Crystal Structures of Type I Dehydroquinate Dehydratase in Complex with Quinate and Shikimate Suggest a Novel Mechanism of Schiff Base Formation

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ABSTRACT: A component of the shikimate biosynthetic pathway, dehydroquinate dehydratase (DHQD) catalyzes the dehydration of 3-dehydroquinate (DHQ) to 3-dehydroshikimate. In the type I DHQD reaction mechanism a lysine forms a Schiff base intermediate with DHQ. The Schiff base acts as an electron sink to facilitate the catalytic dehydration. To address the mechanism of Schiff base formation, we determined structures of the Salmonella enterica wild-type DHQD in complex with the substrate analogue quinate and the product analogue shikimate. In addition, we determined the structure of the K170M mutant (Lys170 being the Schiff base forming residue) in complex with quinate. Combined with nuclear magnetic resonance and isothermal titration calorimetry data that revealed altered binding of the analogue to the K170M mutant, these structures suggest a model of Schiff base formation characterized by the dynamic interplay of opposing forces acting on either side of the substrate. On the side distant from the substrate 3-carbonyl group, closure of the enzyme’s β8–α8 loop is proposed to guide DHQ into the proximity of the Schiff base-forming Lys170. On the 3-carbonyl side of the substrate, Lys170 sterically alters the position of DHQ’s reactive ketone, aligning it at an angle conducive for nucleophilic attack. This study of a type I DHQD reveals the interplay between the enzyme and substrate required for the correct orientation of a functional group constrained within a cyclic substrate.
that is predominantly confined to the substrate, specifically to the bonds adjoining the substrate carbonyl, with little change in the conformation of active site residues. A comparison to related enzymes suggests that this form of substrate conformational change likely represents a common feature of Schiff base formation in enzymes acting on linear ketone substrates.9

However, the Schiff base-forming mechanism observed in transaldolase cannot be extended to type I DHQDs. Unlike transaldolase and the majority of other characterized Schiff base-forming enzymes, the DHQD substrate carbonyl is a carbocyclic ring constituent. This unique property constrains the allowed dihedral change in the carbonyl adjoining bonds, the defining feature associated with transaldolase Schiff base formation, and means that Schiff base formation must qualitatively differ from the process for enzymes that act upon linear substrates.

To understand Schiff base formation in type I DHQDs, we previously attempted to capture the DHQD Michaelis complex. Our strategy was to analyze the structure of the inactive Schiff base-forming lysine to methionine mutant (K170M) cocrystallized with DHQ.10 The resulting complex revealed that DHQ base-forming lysine to methionine mutant (K170M) cocrystallized with DHQ.10 The resulting complex revealed that DHQ base formation occurs between the ε-amino group of Lys170 and C3 of the substrate. The atoms that are a part of Schiff base formation are colored red.

Quinate and shikimate are one biosynthetic step removed from the DHQD substrate and product, respectively. Differing from DHQ and DHS only in containing a hydroxyl rather than a carbonyl at the reactive 3-position (Figure 1), quinate and shikimate should bind noncovalently to DHQD. With the objective of clarifying the mechanism of Schiff base formation, we characterized the binding of quinate and shikimate to the wild-type and K170M mutant DHQD using ITC, NMR, and X-ray crystallography. The data presented here reveal three distinct binding modes of the reaction analogues and demonstrate enhanced affinity in the K170M mutant. Incorporating these findings with previous data, we propose that the dynamic interplay of residues on either side of the active site acts to correctly position the substrate for nucleophilic attack.

### EXPERIMENTAL PROCEDURES

**Protein Expression and Purification.** As previously described, wild-type and K170M constructs were expressed in *Escherichia coli* strain BL21(DE3) in the pMCSG7 vector.10–12 After being inoculated with an overnight culture, cells were grown for 4 h at 37 °C. Then, the temperature was reduced to 25 °C and protein overexpression induced by the addition of isopropyl 1-thio-α-D-galactopyranoside to a final concentration of 0.5 mM. Cells were grown overnight before being harvested by centrifugation, resuspended in a buffer containing 10 mM Tris (pH 8.3), 500 mM NaCl, 10% glycerol, and 5 mM β-mercaptoethanol, and lysed by sonication. Protein was purified by Ni-NTA affinity chromatography and step-eluted with 0.5 M imidazole. To remove the expression tag, purified DHQD was incubated overnight at 4 °C with hexahistidine-tagged TEV protease and repurified by Ni-NTA chromatography.

