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Crystal Structures and Kinetics of Monofunctional Proline Dehydrogenase Provide Insight into Substrate Recognition and Conformational Changes Associated With Flavin Reduction and Product Release

Min Luo[†], Benjamin W. Arentson[#], Dhiraj Srivastava[†], Donald F. Becker[#], and John J. Tanner^{†,‡,*}

[†]Department of Chemistry, University of Missouri-Columbia, Columbia, MO 65211, USA

[#]Department of Biochemistry, University of Nebraska-Lincoln, Lincoln, NE 68588, USA

[‡]Department of Biochemistry, University of Missouri-Columbia, Columbia, MO 65211, USA

Abstract

Proline dehydrogenase catalyzes the FAD-dependent oxidation of proline to Δ^1 -pyrroline-5-carboxylate, which is the first step of proline catabolism. Here, we report the structures of proline dehydrogenase from *Deinococcus radiodurans* in the oxidized state complexed with the proline analog L-tetrahydrofuroic acid and in the reduced state with the proline site vacant. The analog binds against the *si* face of the FAD isoalloxazine and is protected from bulk solvent by the $\alpha 8$ helix and the $\beta 1$ - $\alpha 1$ loop. The FAD ribityl chain adopts two conformations in the E-S complex, which is unprecedented for flavoenzymes. One of the conformations is novel for the PRODH superfamily and may contribute to the low substrate affinity of *Deinococcus* PRODH. Reduction of the crystalline enzyme-inhibitor complex causes profound structural changes, including 20° butterfly bending of the isoalloxazine, crankshaft rotation of the ribityl, shifting of $\alpha 8$ by 1.7 Å, reconfiguration of the $\beta 1$ - $\alpha 1$ loop, and rupture of the Arg291-Glu64 ion pair. These changes dramatically open the active site to facilitate product release and allow electron acceptors access to the reduced flavin. The structures suggest that the ion pair, which is conserved in the PRODH superfamily, functions as the active site gate. Mutagenesis of Glu64 to Ala decreases catalytic efficiency 27-fold, which demonstrates the importance of the gate. Mutation of Gly63 decreases efficiency 140-fold, which suggests that flexibility of the $\beta 1$ - $\alpha 1$ loop is essential for optimal catalysis. The large conformational changes that are required to form the E-S complex suggest that conformational selection plays a role in substrate recognition.

Proline dehydrogenase (PRODH) catalyzes the first reaction of proline catabolism (Figure 1A).¹ PRODH is an FAD-dependent enzyme that catalyzes the oxidation of L-proline to Δ^1 -pyrroline-5-carboxylate (P5C). The electrons stored in the reduced flavin are subsequently transferred to the electron transport chain for eventual ATP production. P5C forms an equilibrium with its hydrolysis product glutamate γ -semialdehyde (GSA), which is oxidized

*Corresponding author: Department of Chemistry, University of Missouri-Columbia, Columbia, MO 65211. Phone: (573) 884-1280. Fax: 573-882-2754. tannerjj@missouri.edu.

Accession Codes

Atomic coordinates and structure factors have been deposited in the Protein Data Bank under the following accession codes: 4H6Q, DrPRODH_O-THFA; 4H6R, DrPRODH_F.

Supporting Information

Electron density evidence for dual FAD conformations in the DrPRODH_O-THFA complex (Figure S1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

to glutamate by the second enzyme of proline catabolism, NAD⁺-dependent P5C dehydrogenase (P5CDH). PRODH and P5CDH are distinct enzymes in eukaryotes and Gram-positive bacteria, whereas the two enzymatic activities are combined into a single polypeptide chain, known as proline utilization A (PutA), in Gram-negative bacteria.^{2, 3}

PRODH is of interest because of its roles in apoptosis, cancer, and schizophrenia. In eukaryotes, PRODH and P5CDH are localized to the inner mitochondrial membrane and mitochondrial matrix respectively, and proline catabolism is important for establishing the mitochondrial redox status.^{4, 5} Seminal work from Phang's group has established that human PRODH (aka proline oxidase or POX) is a tumor suppressor protein.^{5–13} POX expression is induced by the tumor suppressor p53, and POX itself activates intrinsic and extrinsic apoptotic pathways.⁸ Crucial to the role of POX as a tumor suppressor is its ability to generate superoxide.^{5, 8, 14} Also, certain mutations in the gene encoding POX cause type I hyperprolinemia,¹⁵ which is a risk factor for schizophrenia.¹⁶

