Biochemical and Structural Studies of 6-Carboxy-5,6,7,8-tetrahydropterin Synthase Reveal the Molecular Basis of Catalytic Promiscuity within the Tunnel-fold Superfamily*

Zachary D. Miles 1, Sue A. Roberts, Reid M. McCarty 2, and Vahe Bandarian 3

From the Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721

The tunnel-fold (T-fold) superfamily is comprised of a widely distributed group of enzymes that catalyze transformations leading to the production of purines and pterins (1, 2). The mammalian tunnel-fold enzyme 6-pyruvoyltetrahydropterin synthase (PPh₄) 4 synthase (mPTPS) is required for the biosynthesis of 6R-L-erythro-5,6,7,8-tetrahydrobiopterin (BH₄) in eukaryotes (1) (3). BH₄ is an essential cofactor for many enzymes including nitric-oxide synthase and phenylalanine hydroxylase where it can act as either an electron donor or in oxygen insertion (4). In addition, this cofactor has been implicated in a vast array of physiological roles including hyperphenylalaninemia, cellular proliferation, vascular dysfunction, and various neurological disorders (4, 5). mPTPS catalyzes the conversion of 7,8-dihydropterin triphosphate (H₂NTP) to PPh₄, which is subsequently converted to BH₄ by the NADPH-dependent sepiapterin reductase (6, 7). More recently, an alternative pathway has been identified in higher organisms, including humans, which allows for bypassing the enzyme sepiapterin reductase through the combined actions of a carbonyl reductase and an aldose reductase or by members of the aldo-keto reductase family of enzymes (8–11). BH₄ is typically not produced in bacteria, but is present in glycosylated forms in certain cyanobacteria and Chlorobium tepidum (12–16).

Although most bacteria do not possess BH₄, a PTPS homolog is found in virtually all bacterial genomes for production of pterins and deazapurines. Previous studies from our laboratory have established that the Escherichia coli PTPS homolog, 6-carboxy-5,6,7,8-tetrahydropterin synthase, catalyzes the second step in the biosynthesis of pyrrolopyrimidine nucleosides involving the conversion of H₂NTP to 6-carboxy-5,6,7,8-tetrahydropterin (CPh₄) (17). CPh₄ is a precursor to >30 natural products that range from antibiotic and anticancer agents, produced by various strains of Actinomycetes, to the modified tRNA base queuosine, which is found in nearly all kingdoms of life (Fig. 1) (18).

Background: The bacterial homolog, 6-carboxy-5,6,7,8-tetrahydropterin synthase, of the eukaryotic 6-pyruvoyltetrahydropterin synthase enzyme acts on the same substrate but produces different products.

Results: Structural and biochemical studies trace the differential reactivity to four residues.

Conclusion: Differential reactivity between the enzyme homologs is a result of small changes in the enzyme active site.

Significance: This work furthers our understanding of how novel activities may arise from common protein-folds.
Catalytic Promiscuity in the Tunnel-fold

CYP synthase is promiscuous and in addition to H₂NTP converts sepiapterin and PPH₄ to CPH₄ (19). It is not clear how this bacterial protein, which is closely related at the sequence level to its mammalian homolog involved in the biosynthesis of BH₄, catalyzes such distinct transformations. Herein we report a structural and functional investigation of CYP synthase from *E. coli* (QueD, so named for its role in the biosynthesis of queuosine). Comparison of the structure of QueD to its mammalian homolog revealed amino acid substitutions in the active site that may account for the distinct catalytic outcome of the bacterial enzyme. Steady state and rapid kinetic analysis of wild-type CYP synthase and its site-directed variants have led to insights regarding the role of these residues in catalysis. This study highlights how minor changes in this highly conserved active site have led to the emergence of catalytic promiscuity and the evolution of catalytic function to support novel biosynthetic pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—All materials were purchased commercially (unless otherwise noted) and were of the highest purity. All assays were carried out in a Coy anaerobic chamber in an atmosphere of 95–97% N₂, 3–5% H₂. All buffers and materials were deoxygenated in the chamber several days prior to use.

Expression and Purification of *E. coli* QueD and Variants, mPTPS, and Sepiapterin Reductase—All proteins were expressed and purified as described previously (19). Selenomethionine-substituted QueD was obtained by growth in minimal media (20) and purified as described previously (19). Selenomethionine-substituted protein samples were obtained with 5% (v/v) 2-methyl-1,3-propanediol, 0.04 M CaCl₂, 0.1 M sodium acetate (pH 4.5) as precipitant. For the C27A variant (16 mg/ml, 20 mM HEPES (pH 7.5), 10 mM dithiothreitol), the precipitant solution contained 4% (v/v) polyethylene glycol (PEG) 4000 and 0.1 M sodium acetate (pH 4.5). Sepiapterin- or CYP₄-bound structures were obtained by soaking crystals for 1 h in a solution containing 7 mM sepiapterin and 17% (v/v) of appropriate precipitant (either PEG4000 or 2-methyl-1,3-propanediol). Crystals were transferred into a solution containing 35% of PEG 4000 or 2-methyl-1,3-propanediol and frozen in liquid nitrogen.

Diffraction data were collected at SSRL on beam lines 9-2 and 11-1, and the data were reduced and scaled using Crystal Clear (unliganded selenomethionine-substituted protein) or XDS (21, 22). The structure of the unliganded, selenomethionine-substituted protein was solved by molecular replacement with PHASER (23) using PDB structure 2OBA as the search model. Liganded structures were solved in the same manner using the unliganded protein structure (PDB code 4NTN) as the search model. The structures were refined using REFMAC5 (24) and rebuilt using COOT (25). TLS parameters were refined (one TLS group per protein chain) for the liganded and unliganded selenomethionine protein structures. Only protein residues were included in the TLS groups (26). No σ cutoff was used in the refinement. All calculations were performed using programs from the CCP4 package (27). Coordinates for unliganded selenomethionine-substituted QueD (PDB code 4NTN), selenomethionine-substituted protein soaked with sepiapterin (PDB code 4NTM), and the C27A variant of QueD soaked with sepiapterin (PDB code 4NTK) were deposited in the Protein Data Bank. Structure figures were prepared using PYMOL (28).