**Protein Crystallization and Collection of X-ray Data.** Sitting drop crystallization experiments were performed at room temperature using a 1:1 ratio of DHQD (7.5 mg/mL) to reservoir. Wild-type crystals were incubated for ~15 min in 5 M sodium quinate or 2 M sodium shikimate solutions before being frozen. The K170M mutant crystal was soaked for ~15

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Figure 1. Type I DHQD reaction intermediates and analogues. The DHQD-catalyzed reaction is depicted centrally. Quinate and shikimate, boxed on the sides, biosynthetically relate to DHQ and DHS, respectively. Schiff base formation occurs between the ε-amino group of Lys170 and C3 of the substrate. The atoms that are a part of Schiff base formation are colored red.
min in a mother liquor, consisting of reservoir solution supplemented with 5 mM quinate. The wild-type crystals were harvested from a condition containing 0.01 M nickel chloride, 0.1 M Tris (pH 8.5), and 20% poly(ethylene glycol) monomethyl ether 2000. The K170M mutant crystal was harvested from a condition containing 0.01 M nickel chloride, 0.1 M Tris (pH 8.5), and 25% poly(ethylene glycol) 1500. Crystals were frozen in liquid nitrogen, and di-

| Table 1. Data Collection and Refinement Statisticsa |
|---------------------------------|-------|-------|-------|
| PDB entry | WT—quinate | WT—shikimate | K170M—quinate |
| space group | P2₁ | P2₁ | P2₁,2₁,2₁ |
| unit cell dimensions | a = 48.67 Å, b = 74.43 Å, c = 63.13 Å, α = 90°, β = 100.57°, γ = 90° | a = 48.62 Å, b = 74.97 Å, c = 63.04 Å, α = 90°, β = 100.49°, γ = 90° | a = 36.91 Å, b = 72.80 Å, c = 170.91 Å, α = 90°, β = 90°, γ = 90° |
| resolution range (Å) | 30.00−1.78 (1.81−1.78) | 30.00−1.50 (1.53−1.50) | 28.51−1.80 (1.85−1.80) |
| completeness (%) | 97.2 (99.3) | 96.8 (95.0) | 99.2 (95.3) |
| redundancy | 3.0 (2.3) | 3.9 (3.9) | 5.3 (4.2) |
| (I/σ(I)) | 17.5 (2.4) | 25.5 (3.8) | 16.4 (2.8) |
| Rmerge (%) | 5.6 (31.4) | 4.5 (36.7) | 11.0 (55.0) |
| protein | 3619 | 3501 | 3583 |
| water | 239 | 420 | 435 |
| shikimate or quinate | 26 | 24 | 26 |
| average β factor (Å²) | 33.8 | 20.5 | 18.9 |
| protein | 40.3 | 30.8 | 27.8 |
| water | 37.6 | 19.8 | 16.2 |
| bond lengths (Å) | 0.008 | 0.009 | 0.007 |
| bond angles (deg) | 1.35 | 1.42 | 1.26 |
| Ramachandran analysis (%) | favored regions | 98.0 | 98.7 | 97.8 |
| allowed regions | 100 | 100 | 100 |
| disallowed regions | 0 | 0 | 0 |

aData for the highest-resolution shell are given in parentheses. bDefinition of Rwork and Rfree: $R = \sum_{hkl} |F_{\text{calc}}| - |F_{\text{obs}}| / \sum_{hkl} |F_{\text{obs}}|$, where $hkl$ are the reflection indices used in the refinement for Rwork and the 5% not used in the refinement for Rfree. $F_{\text{calc}}$ and $F_{\text{obs}}$ are structure factors deduced from measured intensities and calculated from the model, respectively.