Crystal structures of PRODHs suggest that conformational changes are essential for catalysis, but the details of these conformational changes have not been elucidated. We previously determined structures of the PRODH domain of *Escherichia coli* PutA (PutA86-669) complexed with proline and proline analogs,^{17, 18, 19} as well as a structure of *Bradyrhizobium japonicum* PutA with a sulfate ion bound in the proline site.²⁰ In all of those structures, proline (or the analog) is completely buried, implying that the active site opens to allow product release and closes again in response to substrate binding. Since the structure of the empty PutA PRODH active site is unknown, it has not been possible to deduce these conformational changes for PutA. We also reported a structure of the monofunctional PRODH from *Thermus thermophilus* (TtPRODH) in a substrate-free conformation.²¹ However, the corresponding structure of the closed, substrate-bound active site is unknown. Thus, our understanding of how conformational changes facilitate catalysis has been limited by difficulties in crystallizing the same PRODH in both the open (substrate-free) and closed (substrate-bound) conformations. Although some information has been gained by comparing the structures of PutA669-proline and ligand-free TtPRODH, this comparison is problematic because it is unknown whether PutAs and monofunctional PRODHs bind proline similarly. Another factor that complicates such a comparison is that the PRODH domain of PutA makes tertiary structural contacts that are absent in monofunctional PRODHs, and thus is unlikely that PutA PRODH domains and monofunctional PRODHs exhibit similar degrees of flexibility.

We therefore surveyed several monofunctional PRODHs in search of one that crystallizes in the presence of the proline analog L-tetrahydrofuroic acid (THFA). This survey uncovered *Deinococcus radiodurans* R1 PRODH (DrPRODH) as a suitable enzyme for determining the structural changes that occur in monofunctional PRODHs during the reductive half-reaction (Figure 1B). Herein we report high resolution crystal structures and kinetic studies of DrPRODH, which provide new insight into substrate recognition by monofunctional PRODHs and the conformational changes that accompany flavin reduction and product release.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

The gene encoding DrPRODH (NCBI RefSeq code NP_294538.1, 310 residues) in the vector pMH1F was obtained from the Joint Center for Structural Genomics. The gene was amplified by PCR and ligated into plasmid pKA8H using *NdeI* and *BamHI* sites. The expressed protein includes an N-terminal His₈ tag and Tobacco Etch Virus protease (TEVP) site. The G63A mutant of DrPRODH was created with the QuickChange II site-directed

mutagenesis kit (Agilent) using a forward primer of 5'-GAACCTTGACCTGCTCGCCGAATTTATCGACAGCCCG-3', and a reverse primer of 5'-CGGGCTGTCGATAAATTCGCGAGCAGGTCAAGGTTC-3'. The E64A mutant of DrPROD_H was prepared similarly using a forward primer of 5'-CTTGACCTGCTCGGCGCCTTTATCGACAGCCCGGCC-3', and a reverse primer of 5'-GGCCGGGCTGTCGATAAAGGCGCCGAGCAGGTCAAG-3'. The mutations were confirmed using DNA sequencing.

DrPROD_H and mutants G63A and E64A were expressed in BL21(DE3)pLysS cells as follows. Starter cultures of 10 mL were grown in LB media overnight and used to inoculate 3 L of LB broth. After the culture reached an optical density of OD₆₀₀ = 0.8, 0.5 mM IPTG was added to induce protein expression for 5 hours at 22 °C. The cells were collected by centrifugation, resuspended in 50 mM Tris, 100 mM NaCl, 10 mM imidazole, and 5% glycerol at pH 7.5, and frozen at -80 °C.

DrPROD_H and mutants G63A and E64A were purified as follows. The frozen cells were thawed at 4 °C in the presence of protease inhibitors (0.1 mM TPCK, 0.05 mM AEBSF, 0.1 μM Pepstatin, 0.01 mM Leupeptin, 5 μM E-64) and broken using sonication. The mixture was centrifuged at 16500 rpm in an SS34 rotor for 1 hour at 4 °C, filtered through a 0.45 μm filter (Millipore) and loaded to HisTrap HP column (5 mL) that had been equilibrated in 50 mM Tris, 300 mM NaCl, 10 mM imidazole, and 5% glycerol at pH 7.5. Washing steps with the loading buffer supplemented with 10 mM imidazole followed by 30 mM imidazole were performed. The protein was eluted with 300 mM imidazole. The histidine tag was removed by incubating the protein with 0.2 mg/mL TEVP for 1 hour at 28 °C followed by dialysis at 4 °C against 50 mM Tris, 50 mM NaCl, and 5% glycerol at pH 7.5. The mixture was applied to the HisTrap HP column to separate the cleaved protein, which appeared in the flow-through, from the tag and TEVP. The cleaved protein was dialyzed overnight in the dark at 4 °C into 50 mM Tris, 0.5 mM EDTA, 0.5 mM DTT, and 5% glycerol at pH 7.8 in preparation for anion exchange chromatography (HiTrap Q). The sample was loaded onto the column using a buffer of 50 mM Tris and 5% glycerol at pH 7.8, and a linear NaCl gradient was applied. DrPROD_H eluted in the range 360 – 420 mM NaCl. The protein concentration was estimated using the bicinchoninic acid method (Pierce kit) with bovine serum albumin as the standard. The flavin contents of the purified mutants G63A and E64A were similar to that of DrPROD_H.