Enzymatic Preparation and Purification of H₂NTP—H₂NTP was produced as reported previously (29). The lyophilized material was dissolved in deoxygenated water in the anaerobic chamber and aliquots were frozen at −80 °C.

**HPLC Assays to Detect Turnover of QueD and mPTPS with H₂NTP and Sepiapterin**—The assays (0.1 ml) contained 20 mM PIPES/NaOH (pH 7.4), 10 mM dithiothreitol, 10 mM MgCl₂,
and 50 μM substrate and were initiated by the addition of 10 μM enzyme. The reactions were quenched after 1 h by addition of 30% (w/v) trichloroacetic acid to a final concentration of 10% (v/v). Precipitated protein was removed by centrifugation at 13,000 × g. The supernatant (75 μl) was placed into a glass vial that was sealed inside the anaerobic chamber and analyzed by an ion-pairing HPLC method described previously (19). The eluents were monitored using a diode array detector and the chromatograms were analyzed for appearance of CPH₄ (298 nm) or disappearance of sepiapterin (420 nm) as described above.

**Steady State Kinetics Experiments with QueD—**HPLC-based assays to determine steady state kinetic parameters for the conversion of H₂NTP to CPH₄ were carried out as described above. Velocities were obtained from the linear portion of the reaction at each concentration of H₂NTP. The enzyme concentration for the assays was in the 0.1–1 μM range. Steady state analysis with sepiapterin as substrate was carried out in the same manner as the steady state H₂NTP assays except that conversion of sepiapterin to CPH₄ was monitored (in a total assay volume of 1 ml) by the change in absorbance at 420 nm (ε₂₄₀ = 10.4 mm⁻¹ cm⁻¹) (30).

**Single Turnover Stopped-flow UV-Visible Spectroscopy—**All single turnover experiments were carried out using a BioLogic SFM 400 fitted with a stopped-flow head; a stream of nitrogen gas maintained anaerobicity in the syringe compartment. All solutions were prepared in the anaerobic chamber and loaded into syringes before being transferred to the instrument. The solutions were the same composition as described for the above HPLC assays except one syringe contained substrate, whereas the other contained enzyme, and the two were mixed in a 1:1 ratio. To maintain a single turnover regime, the substrate concentration (50 μM after mixing) was kept below the enzyme concentration (250 μM after mixing) in all experiments. Data obtained with the diode array detector in the 259–701 nm range were analyzed using KinTek Explorer software (version 3.0) (31, 32). In each case, the data were fit to one or more exponentials (as described under “Results”) to analyze the spectral changes corresponding to turnover.

**Single Turnover Quenched-flow Experiments—**These experiments were carried out essentially as described above for the stopped-flow experiments with the exception that the reactions were quenched with 30% (w/v) trichloroacetic acid to a final concentration of 10% (v/v). Samples recovered after the quenches were analyzed by HPLC as described above.

**Assays with mPTPS—**Reactions were conducted in the same manner as the HPLC-monitored experiments described for turnover of E. coli QueD with H₂NTP or sepiapterin at concentrations of mPTPS and substrate indicated in the figure legends.

**RESULTS**

**Overall Structure of QueD—**A ribbon representation of wild-type QueD is shown in Fig. 2, and refinement statistics are summarized in Table 1. QueD is a homohexamer with each monomer containing 121 amino acids (33). QueD crystallizes with the biological assembly, a homohexamer in the asymmetric unit. As with the mPTPS homolog (1, 34), QueD adopts a tunnel-fold common to proteins that bind purine and pterin substrates. The structure consists of four sequential antiparallel β-strands with two antiparallel α-helices on the concave face of the β-sheet between the 2nd and 3rd strands (ββααββ). The subunits assemble into a hollow barrel with an approximate 3-fold axis down the center of the cavity and approximate 2-fold axes in the equatorial plane (Fig. 2). In the structures we are reporting, the packing of hexamers in the unit cell differs between crystal forms. The C27A variant crystallized such that the 3-fold axis of the barrel is parallel to the crystallographic a axis for all hexamers. For selenomethionine-substituted protein, which crystallized under different conditions, the hexamer 3-fold axis is not parallel to a crystallographic axis and adjacent hexamers are oriented with the 3-fold axes rotated by 90 degrees.

**The Active Site Architecture of QueD—**The six active sites of QueD are located at the interface of the monomers that comprise the homohexamer (Fig. 2). As expected from the sequence similarity to the mPTPS homolog, and from biochemical studies showing the presence of ~1.1 eq of zinc divalent cation per monomer (19), we observe electron density consistent with the cation in each active site. The catalytic zinc ions (shown as green spheres) are positioned near the equator and toward the outside of the assembly, near the confluence of three protein chains. As with mPTPS, the zinc divalent cation is coordinated by the imidazole side chains of three histidine residues (His¹⁶, His³¹, and His³³) from a single subunit (35). A water molecule occupies the fourth coordination position of the zinc cation. As in mPTPS, the essential Cys²⁷ residue (Cys⁴² in mPTPS) in the active site of QueD is in a position to interact with an His⁷¹-Asp⁷⁰ dyad (His⁸⁹-Asp⁸⁸ in mPTPS) activating it to catalyze proton abstraction from the substrate to initiate catalysis (29, 35). Interestingly, a second unique dyad, His²⁵-Asp⁴⁵, is also present in QueD, which we hypothesize is responsible for promoting the novel retroaldol cleavage to form CPH₄ by additional interactions with Cys²⁷.