Crystal Structure Determination and Refinement. Data were processed using HKL-3000 for indexing, integration, and scaling. Structures were determined by molecular replacement in Phaser, using the unliganded DHQD structure [Protein Data Bank (PDB) entry 3L2I] as a search model. Structures were iteratively refined with Refmac after working models were displayed in Coot and manually adjusted on the basis of electron density maps. All structure figures were prepared using PyMOL Molecular Graphics System, version 1.3 (Schrödinger, LLC).

Isothermal Titration Calorimetry (ITC). ITC experiments were performed using the MicroCal ITC200 instrument (GE Healthcare) with the jacket temperature set at 25 °C. Wild-type and K170M DHQD samples were prepared by dialysis into a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, and 5 mM 2-mercaptoethanol. Titrations of 10 mM quinate or shikimate dissolved in the same buffer were performed with either buffer or 200 μM wild-type or K170M DHQD. In each case, injections from a 40 μL syringe rotating at 500 rpm were spaced at 2 min intervals. An initial 0.2 μL injection, which was subsequently removed during data analysis, was followed by 17 injections of 2.0 μL each. For K170M DHQD, binding parameters ($K_d$, $ΔH$, and $−TΔS$) were obtained by fitting the resultant data to a single-site binding model using Origin 7 after subtracting heats of dilution into buffer. The $K_d$ was calculated as the inverse of the $K_d$. Nuclear Magnetic Resonance (NMR). NMR experiments were performed on a Bruker 900 MHz AVANCE spectrometer equipped with a cryogenic triple-resonance probe. Experimental conditions were 10 μM DHQD and 500 μM quinate or shikimate in 20 mM PO₄ (pH 7.4) and 100 mM NaCl in 90% 1H₂O and 10% 2H₂O at 25 °C in 3 mm NMR tubes. The WaterLOGSY experiments were performed as previously described. Water was selectively saturated using a 2 ms square-shaped pulse with a mixing time of 2 s and a relaxation
delay of 2.5 s. Spectra were processed by NMRPipe with a 5 Hz line broadening function and analyzed by NMRDraw.18

RESULTS

Wild-Type DHQD—Quinate and Shikimate Complexes. Wild-type DHQD was cocryrstallized with 0.5 M quinate or shikimate. However, no discernible electron density for the compounds was observed at the DHQD active site. Consequently, we resorted to soaking crystals in 5 M quinate or 2 M shikimate prior to data collection. Employing this soaking regime, structures determined to 1.78 and 1.50 Å resolution (Table 1) revealed clear active site electron density for quinate and shikimate, respectively.

As in previously described S. enterica DHQD structures, the crystallographic asymmetric units of the shikimate and quinate complexes each contain the physiological homodimer (Figure 2a). Interestingly, in both the quinate and shikimate complexes, the two protomers in the asymmetric unit, here termed protomer A and protomer B, exhibit disparate conformational behaviors.

The first difference between protomers A and B concerns the conformation adopted by the Val228-to-Gln236 loop that connects β-strand 8 to α-helix 8 (β8–α8 loop). The β8–α8 loop has previously been observed open and partially disordered in the unliganded state but closed over the active site and hydrogen bonding with DHQ in the K170M mutant and Schiff base reaction intermediate structures in the wild-type enzyme.10,11 In protomer A of both the quinate and shikimate complex structures, the β8–α8 loop adopts the closed conformational state (colored yellow in Figure 2b). By contrast, in protomer B of these complexes the loop adopts the open conformational state (colored orange in Figure 2b).

The second difference between protomers A and B concerns the mode of ligand binding. Correlating with the difference in β8–α8 loop conformation, quinate and shikimate exhibit distinct binding modes within the two protomers (Figure 2b).

