules | 2                        |     |              |          |
| HPP molecules       | 2                              |     |              |          |
| Solvent molecules   | 259                            | 140 | 348          |          |

**FIGURE 1.** A, metabolic routes from chorismate leading to the synthesis of L-tyrosine and L-phenylalanine. In the arogenate, 4-hydroxyphenylpyruvate, or phenylpyruvate route, prephenate and arogenate are branch point intermediates in both L-tyrosine and L-phenylalanine biosynthesis. Prephenate dehydrogenase catalyzes the oxidative decarboxylation of prephenate with NAD$^+$ to produce hydroxyphenylpyruvate, NADH, and CO$_2$ (40). B, a comparison of the chemical structure of the three ligands, HPP, HPpropionate, and tyrosine, used in the crystallization of A. aeolicus prephenate dehydrogenase. These ligands all have an -OH at the C4 position and a propionyl side chain at the C1 position of the ring.
analogues in this case, to the unpaired monomer. Mainly because the active site residues in the open monomer are no longer in their optimum position to H-bond with the ligands tested.

The binding of the product analogue induces structural changes within the PD subunits (Fig. 3, A and B, inset). A comparison of the active sites shows that most interactions are conserved among the three ligands; in contrast, their conformations are markedly different. The position of the water molecule (WATT) is also well conserved in all three structures containing HPpropionate, HPP, or tyrosine.

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that the ionic interaction with Arg-250 is maintained with sub-
strate binding, whereupon the release of the product this inter-
action is lost.

Other secondary structures that have shifted upon ligand
binding include α8, α9, α10, and α11 (residues 214–266). Two functionally important residues, His-217 and Arg-250, are con-
tained within these regions. With respect to the binary subunit,
the side chain of Arg-250 in the paired subunit is shifted 1.3 Å
closer to the active site pocket and closer to the side-chain car-
boxylate of the respective ligand.

Location of the A. aeolicus PD Active Site—A comparison of
the structures of A. aeolicus PD with each of the three ligands,
HPP, HPpropionate, and L-tyrosine, helps to identify residues
involved in substrate binding and catalysis, and in the regula-
tion of enzyme activity by inhibitor, L-tyrosine. All of the ligands studied have a common C4-hydroxyl group, but they vary
in the properties of the side chain at the C1 position. These
variations at the side chain dictate the binding properties of
the different ligands to A. aeolicus PD (Fig. 1B).

HPP, the immediate product, acts as a linear competitive
inhibitor with respect to prephenate for the reaction catalyzed
by Δ19PD (data not shown). The dissociation constant of HPP
from the enzyme-NAD\(^{+}\) complex of 118 ± 14 \(\mu M\) is essentially
identical to the Michaelis constant for prephenate (\(K_m\) of 135 ±
12 \(\mu M\)) even though HPP is aromatic rather than a cylohexa-
diene and lacks the ring carboxyl group associated with pre-
phenate (Table 4). Based on the structural similarities between
these two compounds, we propose that the interactions
between HPP and active site residues of PD should closely
reflect those with prephenate. Initial velocity data obtained by
varying prephenate (102–680 \(\mu M\)) in the presence of 2 mM
NAD\(^{+}\), and increasing concentrations of L-tyrosine also fit well
to the equation for linear competitive inhibition (data not
shown), with a \(K_i\) of 15.9 ± 1.3 \(\mu M\) for tyrosine.

Architecture of the Substrate Binding Site—The position of
the active site of A. aeolicus PD is consistent with our previous
report from modeling with prephenate (29). The active site lies
adjacent to the nicotinamide moiety of NAD\(^{+}\), which is
expected for efficient hydride transfer from prephenate to the
C4 position of the nicotinamide ring. Amino acids from both
subunits of the dimer contribute to the prephenate binding site;
however, a large portion of the binding pocket is contained
within one subunit, specifically at the inter-domain cleft (29).

The active site can be arbitrarily divided into three regions
based on residue composition and interactions with the prod-
uct analogues; a polar region consisting of residues His-147,
Ser-126, His-214, Ser-213, Phe-209, and His-205, a hydropho-
bic region consisting of residues Ile-149, Ala-150, Gly-151, Thr-
152, His-217, Phe-221, Met-258, and Trp-259, and an ionic
region consisting of residues Glu-153, Arg-250, Ile-251, and
Asp-247 (Figs. 4A, 4B, 4C, and 5A). Collectively, the physio-
chemical properties of these three regions in the active site
reflect the interactions with the different groups of the sub-
strate. The C-4 hydroxyl group of the ligand is located in the
polar region, whereas the side chain participates in interactions
with residues in the ionic region.