In addition to the structure of the wild-type enzyme, we also determined the structure of QueD with the product CPH₄ in the active site (PDB code 4NTM), and the QueD variant C27A with the substrate sepiapterin in the active site (PDB code 4NTK) (Table 1). Illustrations of the active site residues and corresponding ligand electron density are shown in Fig. 3, A and B. The electron density that is observed when sepiapterin is soaked into selenomethionine-substituted QueD is consistent with formation of the product CPH₄ that clearly has a shorter side chain than sepiapterin (Fig. 3A), demonstrating that the crystals are catalytically active and convert sepiapterin to product.

By contrast, electron density after soaking sepiapterin into crystals of the catalytically inactive C27A variant shows the side chain to be long enough to be uncleaved (Fig. 3B). Therefore, we initially built sepiapterin into the electron density in each of the active sites. However, after refinement, the terminal methyl group of the side chain was out of the electron density in all six active sites. Moreover, a negative difference density peak appeared centered on the methyl carbon in several, but not all of the active sites. The lack of electron density for the methyl group and presence of difference peaks persisted through several refinement cycles. Although kinetic results (discussed below) led us to believe that no reaction should occur in crystals.
of the C27A variant, we replaced sepiapterin with CPH₄ in the model. Two positive difference peaks appeared, one 2.3 Å from the zinc atom, corresponding to the 2’-oxygen of the sepiapterin side chain, and the second in the plane of the O-C-C-O of the side chain corresponding to the methyl group. This placement of the methyl carbon requires the second carbon to be sp² hybridized. Subsequent refinement modeling the ligand as the enol form of sepiapterin resulted in the loss of the difference density peaks and led to electron density that covered the methyl group, which is planar with the OCCO of the side chain. The electron density for the methyl carbon is slightly weaker than that of the surrounding atoms, which may imply some structural heterogeneity in the bound ligand. Therefore, it appears that whereas the C27A variant is catalytically inactive, the predominant form of bound sepiapterin is the deprotonated enolate form with both oxygen atoms coordinated, asymmetrically, to the zinc cation. Stopped-flow evidence supporting this tautomerization of sepiapterin will be presented below.

The bound ligands have an extensive array of hydrogen bonds that satisfy each of the allowed hydrogen bond donor/acceptor sites of the pyrimidine ring (see Fig. 4). All six active sites appear to be fully occupied with ligand in both soaked structures. No large protein conformational changes occur upon sepiapterin binding the protein. The core root mean square deviation is 0.42 Å when unliganded and liganded selenomethionine structures are overlaid using the SSM algorithm as implemented in COOT (36).

Although ligand binding induces no overall changes, there are active site adjustments when ligands bind. The largest movements are of the side chains of Glu⁵⁴ and Phe⁵⁵, which move ~2 Å into the active site. The side chain of Glu⁵⁴ forms a hydrogen bond with N1 of the pterin ring and Phe⁵⁵ stacks with the pterin ring of the ligand. Crystal contacts for the selenomethionine protein-CPH₄ complex are different from those of the C27A protein-sepiapterin complex, so these small conformational changes that are the same in both structures are presumably not an artifact of crystal contacts. Structures of unliganded and sepiapterin-soaked native QueD have been deposited in the Protein Data Bank (PDB codes 3QN9, 3QN0, and 3QNA). These structures show the same small adjustments in active site conformation upon ligand binding. It is important to note that sepiapterin is not the natural substrate of the enzyme and is used as a model for the second step in the reaction catalyzed by QueD, as described in this article. Therefore, we cannot exclude the possibility that there may be more significant conforma-

FIGURE 2. The ribbon representation of QueD. The six active sites in the homohexamer lie at trimer interfaces and house a catalytic zinc divalent cation (green sphere). A close-up of the active site is shown to illustrate the contributions from adjacent subunits.
tional changes that occur on native substrate binding during the first half-reaction.

The other significant difference in the active site is rotation of the Cys27 sulfur toward the position of His25 causing its rotation away from the active site. Both Cys27 and His25 occupy alternate conformations in the CPH4-containing crystal (Fig. 3A); in conformation A, the sulfur of Cys27 and the nitrogen of His25 are separated by 3.4 Å. In contrast, the distance between these atoms is 4.1 Å in the unliganded structure.

The structural data highlight the substantial similarities of mPTPS and QueD, as well as the conspicuous His25–Asp54 dyad in the vicinity of the substrate, which may be driving the functional differences. In the remainder of this article we focus on biochemical studies of residues Cys27, His25, Asp54, Asp70, and His71 to better understand their role in catalysis.

Steady State Kinetic Characterization of QueD and Site-directed Variants—To understand how the difference in reactivity between QueD and mPTPS may relate to the structural differences, we examined the effect of mutations to either the conserved or unique dyad on the activity of QueD. In initial end point assays to assess activity, QueD was incubated with sepiapterin or H2NTP and the reaction mixtures were examined by

### TABLE 1

**Data collection and refinement statistics**

| Data collection statistics | 4NTN | 4NTM | 4NTK |
|---------------------------|------|------|------|
| **PDB ID**                | 4NTN | 4NTM | 4NTK |
| **Modification, ligand**  | SeMet, no ligand | SeMet, CPH4 | C27A, sepiapterin |
| **Beamline**              | SSRL 9–2 | SSRL 9–2 | SSRL 11–1 |
| **Wavelength (Å)**        | 0.97915 | 1.0000 | 0.9795 |
| **Crystal class, space group** | Orthorhombic, f222 | Tetragonal, P42,2,2 | Orthorhombic, P212121 |
| **Z**                     | 6    | 6    | 6    |
| **Unit cell parameters**  |      |      |      |
| **a (Å)**                 | 107.08 | 111.30 | 111.30 |
| **b (Å)**                 | 111.76 | 111.30 | 126.05 |
| **c (Å)**                 | 161.44 | 112.60 | 115.09 |
| **Resolution limits (Å)** | 56.0–2.00 (2.10–2.00) | 39.0–2.05 (2.10–2.05) | 38.4–1.60 (1.69–1.60) |
| **No. independent reflections** | 66,155 (9,592) | 50,332 (3,709) | 98,203 (13,940) |
| **Completeness (%)**      | 99.9 (100.0) | 99.6 (95.6) | 98.4 (97.0) |
| **Redundancy**            | 4.9 (4.7) | 7.2 (6.9) | 3.3 (3.4) |
| **Avg I/Avg σ(I)**        | 9.2 (2.0) | 33.2 (4.2) | 18.1 (2.1) |
| **Rsym**                  | 0.087 (0.635) | 0.031 (0.577) | 0.033 (0.546) |
| **Rpim**                  | 0.079 (0.635) | 0.031 (0.577) | 0.033 (0.546) |