Binding of Quinate and Shikimate to Protomer A of Wild-Type DHQD. Quinate (Figure 3a) and shikimate (Figure 3b) assume similar binding modes within protomer A of their respective complexes. With the closed β8–α8 loop, quinate and quinate are positioned within the enclosed active site like the covalent reaction intermediate observed in a previous wild-type complex with DHQ and noncovalently bound DHQ in the K170M–DHQ complex (Figure 3c).10

Establishing essentially the same interactions that have been observed for DHQ in the K170M–DHQ complex, the presence of Lys170 represents the principal feature differentiating the wild-type quinate and shikimate complex structures from the mutant complex. Within both quinate and shikimate structures, the Lys170 side chain has retracted, with its Nε atom displaced 1.5−1.9 Å from its extended unliganded state position (Figure 3d). This retraction of Lys170 is rationalized by a superposition with the unliganded structure, which reveals that the unliganded state Lys170 conformation would otherwise sterically clash with the quinate or shikimate 3-hydroxyl (dashed red line in Figure 3d). This indicates that the change in the Lys170 conformation, from fully extended to retracted, is necessitated by the proximity of the bound ligand.

Binding of Quinate and Shikimate to Protomer B of Wild-Type DHQD. The ligand electron density is not as well-resolved within the respective forms of protomer B but is nonetheless of sufficient quality to allow quinate (not shown) and shikimate (Figure 4a) to be confidently modeled with partial occupancy. Unlike the similar mode of quinate and shikimate binding exhibited in protomer A, dissimilar binding modes are exhibited between protomer B of the quinate and shikimate complexes (not shown). This distinction persists despite nearly identical open β8–α8 loop conformational states in the two protomers.

In protomer B of the quinate complex, the carbocyclic quinate ring is flipped relative to its protomer A conformation (Figure 2b). As a result of this flipped orientation, the carbocyclic moiety interacts with Lys170 and the C3 atom that corresponds to the reactive atom in DHQ is located ~7.5 Å from the Lys170 Nε atom (not shown). This mode of binding is similar to the mode of DHS binding observed in the previously described E86A mutant DHS complex.12 As the functional implications of this presumably nonproductive binding mode have been thoroughly discussed in a recent publication12 and do not pertain to the mechanism of Schiff base formation, we will leave discussion of quinate complex protomer B here.

In contrast to the quinate protomer B, shikimate in protomer B (light gray in Figure 4b) retains its protomer A orientation (dark gray in Figure 4b). However, the carbocyclic shikimate ring has shifted from its protomer A conformation (arrow 1 in Figure 4b). Like in protomer A, the shikimate 1-carboxyl group forms a bidentate salt bridge with Arg213. The difference in the protomer B shikimate position is related to the distinct conformation of Arg213 (arrow 2 in Figure 4b). Arg213 has previously been observed to undergo a coordinated conformational change upon β8–α8 loop closure.11 The Arg213 guanidinium group points away from Lys170 in its open loop.
conformation but swivels to point toward Lys170 following loop closure (Figure 4b). Tracking with the open loop conformation of Arg213, shikimate is shifted away from Lys170 and its protomer A position in protomer B (Figure 4b).

**Binding of Quinate to the K170M Variant.** To obtain the previously described K170M–DHQ complex. To determine if this difference in required ligand concentration resulted from the K170M mutation, diffraction data were collected from a preformed unliganded K170M crystal soaked in 5 mM quinate.

Indeed, a 1.80 Å crystal structure (Table 1) revealed that, in contrast to wild-type DHQD, a low millimolar quinate concentration was sufficient to generate unambiguous ligand

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Figure 3. Forms of protomer A of the quinate and shikimate complexes. (a) Stick model of the quinate complex active site (protomer A). (b) Stick model of the shikimate complex active site (protomer A). The $F_o - F_c$ map was calculated with ligand omitted and is contoured at 3σ. (c) Superposition of forms of protomer A of quinate (blue) and shikimate (gray) to the K170M–DHQ complex (pink, PDB entry 3NNT) illustrates the similar mode of ligand binding and closed loop conformational states. (d) Superposition of forms of protomer A of quinate (rmsd = 0.31 Å over 196 Cα atoms) and shikimate (rmsd = 0.20 Å over 196 Cα atoms) to the unliganded structure (yellow, PDB entry 3L2I). The retraction of Lys170 from its unliganded state conformation prevents a clash (dashed red line) with quinate or shikimate.