Role of His-147 in the Reaction Mechanism—Interactions
between the C4-hydroxyl group of the different ligands (HPP,
HPpropionate, and L-tyrosine) and active site residues are con-
served. The C4-hydroxyl hydrogen bonds with His-147, a
highly conserved catalytic group, which is part of a hydrogen
bonding network between Ser-126 and Ser-213 and a highly
conserved water molecule (WAT1) (Figs. 2A, 2B, 2C, 4A, 4B,
and 4C). Additionally, C4 of the ligand is also located within 2.5
Å from the N1 portion of the nicotinamide ring of NAD\(^{+}\).

These interactions are consistent with kinetic data from E. coli
CM-PD, which implicates His-197 (corresponding to A. aeoli-
cus His-147) as a key catalytic group. As observed for the E. coli
enzyme, pH activity profiles of Δ19PD revealed that a deproto-
nated group, most likely His-147 with a \(pK_a\) of \(\sim 6.8\), is required
for catalysis (data not shown). Kinetic analysis was also con-
ducted on the A. aeolicus PD H147N mutant to confirm its role
in catalysis (Table 4). The H147N mutant is essentially inactive
but binds prephenate with apparent affinity similar to the wild-
type enzyme, as determined by its kinetic parameters and by
thermodynamic measurements of the quenching of tryptophan
fluorescence emission by prephenate (data not shown) (Table 4).
These findings further support the catalytic role of His-147
in polarization the C4-hydroxyl group of prephenate to facili-
tate hydride transfer from prephenate to NAD\(^{+}\).

Role of Ser-126 in the Reaction Mechanism—The role of Ser-
126 was further investigated by mutagenesis and kinetic analy-
ses. Ser-126 coordinates an H-bonding network with His-147,
the N1 atom of NAD\(^{+}\) and C4-hydroxyl group, and thus may
play a role directly in catalysis by bringing together these
groups in a catalytically competent conformation. In addi-

### Table 4

Summary of kinetics parameters for wild-type Δ19PD and variant dehydrogenases at pH 7.4 and 55 °C

| Protein | Variable substrate | NAD\(^{+}\) |
|---------|--------------------|-----------|
|         | \(K_m\)  \(\mu M\) | \(k_{cat}\)  \(s^{-1}\) | \(k_{cat}/K_m\)  \(s^{-1}\mu M^{-1}\) | \(K_m\)  \(\mu M\) | \(k_{cat}\)  \(s^{-1}\) | \(k_{cat}/K_m\)  \(s^{-1}\mu M^{-1}\) |
| WT Δ19PD | 135 ± 12 | 13.0 ± 0.3 | 9.6 \(\times 10^4\) \(s^{-1}\mu M^{-1}\) | 71 ± 3.2 | 11.5 ± 0.3 | 1.6 \(\times 10^5\) \(s^{-1}\mu M^{-1}\) |
| S126A | 1335 ± 39 | 0.8 ± 0.01 | 1.4 \(\times 10^3\) \(s^{-1}\mu M^{-1}\) | 99 ± 6 | 0.8 ± 0.02 | 8.0 \(\times 10^4\) \(s^{-1}\mu M^{-1}\) |
| H147N<sup>b</sup> | 104 ± 11 | 3.4 \(\times 10^{-3}\) | 3.3 \(\times 10^4\) \(s^{-1}\mu M^{-1}\) | ND<sup>c</sup> | ND<sup>c</sup> | ND<sup>c</sup> |
| H217A | 4132 ± 277 | 0.8 ± 0.03 | 1.9 \(\times 10^4\) \(s^{-1}\mu M^{-1}\) | 10 ± 0.5 | 0.6 ± 0.004 | 6.0 \(\times 10^4\) \(s^{-1}\mu M^{-1}\) |
| H217N | 3213 ± 445 | 0.5 ± 0.02 | 1.5 \(\times 10^4\) \(s^{-1}\mu M^{-1}\) | 12 ± 0.2 | 0.3 ± 0.01 | 2.5 \(\times 10^4\) \(s^{-1}\mu M^{-1}\) |
| R250Q | 1185 ± 118 | 9.9 ± 0.42 | 8.4 \(\times 10^4\) \(s^{-1}\mu M^{-1}\) | 89 ± 12 | 11.6 ± 0.6 | 1.3 \(\times 10^5\) \(s^{-1}\mu M^{-1}\) |

<sup>a</sup> Values were calculated from initial rates using a minimum of five NAD\(^{+}\) concentrations ranging from one-half \(K_m\) up to 10-fold \(K_m\) for the variable substrate. When prephenate was the variable substrate NAD\(^{+}\) was fixed at 2 mM, while when NAD\(^{+}\) was the variable substrate, prephenate was fixed at 11 mM (WT, S126A), 18 mM (H217A), 16 mM (H217N), and 6 mM (R250Q).