| Refinement statistics     | 4NTN | 4NTM | 4NTK |
|---------------------------|------|------|------|
| **Resolution limits (Å)** | 55.90–1.99 | 33.92–2.05 | 55.90–1.99 |
| **R-factor**              | 0.204/66142 | 0.226/50256 | 0.187/98125 |
| **R-factor (working)**    | 0.203/62782 | 0.225/47712 | 0.185/93200 |
| **R-factor (free)**       | 0.222/3360 | 0.255/2544 | 0.217/4925 |
| **R.m.s. deviation**      | 0.006 | 0.006 | 0.014 |
| **Bond angles (°)**       | 1.145 | 1.13 | 1.725 |
| **General planes (Å)**    | 0.004 | 0.010 | 0.011 |
| **Ramachandran plot**     | 98.9 | 97.8 | 98.3 |
| **Favored region (%)**    | 1.1 | 2.1 | 1.7 |
| **Allowed region (%)**    | 0 | 1 | 0 |
| **Outliers (No. of residues)** | 0 | 1 | 0 |
| **Average B values**      | 50.5 | 61.1 | 27.9 |
| **Protein atoms (Å2)**    | 52.7 | 47.5 | 27 |
| **Ligand (Å2)**           | 50.75 | 48.3 | 37.8 |
| **Solvent (Å2)**          | 27.3 | 49.2 | 24 |
| **Wilson B factor (Å2)**  | 52.7 | 47.5 | 27 |

* Numbers in parentheses refer to the respective highest resolution data shell in the data set.

* Rsym = (∑|I − F|/ΣF), where F is the observed structure-factor amplitude and F is the calculated structure-factor amplitude.

* As calculated using Molprobity (46).

FIGURE 3. **Structures of QueD with bound ligands.** The electron density maps used to prepare the figures are those obtained after molecular replacement and refinement but before any ligand was built into the active sites. A, the active site of selenomethionine-substituted QueD with the product CPH4 bound. B, the active site of the C27A inactive variant of QueD. In B, sepiapterin soaked into the crystal has undergone deprotonation and tautomerization to the enolate form. For both figures, the Fc − F Electron density maps are contoured at 3.0 o a.
HPLC for CPH₄ production. The data (Fig. 5) show that, as expected, the C27A variant of QueD cannot convert either substrate to CPH₄ confirming the essential role of this residue in catalysis and the lack of turnover in the crystal (see above). Mutation of the conserved dyad (D70N/H71A) greatly diminished CPH₄ production from H₂NTP, whereas mutation of the unique dyad (H25A/D54N) completely abolished this activity. Interestingly, both dyad mutants were able to produce CPH₄ from sepiapterin. The quadruple variant (H25A/D54N/D70N/H71A), where both dyads are deleted, is completely inactive under all conditions tested.

Steady state kinetic analyses of QueD and variants were carried out with H₂NTP and sepiapterin to quantify the roles of the conserved residues. Unfortunately, we were not able to obtain reliable rates at very low concentrations of substrate, hampering determination of accurate K_m values. Therefore, for the foregoing discussion of the stopped-flow data, we only use the data in Fig. 6 to estimate the lower limit of the turnover number of wild-type enzyme with H₂NTP, and of wild-type H25A/D54N and D70N/H71A variants with sepiapterin. The C27A variant is catalytically inactive under all conditions examined.

Pre-steady State Kinetic Characterization of Wild-type QueD and Site-directed Variants—To gain additional insights into the role(s) of the conserved residues, single turnover stopped-flow experiments were undertaken with H₂NTP and sepiapterin as substrates. In experiments where QueD is mixed with H₂NTP, an intermediate with a λ_max at ~440 nm builds in an interval of 4.5 s after mixing and disappears in the next ~50 s (Fig. 7A). The data are consistent with a model that included steps for formation and disappearance of an intermediate. The data are best fit with three exponentials, the first two corresponding to the formation (1.5 and 0.6 s⁻¹) and the third to the disappearance (0.1 s⁻¹) of the 440 nm transient (Fig. 7B). Because the turnover number of the enzyme with H₂NTP is 0.013 s⁻¹ the two phases are fast enough to correspond to formation and disappearance of a kinetically competent intermediate.

Quenched-flow experiments were carried out to further probe the nature of the 440-nm intermediate. In these experiments, QueD was mixed with H₂NTP and samples were quenched at various times (0.5 to 60 s) after mixing and analyzed by HPLC. The samples reveal clear accumulation and disappearance of a peak at ~8.0 min whose UV-visible spectrum and retention time are identical to those of commercially available sepiapterin. The intermediate reaches maximal concentration in nearly the same time frame (14 s) as the stopped-flow detected 440-nm intermediate (4.3 s), strongly supporting the notion that this intermediate is sepiapterin or a close structural analog.