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Figure 4. Protomer B of the shikimate complex. (a) Stick model of the protomer B active site of the quinate complex. The $F_o - F_c$ electron density map was calculated and contoured as described in the legend of Figure 3. (b) Superposition of protomers A (light gray) and B (dark gray) of the shikimate complex. The difference in shikimate position (arrow 1) tracks with the conformational change of Arg213 (arrow 2).
Figure 5. K170M–quinate complex. (a) Stick model of the K170M mutant–quinate complex active site (protomer A). Density map calculated and contoured as in Figure 2. (b) Overlay of the K170M mutant–quinate (green) and wild-type–quinate (blue) complexes.

**DISCUSSION**

**Significance of Quinate and Shikimate Binding Modes Observed in Protomer A.** While quinate and shikimate complexes were pursued with the objective of clarifying the noncovalent basis of substrate and product binding, it may be more instructive to view protomer A binding (Figure 3) in the context of analyzing the carbinolamine intermediates. In the forward direction of the reaction, the carbinolamine #1 intermediate precedes Schiff base formation, whereas the carbinolamine #2 intermediate follows Schiff base hydrolysis (Figure 1). The quinate and shikimate 3-hydroxyls contain the sp3 geometry and stereochemistry that defines these intermediate states (Figure 1). Quinate, in particular, is positioned very much like the Schiff base-bound dehydroquininate, with C3 of quinate shifted 0.3 Å from C3 of the Lys170-bound dehydroquininate. It thus stands to reason that quinate should form interactions characteristic of the carbinolamine intermediates.

Interestingly, the quinate 3-hydroxyl interacts with His143 and Arg82 (Figure 3a). Recent computational studies of Schiff base formation and hydrolysis predicted such an interaction, as well as a key role for these residues in coordinating the attacking water in Schiff base hydrolysis.19 Thus, this experimentally observed interaction combined with recent computational predictions makes an emerging case for an important role for Arg82 in DHQD catalysis.

**A Proposal for the Mechanism of Schiff Base Formation in Type I DHQDs.** The first insight into Schiff base formation in type I DHQDs came from the structure of the K170M mutant in complex with DHQ.10 In that structure, DHQ assumes an orientation similar to that of the Schiff base-bound intermediate states within the wild-type enzyme. Notably, such a DHQ orientation vis-à-vis Lys170 is inconsistent with the required nucleophilic approach trajectory for Schiff base formation. This observation combined with the enhanced affinity of the K170M variant for substrate and product reaction analogues makes a compelling case that the K170M–DHQ complex does not accurately represent the Michaelis state.

We are aware that the high ligand concentration necessary to obtain the shikimate and quinate complexes raises questions about the functional relevance of the binding modes observed in these structures. Nevertheless, the binding mode observed in protomer B of the shikimate structure is intriguing. Previous structural and kinetic data make it clear that the β8–α8 loop closure and the accompanying Arg213 conformational change fundamentally relate to substrate binding.11 In protomer A of the wild-type DHQD complex with shikimate, the β8–α8 loop is open and Arg213 adopts its unliganded conformation (Figure 7a,b). Because Arg213 engages the shikimate carboxyl group in a fashion similar to that in closed β8–α8 loop complexes, it is easy to envision how loop closure and the accompanying Arg213 conformational change would cause the substrate to shift toward Lys170 (Figure 4b). Importantly, as these conformational changes (of the β8–α8 loop and Arg213) push the substrate deeper into the active site, some rearrangement must be necessary to avoid a steric clash between Lys170 and substrate carbonyl (Figure 7c,d) and differentiate this state from the one observed in the K170M–DHQ complex structure (Figure 7, box).