<sup>b</sup> NAD\(^{+}\) was kept at 2 mM, and prephenate was varied from 50 to 600 \(\mu M\) using 630 \(\mu g\) of protein. Parameters for NAD\(^{+}\) were not determined.

<sup>c</sup> ND, not determined.
**Role of Wat-1 in the Reaction Mechanism**—Wat-1 is a highly conserved water molecule that participates in the H-bonding network with the ligand. Interestingly, this water molecule is only observed in the ternary complex of the enzyme with NAD\(^+\) and the ligands studied. Specifically, Wat-1 is shown to bridge the interaction between Ser-113, Ser-126, and the C-4 hydroxyl of the ligand (Figs. 2A, 2B, 2C, 4A, 4B, and 4C). We propose that Wat-1 may serve two mechanistic roles: Wat-1 may be participating solely in the binding interaction with the ligand and/or Wat-1 could participate in modulating the properties of His-147 in the catalytic mechanism.

**Role of Arg-250 in the Reaction Mechanism**—The carboxylate of the propionyl side chain is conserved between HPP, HPpropionate, and \(\alpha\)-tyrosine (Fig. 1B). Analysis of the \(\Delta 19\)PD structure in complex with each of the three ligands revealed that this carboxylate directly interacts with the guanidinium group of Arg-250 and is positioned in proximity of this group by an ionic network (Fig. 4, A–C).

In these PD-ligand structures, Arg-250 is ordered with excellent electron density. In the absence of a ligand, Arg-250 is disordered judging by the lack of its representative electron density thus suggesting that it has an important binding role. Kinetic analysis of the R2\(50\)Q mutant displayed a 10-fold increase in the \(K_m\) for prephenate and a 20-fold increase in \(K_p\), 370 ± 42 \(\mu\)M, for tyrosine relative to the wild-type enzyme without significant change in the enzyme’s affinity for NAD\(^+\) or its turnover rate (Table 4). Together these findings are in agreement with Arg-250 being important, but it is not critical, for the binding of the different ligands via the side-chain carboxylate group. Mutating Arg-250 did not eliminate prephenate or \(\alpha\)-tyrosine binding. This is indicative that one or more other active site residues are also important for ligand binding.

**Role of His-217 in the Reaction Mechanism**—The most important differences between the C1-side chain of the ligands are: HPP has a keto group in addition to the conserved carboxyl, \(\alpha\)-tyrosine possesses an amino group, and HPpropionate is lacking a group at the corresponding position. The structure of the \(\Delta 19\)PD-NADH-HPP complex shows that the keto group on this propionyl side chain can potentially make H-bonding interaction with the main-chain amide of Gly-244 and is also positioned near a H-bonding network, which is mediated by a water molecule, Wat-2 (Fig. 4A). This H-bonding network includes Ne2 of His-217, -OH of Ser-254, the keto and the carboxyl groups of HPP propionyl side chain and Wat-2 (Fig. 4A). The N81 of His-217 is also H-bonding to the main-chain carbonyl of Ser-213. These H-bonding interactions by both N81 and Ne2 of His-217 would indicate that it is in the protonated state. To further understand the role of His-217, H217A and H217N mutants were designed and kinetically analyzed. Substitutions of His-217 to either alanine or asparagine produce mutant enzymes that are not inhibited by tyrosine but also have significant changes in their kinetic parameters for the \(\Delta 19\)PD-catalyzed reaction. The \(K_m\) for prephenate was increased, by 40-fold and 30-fold, for the H217A and H217N mutants, respectively, thus indicating the importance of His-217 in prephenate selectivity (Table 4). However, these substitutions also coincided with 10- to 20-fold decrease in \(k_{cat}\) thus indicating that this interaction with the keto group of the substrate may assist in
Interestingly, this E. coli the k-meric structures. We determined that the binding of NAD+/H11001 was lowered by 7-fold for both His-217 variants (His-217A and His-217N) from this organism could explain the varying levels of perturbations in kinetic properties between the H217A and H257A mutants from A. aeolicus PD and E. coli CM-PD.