The C27A variant is clearly inactive in overall turnover (Fig. 5). Nevertheless, we carried out a stopped-flow analysis of this variant with H₂NTP and sepiapterin. We observe no detectable change in the UV-visible spectrum with H₂NTP. However, the UV-visible spectrum of sepiapterin undergoes a red shift upon mixing with the enzyme leading to a 440-nm species, which is indistinguishable from that formed during turnover with H₂NTP (Fig. 8A). The spectral change at 440 nm is fit to a single exponential yielding a rate constant of ~0.4 s⁻¹ (Fig. 8B). Therefore, whereas the variant is inactive with respect to overall turnover, it is capable of binding sepiapterin and catalyzing formation of the transient that we observed with H₂NTP and the catalytically active QueD variants. The slower rate constant for formation of sepiapterin observed in the rapid quench assays with H₂NTP as substrate may reflect the rate of conversion of this transient enolate species to sepiapterin in solution upon being released from the active site after the quench.

The two dyad variants are differentially affected in turnover with H₂NTP. The unique dyad H25A/D54N variant clearly forms the same 440-nm intermediate, but this intermediate is not turned over further (Fig. 7B). Analysis of the data at 440 nm reveals two phases with rate constants of 0.23 and 0.05 s⁻¹. Therefore, the absence of production of CPH₄ with the unique dyad (Fig. 5) indicates loss of ability to convert the 440-nm transient to product and not a loss of catalytic activity. We were unable to identify distinct intermediates by HPLC with this variant, presumably because of enzymatic or non-enzymatic conversion of the unstable sepiapterin-like intermediate to other compounds. By contrast, we did not observe any transient species accumulate when the conserved dyad D70N/H71A var-
mPTPS and QueD both utilize H$_2$NTP as substrate, but despite a strikingly similar overall-fold and active site archite-
cructure, they exhibit differential activity toward sepiapterin and H$_2$NTP. Under the HPLC conditions employed, CPH$_4$, sepiapterin, and H$_2$NTP elute at 3.8, 8.0, and 15.5 min, respectively. Note that in the chromatograms H$_2$NTP is detected at 330 nm, whereas CPH$_4$ is detected at 298 nm. Traces of the reaction, except for the H$_2$NTP control, are shown at this $\lambda_{\text{max}}$ of CPH$_4$ for clarity. The trace for H$_2$NTP as a control was generated without the quench due to the instability of H$_2$NTP in acidic conditions.

The stopped-flow data with H$_2$NTP as substrate support the hypothesis that a sepiapterin-like molecule is an intermediate of the reaction catalyzed by QueD. When the wild-type enzyme is mixed with sepiapterin, the 420-nm peak corresponding to the substrate is lost with concomitant build-up of a species with a $\lambda_{\text{max}}$ at $\sim$340 nm (Fig. 9A). This 340-nm intermediate then disappears along with the spectral features near 420 nm over $\sim$60 s. The fits of the data at 420 and 340 nm yield rate constants of 0.6 and 0.004 s$^{-1}$ corresponding to conversion of sepiapterin to the 340-nm intermediate and its subsequent conversion to product (Fig. 9B). As with turnover of the wild-type enzyme with H$_2$NTP, these rates are of the same order of magnitude as the turnover number of the enzyme with sepiapterin as substrate (0.01 s$^{-1}$). Interestingly, the conserved dyad D70N/H71A variant exhibits nearly overlapping kinetic profiles that are also fit by similar rate constants (0.4 and 0.1 s$^{-1}$, respectively) (Fig. 9B). However, whereas a disappearance of the substrate (at 420 nm) is observed with the H25A/D54N variant, consistent with the fact that this variant turns over with sepiapterin, no intermediates build up at 340 nm during the process (Fig. 9B).

**DISCUSSION**

mPTPS and QueD both utilize H$_2$NTP as substrate, but despite a strikingly similar overall-fold and active site architec-
ture they produce distinctly different major products. The active sites of both proteins are composed of a constellation of residues that are contributed by adjacent subunits in the biological assembly (34). The substrate in each active site is bound to a conserved zinc divalent cation via the C1/His and C2/His hydroxyl groups and the proteins retain similar binding interactions with the substrate (35). Each active site houses an essential cysteine residue whose activation with a conserved Asp/His dyad is proposed to initiate catalysis (29). The most conspicuous structural difference between the two active sites is the presence of an additional His/Asp dyad in the bacterial enzyme on the opposite face of the Cys residue relative to the conserved dyad.

**FIGURE 6.** Steady state kinetic analysis of: A, QueD with H2NTP as substrate; B, QueD with sepiapterin as a substrate; C, D70N/H71A variant of QueD with sepiapterin as a substrate; D, H25A/D54N variant of QueD with sepiapterin as substrate.

**FIGURE 7.** Pre-steady state analysis of the reaction catalyzed by QueD and variants with H2NTP as substrate. A, UV-visible spectra from a single turnover stopped-flow reaction of QueD with H2NTP. There is a clear buildup and disappearance of an intermediate at 440 nm that reaches a maximum absorbance at 4.25 s. The initial trace is in black, whereas the final trace is in red within the corresponding time frame. B, the UV-visible spectra from a single turnover stopped-flow experiment of wild-type and variants of QueD reacted with H2NTP and monitored at 440 nm over time. The trace for the wild-type enzyme is in blue, whereas the traces for the H25A/D54N variant and D70N/H71A variants are in orange and green, respectively. There is formation and disappearance of the 440-nm intermediate described in A for wild-type QueD. Fits to the data obtained from kinetic analysis are overlaid in black.
Therefore, we investigated site-directed variants of QueD to determine the contribution(s) of each dyad to the functional differences between QueD and mPTPS.

Although the biological role of QueD is to convert H$_2$NTP to CPH$_4$, we have shown previously that the enzyme also utilizes sepiapterin as substrate converting it to CPH$_4$ (19). Our working hypothesis was that the reaction likely involves intermediates that resemble this alternate substrate. Indeed time-resolved studies of QueD reveal the build up and disappearance of a kinetically competent intermediate with a $\lambda_{max}$ of 440 nm. The identity of the intermediate is difficult to establish unambiguously; however, rapid quench studies reveal transient formation of sepiapterin in the same time frame as that of the 440-nm transient. The differences between the 440-nm transient observed by stopped-flow and the solution spectrum of sepiapterin may represent tautomerization to the enolate form, which is also observed in the crystal structure of the C27A variant, despite the inability of this variant to catalyze the overall reaction.