In protomer A of the quinate and shikimate complexes, such a steric clash is avoided by the retraction of the Lys170 side chain (Figure 3d). However, the high concentration of ligand...
Figure 6. Binding of quinate and shikimate to wild-type and K170M DHQD. (a) Data from ITC experiments in which 10 mM quinate was injected into 200 μM wild-type (red) or K170M (black) DHQD are shown. The top panel shows the direct heat generated per injection, while the bottom panel shows the integrated and normalized heat data with a curve fit to the K170M DHQD data. (b) Data from ITC experiments in which 10 mM shikimate was injected into 200 μM wild-type (red) or K170M (black) DHQD. The experimental setup and presentation are the same as in panel a. (c) WaterLOGSY NMR spectrum of quinate in the presence of wild-type DHQD (top) or K170M DHQD (bottom). Red arrows denote the resonances corresponding to those of quinate. The negative sign of the quinate peaks in the wild-type spectrum indicates little or no binding. The positive sign of the quinate peaks in the K170M spectrum indicates binding. (d) WaterLOGSY NMR spectrum of shikimate in the presence of wild-type DHQD (top) or K170M DHQD (bottom). Red arrows denote the resonances corresponding to those of shikimate. The negative sign of the shikimate peaks in the wild-type spectrum indicates little or no binding. The positive sign of the shikimate peaks in the K170M spectrum indicates binding.
The notion that the reactive portion of the DHQ ring might pivot away from Lys170 falls in line with recent docking/molecular dynamic experiments, which, performed using the closed β8–α8 loop conformational state as a starting model, identified a stable binding mode for DHQ similar to its position in the K170M structure but differing in that the 3-carbonyl has rotated away from Lys170. When this model was subjected to quantum chemical calculations, small movements of the substrate and/or Lys170 side chain positioned the reactive components at a reasonable nucleophilic approach angle. Taken together with the computational findings, the new structural data suggest that, following the initial binding event, β8–α8 loop closure helps guide the substrate into the active site. Here, the DHQ 3-carbonyl pivots away from the K170M binding mode to prevent a steric clash and to allow nucleophilic approach to follow a favorable trajectory (Figure 7).

In summary, the most striking feature of this model of Schiff base formation concerns the interplay of residues on either side of the substrate in promoting Schiff base formation. On the external side of the active site cavity, the β8–α8 loop critically interacts with the substrate, effectively pushing it into sufficient proximity of Lys170 for covalent bond formation to occur. The direct contribution of the β8–α8 loop to the catalytic rate is confirmed by studies of loop mutant Q236A, which reveal a 30-fold decrease in $k_{cat}$. On the other side of the substrate, as evidenced by structural revelations demonstrating perturbed substrate binding to the K170M mutant, Lys170 sterically orients the carbonyl, presumably positioning it at a favorable nucleophilic approach angle. In this way, the β8–α8 loop and Lys170 act in concert, pressing the substrate from opposing sides, drawing it in and positioning it for nucleophilic attack. Importantly, Lys170 causes DHQ to rotate away from the Schiff base-forming residue, exposing it to a favorable nucleophilic approach by the lysine amino group, a reaction that results in Schiff base formation. These results reveal the various substrate binding modes that must occur during the catalytic cycle, and it is the mimicking of these binding modes that can be used by inhibitors to prevent the dehydration reaction.

Figure 7. Proposed mechanism of Schiff base formation. (a) DHQD adopts an open loop conformation in the absence of ligand. (b) DHQ binds to the open loop conformational state, as observed in protomer B of the shikimate complex. (c) Closure of the loop requires Arg213 and DHQ to shift deeper into the active site. The presence of Lys170 in the wild-type enzyme sterically disallows the K170M binding mode (black box), forcing the DHQ 3-carbonyl to pivot away from Lys170 and generating a prenucleophilic attack state like that described by Pan et al. (d) The reactive carbonyl is positioned at a stereoelectronically reasonable nucleophilic approach angle in the prenucleophilic attack state that allows reaction with Lys170 to generate the Schiff base intermediate.

necessary to generate these structures as compared to the lower concentration required for the K170M mutant suggests that this retraction is energetically unfavorable. An alternative mechanism by which the steric clash could be resolved would involve the substrate, or a portion of its carbocyclic ring, pivoting away from Lys170. In addition to preventing a clash, such a rearrangement has the potential advantage of exposing the carbonyl to an energetically favorable nucleophilic approach angle and thus promoting Schiff base formation.

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**ABBREVIATIONS**

DHQ, dehydroquinate; DHQD, dehydroquininate dehydratase; DHS, dehydroshikimate; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance.

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