The importance of His-217 is further illustrated by the co-crystal structure of HPpropionate with \(\Delta 19\)PD. HPpropionate lacks the side-chain keto group and as such cannot participate in the hydrogen-bonding interaction at this position (Fig. 4B). Superimposition of HPP and HPpropionate in the active site clearly show that the propionyl side chains are not superimposable; the HPpropionate side chain is slightly shifted closer to His-217 (Fig. 5A). Kinetic constants for the interaction of HPpropionate to E. coli PD is ten times better than that for HPpropionate, 0.18 mM versus 1.8 mM (26). These findings are in agreement with our hypothesis that one or more residues in the microenvironment of His-217 are functioning as a determinant for the selective binding of ligands to PD active site. Ligands lacking the keto group can still bind but with lower affinity because of increased dynamics of their side chain.

The structure of \(\Delta 19\)PD in complex with tyrosine revealed a different set of H-bonding interactions with the amino group of tyrosine compared with those with the keto group of HPP (Fig. 4, A and C). In this complex, the amino group of L-tyrosine is pointing in the opposite direction compared with that of the corresponding keto group of HPP. As a result, the amino group of L-tyrosine is directed away from His-217 and from the Gly-244 amide backbone by approximately 180 degrees and instead is interacting with the main-chain carbonyl group of Thr-152 (Figs. 4A, 4C, and 5A). Superimposition of the ligands in the active site of PD revealed that the side chain of L-tyrosine has been shifted away from His-217 by 3.2 Å, compared with the propionyl side chain of HPP, and the resulting distance between the amino group of the alanyl side chain and His-217 is 4.1 Å.

H217A/N Mutants Are Insensitive to Tyrosine Inhibition—Superimposition of the three ligands in the A. aeolicus PD active site revealed that the propionyl side chains adopt a different conformation depending on the presence of a keto, HPP, or an amino group, tyrosine. This observation in conjunction with the kinetic studies of the His-217 mutants led us to speculate that the micro-

FIGURE 5. Superimposition of ligands in the active site of PD. A, superimposition of HPP (green), HPpropionate (yellow), and tyrosine (silver) illustrates the substrate-specific interactions. The keto group of HPP is directly pointing toward His-217. The amino group of Tyr is pointing away from His-217 and is interaction with main-chain carbonyl of Thr-152. B, the PDH active site (yellow) with a bound HPP molecule (green) superimposed with the putative ADH active site (silver). The functionally important active site residues identified in PDH are conserved in ADH, in parentheses. A major difference between the two active sites is the presence/absence of His-217 and of bulky groups adjacent to the substrate-binding site. The absence of these bulky groups in the ADH active site produces a large pocket. C, a Cu+ superimposition of the PDH (blue) and ADH (magenta) monomeric structures.
environment around His-217 is important for tyrosine binding. We further speculated that the ionization state of His-217 and its interaction with other active site residues might dictate the nature of ligands that can bind to the active site of PD. Based on the H-bonding interactions at both Nδ1 and Nε2 discussed above for His-217, we speculated that the side chain of this histidine is in the protonated state. This hypothesis is consistent with our observation for the HPP-bound and L-tyrosine-bound structures, in which the keto group of HPP is pointing toward the Nε1 of His-217 and the amino group of L-tyrosine is pointing away from the histidine and instead is interacting with the main-chain carbonyl of Thr-152 (Fig. 4C, 5A). It is likely that this charge state of His-217 would produce a repulsive effect, which directs the interaction between the main chain carbonyl of Thr-152 and the amino group of the bound tyrosine.

Tyrosine inhibition studies were conducted to evaluate the role of His-217 and other residues in the binding of L-tyrosine to PD. L-Tyrosine was shown to have an inhibitory effect on both wild-type enzyme and on S126A, but had a reduced inhibitory effect on the activity of R250Q (Fig. 6). R250Q activity is inhibited by L-tyrosine less effectively than the WT enzyme, because the interaction is absent between the carboxylate of the bound tyrosine and Arg-250. The most interesting finding though is that the inhibitory effect of L-tyrosine was completely eliminated by mutating His-217 to either an alanine or an asparagine (Fig. 6). Indeed, the binding of prephenate is also affected by the alanine and asparagine substitutions, which may suggest that mutating His-217 could result in structural perturbation of the active site, which has a more pronounced effect on tyrosine binding. In addition, our results from mutational analysis of the E. coli enzyme show that the H257A mutation (equivalent to H217A in A. aeolicus) also completely eliminates feedback inhibition by L-tyrosine, and its Michaelis constant for prephenate is relatively unperturbed. We envision that removing His-217 eliminates the repulsive effect from the microenvironment, which is responsible for directing the interaction between the amino group of tyrosine and the main-chain carbonyl of Thr-152.