We hypothesize that the role of the conserved dyad is to initiate catalysis with H$_2$NTP as substrate, as mutation of these residues leads to $\sim$80-fold decrease in overall turnover with H$_2$NTP without effecting turnover parameters regarding catalysis with sepiapterin. By contrast, mutation of the unique dyad

FIGURE 8. Pre-steady state analysis of sepiapterin binding to the C27A variant of QueD. A, UV-visible spectra from a single turnover stopped-flow reaction show binding of sepiapterin to the C27A variant of QueD. Upon binding to the C27A variant, the spectrum of sepiapterin undergoes a red shift as well as a change in extinction coefficient. The initial trace is in black, whereas the final trace is in red within the corresponding time frame. B, the UV-visible spectra from the same single turnover stopped-flow experiment monitored at 440 nm over time. The spectral change is best fit by a single exponential with a rate constant of 0.4 s$^{-1}$. Fits to the data obtained from the kinetic analysis are overlaid in black.

FIGURE 9. Pre-steady state analysis of the reaction catalyzed by QueD and variants with sepiapterin as substrate. A, UV-visible spectra from a single turnover stopped-flow reaction of QueD with sepiapterin. There is a loss of absorbance at 420 nm corresponding to the loss of sepiapterin substrate with a concomitant buildup of an intermediate at $\sim$340 nm during the first 6 s. Then, both disappear over the remainder of the experiment. The initial trace is in black, whereas the final trace is in red within the corresponding time frame. B, the UV-visible absorbance from a single turnover stopped-flow experiment of QueD and site-directed variants reacted with sepiapterin and monitored at either 420 or 340 nm over time. The trace for wild-type QueD is in blue, whereas the data for the H25A/D54N and D70N/H71A variants are shown in orange and green, respectively. Both QueD and the D70N/H71A variant turnover sepiapterin and build and resolve the 340-nm intermediate in a similar fashion. Conversely, the H25A/D54N variant slowly depletes the initial sepiapterin substrate and does not build an appreciable 340-nm intermediate. Fits to the data obtained from the global kinetic analysis are overlaid in black.
leads to a \( \sim 160 \)-fold drop in activity of the enzyme with sepiapterin and buildup of the 440-nm intermediate when assayed with H\(_2\)NTP, which is not carried through further to CPH\(_4\). As described above, the time-resolved data clearly show that a sepiapterin analog is an intermediate in the reaction. This observation, in the context of the fact that sepiapterin is a substrate, suggests that the conversion of H\(_2\)NTP to CPH\(_4\) is a multistep process. Although we cannot assign specific roles to these residues, the dyads clearly have differential contributions at distinct stages in the catalytic cycle.

A mechanistic paradigm for QueD is shown in Fig. 11. The proposed mechanism has many features in common with those...
proposed for mPTPS. Specifically, both enzymes have an essential active site cysteine residue, a divalent zinc ion, and a conserved dyad within hydrogen bonding distance to the conserved Cys. The first step in the reaction is the binding of the substrate to the active site via hydrogen bonding and coordination of the 1'- and 2'-hydroxyls to the active site zinc divalent cation. In the structure of the C27A variant of QueD complexed with sepiapterin, the hydroxyl oxygen atoms are bound asymmetrically and at distances of ~2 and ~2.3 Å from the zinc ion. In the catalytic cycle the required Cys residue, which is activated by interaction with the conserved Asp70, His71 dyad, abstracts a proton from C2' eliminating the triphosphate and is subsequently tautomerized to a sepiapterin-like intermediate.

The unique dyad, either directly or in concert with the catalytically essential Cys27, activates a water molecule for the next half-reaction, which entails the elimination of the C2'-C3' as acetaldehyde to form the CPH4 product. We favor a role for Cys27 in this half-reaction as well because the C27A variant is completely inactive in conversion of sepiapterin to CPH4. Again, the C27A variant appears to catalyze a change in the conjugation of sepiapterin upon binding leading to a 440-nm species, corroborating the planar geometry for sepiapterin observed in the x-ray crystal structure of the C27A variant. This mechanism accounts for all our observations on QueD as well as the literature on the mechanism of the mPTPS enzyme (38–42).

Recent studies have shown that the mPTPS enzyme can complement production of queuosine in a ΔqueD strain of E. coli (37). This has been somewhat puzzling, as mPTPS had never been shown to catalyze the conversion of H2NTP to CPH4. Our biochemical data with purified mPTPS provide an experimental basis of the complementation. As shown in Fig. 10, in addition to converting H2NTP to PPH4, mPTPS also catalyzes conversion of PPH4 to CPH4 albeit at vastly slower rates. The extent of modification of tRNA to queuosine under normal growth conditions is not known, but the overexpression of the mammalian protein is likely to produce the necessary pool of CPH4 to support the tRNA modification. As is clear in the proposed mechanism, the active site zinc cation should be able to promote a number of tautomerizations and one can readily propose mechanisms by which zinc-bound PPH4 would be converted to CPH4. We note that whereas the unique dyad of QueD appears to be necessary for conversion of sepiapterin to CPH4, it is not essential. The active site of mPTPS appears to have been set up to permit zinc-mediated tautomerizations and small variations in active site environment, such as the introduction of the unique dyad, are sufficient to amplify rates of the reactions that produce CPH4.