Structural Comparisons of AD and PD—The availability of an AD structure permits structural comparisons between L-arogenate-specific and prephenate-specific TyrA dehydrogenases for better understanding of the molecular mechanism responsible for substrate binding in the TyrA family of proteins. To this end, we compared the ternary complex 19PD-NADH-HPP, 19PD-NADH-HPropionate, and 19PD-NADH-tyrosine structures with that of Synechocystis sp and superimposed the structure of 19PD-NADH-HPP with the Synechocystis sp. AD structure (PDB ID: 2F1K) (28) (Fig. 5, A–C).

The monomers superposed over 272 equivalent Cα atoms and have an root mean square deviation value of 2.4 Å. The overall structures PD and AD are quite similar, because their secondary structures have been maintained (Fig. 5C). Regions exhibiting significant deviations include portions of the loop between β6 and α6 and helices αβ, α9, α10, and α11 (28, 29) (Fig. 5C). Overall, the active site of AD is more open and accessible, relative to that of binary 19PD-NADH-tyrosine complex (Fig. 5B). This is due to the fact that α10, α11, and the β6–α6 loop region, which comprise the base and wall of the pocket, respectively, are shifted ~3.1–6.5 Å away from the active site (Fig. 5C) (28, 29). Analysis of the PD and AD active sites reveals that functionally important residues are conserved (Fig. 5, A and B). For example, the catalytic histidine, His-147 in PD, and the important binding arginine, Arg-250 in PD, are equivalently positioned in both structures. Moreover, the serine residues that are shown to bind to the C4-hydroxyl group of prephenate are also spatially conserved. Other common active site residues include Gly-151, Thr-152, His-205, Ser-213, and His-214 (numbering corresponds to the 19PD from A. aeolicus) (Fig. 5B).

Given the importance of the backbone carbonyl of Thr-152 in the binding of L-Tyr, perhaps this group may also bind the amine moiety of L-arogenate. The conservation of functionally important residues indicates that L-arogenate may bind to the AD active site in a similar fashion to that of prephenate in PD. Superimposing the HPP molecule from the 19PD-NADH-HPP structure into the AD active site supports this hypothesis. Based on the latter study, we infer that it is indeed possible for L-arogenate to interact with Ser-92, His-112, Arg-217, and Arg-250, which correspond to Ser-126, His-147, and Arg-250 in 19PD. Our proposed interaction with Arg-250 contradicts Legrand et al. (28) who have suggested that this arginine is too far from the active site pocket to play a role in substrate binding.

Despite these similarities in the active site, there are notable differences. Most notably, there is a large pocket adjacent to the superposed HPP molecule in the AD active site (Fig. 5, A and B). This pocket results from the absence of bulky Trp and His residues (Trp-259 and His-217 in PD) and the position of α10 and α11 in the AD structure (Fig. 5, A–C). These missing residues could provide the required binding interactions for prephenate. Interestingly, Synechocystis sp. AD is lacking a histidine corre-
sponding to His-217 and is not inhibited by tyrosine. This finding is completely in line with our speculative role of His-217 in conferring selectivity for tyrosine binding.

Biological and Biochemical Relevance—This study describes the functional role of active site residues of proteins in the TyrA family, mainly, residues that are involved in ligand selectivity, binding, and catalysis. Comparative structural analysis with existing structures in the PDB revealed that the catalytic histidine residue is conserved among proteins in the TyrA family. The arginine residue that is involved in binding the different ligands through the side-chain carboxylate is also conserved. The most significant finding, however, is that the mutation of ligands through the side-chain carboxylate is also conserved.

Structure of Prephenate Dehydrogenase

The mutation of residue the equivalent His-217 could dictate the selective binding of different ligands to the PD active site. Notably, the presence of His-217 allows l-tyrosine to function as a competitive inhibitor with respect to prephenate. Substitution of the histidine residue at position 217 completely eliminated the inhibitory effect of l-tyrosine on A. aeolicus PD activity. This novel finding has direct implications on future metabolic engineering of downstream pathways in plants. It is likely that tyrosine could function as a feedback regulator by inhibiting PD at elevated intracellular concentrations. The mutation of residue the equivalent His-217 could produce a constitutively active PD, which will result in the unregulated production of HPP and subsequently tyrosine. Tyrosine, aside from being an essential component in proteins, is known to be an essential precursor of the biosynthesis of benzylisoquinoline alkaloids. Benzylisoquinoline alkaloid is diversified toward the production of >2500 secondary metabolites, and in addition benzylisoquinoline alkaloid itself is used as a precursor for the synthesis of pharmaceuticals, including morphine and codeine (39).

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