The differences in reactivity between the highly similar mPTPS and bacterial QueD homologs provide an interesting case of evolution of new catalytic activities in existing enzyme folds. The conversion of H2NTP to CPH4, albeit at very low levels, is clearly a promiscuous activity as the biosynthesis of queuosine is only carried out in prokaryotes. The concept of enzyme promiscuity leading to novel metabolic pathways was first proposed by Jensen (43) (for a review, see Ref. 44). Following a gene duplication event, enzymes that possess some low-level secondary activity will have a selective advantage toward evolution of a new activity as it will theoretically require fewer advantageous mutations to elevate this ability to a prominent component of a metabolic pathway. An enzyme possessing this low-level catalytic promiscuity will therefore have an evolutionary “head start.” Recently, a model regarding the evolution of new metabolic pathways, the innovation-amplification-divergence model, describes quite exquisitely how these new activities could be borne of previous weak activities in ancestral enzymes (45). QueD and mPTPS may represent naturally occurring examples of the evolutionary models that posit promiscuous activity is required for emergence of new biological function(s).

Acknowledgments—Portions of this research were carried out at the Stanford Synchrotron Radiation Lightsource, a Directorate of SLAC National Accelerator Laboratory and an Office of Science User Facility operated for the United States Department of Energy Office of Science by Stanford University. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and National Institutes of Health, NIGMS Grant P41GM103393.

REFERENCES

1. Colloch, N., Poupon, A., and Moron, J. P. (2000) Sequence and structural features of the T-fold, an original tunnelling building unit. Proteins 39, 142–154.
2. Thöny, B., Auerbach, G., and Blau, N. (2000) Tetrahydrobiopterin biosynthesis, regeneration and functions. Biochem. J. 347, 1–16.
3. Takikawa, S., Curtius, H. C., Redweik, U., Leimbacher, W., and Ghisla, S. (1986) Biosynthesis of tetrahydrobiopterin: Purification and characterization of 6-pyruvoyl-tetrahydropterin synthase from human liver. Eur. J. Biochem. 161, 295–302.
4. Werner, E. R., Blau, N., and Thöny, B. (2011) Tetrahydrobiopterin: biochemistry and pathophysiology. Biochem. J. 438, 397–414.
5. Kaufman, S. (1993) New tetrahydrobiopterin-dependent systems. Annu. Rev. Nutr. 13, 261–286.
6. Smith, G. K. (1987) On the role of sepiapterin reductase in the biosynthesis of tetrahydrobiopterin. Arch. Biochem. Biophys. 255, 254–266.
7. Switchenko, A. C., Primus, J. P., and Brown, G. M. (1984) Intermediates in the enzymic synthesis of tetrahydrobiopterin in Drosophila melanogaster. Biochem. Biophys. Res. Commun. 120, 754–760.
8. Milstien, S., and Kaufman, S. (1989) Immunological studies on the participation of 6-pyruvoyl tetrahydropterin (2'-oxo) reductase, an aldose reductase, in tetrahydrobiopterin biosynthesis. Biochem. Biophys. Res. Commun. 165, 845–850.
9. Park, Y. S., Heizmann, C. W., Wermuth, B., Levine, R. A., Steinerstauch, P., Guzman, J., and Blau, N. (1991) Human carbonyl and aldose reductases: new catalytic functions in tetrahydrobiopterin biosynthesis. Biochem. Biophys. Res. Commun. 175, 738–744.
10. Iino, T., Takikawa, S. I., Yamamoto, T., and Sawada, H. (2000) The enzyme that synthesizes tetrahydrobiopterin from 6-pyruvoyl-tetrahydropterin in the lemon mutant silkworm consists of two carbonyl reductases. Arch. Biochem. Biophys. 373, 442–446.
11. Iino, T., Tabata, M., Takikawa, S., Sawada, H., Shintaku, H., Ishikura, S., and Harra, A. (2003) Tetrahydrobiopterin is synthesized from 6-pyruvoyl-tetrahydropterin by the human aldo-keto reductase AKR1 family members. Arch. Biochem. Biophys. 416, 180–187.
12. Kong, J. S., Kang, J. Y., Kim, H. L., Kwon, O. S., Lee, K. H., and Park, Y. S. (2006) 6-Pyruvoyl-tetrahydropterin synthase orthologs of either a single or dual domain structure are responsible for tetrahydrobiopterin synthesis in bacteria. FEBS Lett. 580, 4900–4904.
13. Choi, Y. K., Hwang, Y. K., and Park, Y. S. (2001) Molecular cloning and disruption of a novel gene encoding UDP-glucose: tetrahydrobiopterin α-glucosyltransferase in the cyanobacterium Synechococcus sp. PCC 7942.
Catalytic Promiscuity in the Tunnel-fold

14. Lee, Y. G., Kim, A. H., Park, M. B., Kim, H. L., Lee, K. H., and Park, Y. S. (2010) Molecular cloning of cyanobacterial pteridine glycosyltransferases that catalyze the transfer of either glucose or xylose to tetrahydrobioppterin. *Appl. Environ. Microbiol.*, 76, 7658–7661

15. Chung, H. J., Kim, Y. A., Kim, Y. J., Hwang, Y. K., and Park, Y. S. (2000) Purification and characterization of UDP-glucose: tetrahydrobioppterin glucosyltransferase from *Synechococcus* sp. PCC 7942. *Biochim. Biophys. Acta* 1524, 183–188

16. Cho, S. H., Na, J. U., Youn, H., Hwang, C. S., Lee, H. C., and Kang, S. O. (1998) Tepidopterin, 1-O-(t-threo-bioterin-2’-yl)-(N-acetylglucosamine from *Chlorobium tepidum*. *Biochim. Biophys. Acta* 1379, 53–60

17. McCarty, R. M., Somogyi, A., Lin, G., Jacobsen, N. E., and Bandarian, V. (2009) The deazapurine biosynthetic pathway revealed: *in vitro* enzymatic synthesis of PreQ(0) from guanosine 5’-triphosphate in four steps. *Biochemistry* 48, 3847–3852

18. McCarty, R. M., and Bandarian, V. (2012) Biosynthesis of pyrrolopyrimidines. *Bioorg. Chem.* 43, 15–25

19. McCarty, R. M., Somogyi, A., and Bandarian, V. (2009) *Escherichia coli* QueD is a 6-carboxy-5,6,7,8-tetrahydropterin synthase. *Biochemistry* 48, 2301–2309

20. Doublé, S. (1997) Preparation of selenenomethyl proteins for phase determination. *Methods Enzymol.* 276, 523–530

21. Pflugrath, J. W. (1999) The finer things in x-ray diffraction data collection. *Acta Crystallogr. D Biol. Crystallogr.* 55, 1718–1725

22. Kabsch, W. (2010) Xds. *Acta Crystallogr. D Biol. Crystallogr.* 66, 125–132

23. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* 40, 658–674

24. Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F., and Vagin, A. A. (2011) REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr. D Biol. Crystallogr.* 67, 355–367

25. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* 66, 486–501

26. Schomaker, Y., and Trueblood, K. N. (1998) Correlation of internal torsional motion with overall molecular motion in crystals. *Acta Crystallogr. B Struct. Sci.* 54, 507–514

27. Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. M., Krissinel, E. B., Leslie, A. G., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterton, E. A., Powell, H. R., Read, R. J., Vagin, A., and Wilson, K. S. (2011) Overview of the CCP4 suite and current developments. *Acta Crystallogr. D Biol. Crystallogr.* 67, 235–242

28. Delano, W. L. (2002) The PyMol Molecular Graphics System. Delano Scientific, San Carlos, CA

29. Ploom, T., Thöny, B., Yim, J., Lee, S., Nar, H., Leimbacher, W., Richardson, J., Huber, R., and Auerbach, G. (1999) Crystallographic and kinetic investigations on the mechanism of 6-pyruvoyltetrahydropterin synthase. *J. Mol. Biol.* 286, 851–860

30. Matsubara, M., Katoh, S., Akino, M., and Kaufman, S. (1966) Sepiapterin reductase. *Biochim. Biophys. Acta* 122, 202–212

31. Johnson, K. A., Simpson, Z. B., and Blom, T. (2009) Global kinetic explorer: a new computer program for dynamic simulation and fitting of kinetic data. *Anal. Biochem.* 387, 20–29

32. Johnson, K. A., Simpson, Z. B., and Blom, T. (2009) FitSpace explorer: an algorithm to evaluate multidimensional parameter space in fitting kinetic data. *Anal. Biochem.* 387, 30–41

33. Reader, J. S., Metzgar, D., Schimmel, P., and de Crécy-Lagard, V. (2004) Identification of four genes necessary for biosynthesis of the modified nucleoside queuosine. *J. Biol. Chem.* 279, 6280–6285

34. Nar, H., Huber, R., Heizmann, C. W., Thöny, B., and Bürgisser, D. (1994) Three-dimensional structure of 6-pyruvoyltetrahydropterin synthase, an enzyme involved in tetrahydropterin biosynthesis. *EMBO J.* 13, 1255–1262

35. Bürgisser, D. M., Thöny, B., Redweik, U., Hess, D., Heizmann, C. W., Huber, R., and Nar, H. (1995) 6-Pyruvoyltetrahydropterin synthase, an enzyme with a novel type of active site involving both zinc binding and an intersubunit catalytic triad motif: site-directed mutagenesis of the proposed active center, characterization of the metal binding site and modelling of substrate binding. *J. Mol. Biol.* 253, 358–369

36. Krissinel, E., and Henrick, K. (2004) Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallogr. D Biol. Crystallogr.* 60, 2256–2268

37. Phillips, G., Grochowski, L. L., Bonnett, S., Xu, H., Bailly, M., Blaby-Haas, C., El Yacoubi, B., Iwata-Reuyl, D., White, R. H., and de Crécy-Lagard, V. (2012) Functional promiscuity of the COG0720 family. *ACS Chem. Biol.* 7, 197–209

38. Deng, H., Callender, R., and Dale, G. E. (2000) A vibrational structure of 7,8-dihydrobioppterin bound to dihydropterin aldolase. *J. Biol. Chem.* 275, 30139–30143

39. Ghisla, S., Kuster, T., Steinerstau, P., Leimbacher, W., Richter, W. J., Raschdorf, F., Dahinden, R., and Curtius, H. C. (1990) 1H NMR and mass spectrometric studies of tetrahydropterins: evidence for the structure of 6-pyruvoyltetrahydropterin, an intermediate in the biosynthesis of tetrahydrobioppterin. *Eur. J. Biochem.* 187, 651–656

40. Le Van, Q., Katzenmeier, G., Schwarzkopf, B., Schmid, C., and Bacher, A. (1988) Biosynthesis of bioppterin: studies on the mechanism of 6-pyruvoyltetrahydropteridene synthase. *Biochem. Biophys. Res. Commun.* 151, 512–517

41. Maharaj, G., Selinsky, B. S., Appleman, J. R., Perlm, M., London, R. E., and Blakely, R. L. (1990) Dissociation constants for dihydrofolic acid and dihydrobioppterin and implications for mechanistic models for dihydrofolate reductase. *Biochemistry* 29, 4554–4560

42. Bracher, A., Eisenreich, W., Schramek, N., Ritz, H., Götzte, E., Herrmann, A., Gültlich, M., and Bacher, A. (1998) Biosynthesis of pteridines: NMR studies on the reaction mechanisms of GTP cyclohydrolase I, pyruvoyltetrahydropterin synthase, and sepiapterin reductase. *J. Biol. Chem.* 273, 28132–28141

43. Jensen, R. A. (1976) Enzyme recruitment in evolution of new function. *Annu. Rev. Microbiol.* 30, 409–425

44. O’Brien, P. J., and Herschlag, D. (1999) Catalytic promiscuity and the evolution of new enzymatic activities. *Chembio* 6, R91–R105

45. Näsvall, J., Sun, L., Roth, J. R., and Andersson, D. I. (2012) Real-time evolution of new genes by innovation, amplification, and divergence. *Science* 338, 384–387

46. Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr. D Biol. Crystallogr.* 66, 12–21