Crystallization and Crystal Soaking Experiments

All crystallization experiments were performed at 22 °C using the sitting-drop method of vapor diffusion with drops formed by mixing equal volumes of the reservoir and the protein stock solutions. The latter solution consisted of 2.8 mg/mL DrPROD_H in 50 mM Tris, 50 mM NaCl, 0.5 mM THP, 0.5 mM EDTA, 5% glycerol, and 400 mM THFA at pH 7.5. Initial conditions were identified using commercially available crystal screens (Hampton Research). Optimized crystals were grown using a reservoir solution of 0.2 M MgCl₂, 25% (w/v) PEG 3350, and 0.1 M Bis-tris at pH 5.8. The crystals were cryoprotected with the reservoir solution supplemented with 25% (v/v) PEG 200, picked up with Hampton loops, and flash-cooled in liquid nitrogen. The space group is $P2_12_12_1$ with unit cell dimensions of $a = 44 \text{ \AA}$, $b = 95 \text{ \AA}$, and $c = 136 \text{ \AA}$. The asymmetric unit includes two enzyme-THFA complexes and 40% solvent.

Crystals of DrPROD_H with the FAD reduced (DrPROD_H_r) were obtained by soaking the aforementioned crystals for ~17 minutes in 50 mM sodium dithionite, 0.2 M MgCl₂, 25% PEG 3350, 0.1 M Bis-Tris pH 5.5, and 25% PEG 200. The crystal color changed from deep yellow to colorless during soaking, which is consistent with reduction of the FAD. The crystals were flash cooled in liquid nitrogen to trap the reduced enzyme conformation.

X-ray Diffraction Data Collection, Processing, and Refinement

Diffraction data were collected on beamlines 4.2.2 of the Advanced Light Source and 24-ID-C of the Advanced Photon Source. The reflections were integrated with XDS²² and scaled with SCALA²³ (Table 1). The phase problem was solved using molecular replacement as implemented in MOLREP.²⁴ The search model was the ($\beta\alpha$)₈ barrel of *Thermus thermophilus* PRODH (residues 37 – 279 of chain A of PDB code 2G37, 47 % identical to DrPRODH). The correct solution had a correlation coefficient of 0.36 with two molecules in the $P2_12_12_1$ asymmetric unit. For reference, molecular replacement calculations that assumed other primitive orthorhombic space groups yielded correlation coefficients of 0.26 – 0.31. The model from molecular replacement was used as the starting point for several rounds of model building with COOT²⁵ and refinement with PHENIX.²⁶ Table 1 lists refinement statistics.

The oxidized flavin exhibits two conformations in the THFA complex (Figure S1 of Supporting Information). The two conformations have occupancies of 0.56 (conformation A) and 0.44 (conformation B) and differ in the orientations of the 2' and 3' hydroxyl groups of the ribityl chain. Justification for building two conformations was obtained by refining the structure with single conformations of the FAD. For example, the $F_o - F_c$ map calculated after a refinement that included only conformation A at occupancy of 1.0 showed strong features for the 2'-OH and 3'-OH ribityl groups of conformation B (Figure S1, blue cage). Conversely, the map calculated after a refinement that included just conformation B at occupancy of 1.0 showed strong features for the 2'-OH and 3'-OH ribityl groups of conformation A (Figure S1, green cage).

Enzyme Activity Assays

All chemicals used during kinetic characterization were purchased from Fischer Scientific or Sigma-Aldrich. All steady-state assays were conducted at 23 °C in 50 mM potassium phosphate, 25 mM NaCl at pH 7.5. For all assays, Coenzyme Q1 (CoQ1) was used as the electron acceptor with CoQ1 reduction monitored by the decrease in absorbance at 278 nm using a molar extinction coefficient of 14.5 mM⁻¹ cm⁻¹.²⁷ The K_m for proline and k_{cat} for wild-type DrPRODH (0.25 μM) were determined by varying the concentration of proline (0 – 500 mM) while keeping CoQ1 constant (200 μM). Inhibition of wild-type DrPRODH (0.25 μM) by THFA was analyzed by varying proline (10 – 500 mM) and THFA (0 – 200 mM), while keeping CoQ1 constant (200 μM). These assays were performed in a total volume of 200 μl per assay using a Powerwave XS microplate spectrophotometer (Bio-Tek). The K_m for CoQ1 and k_{cat} for wild-type DrPRODH (0.25 μM) were determined by varying CoQ1 (0 – 450 μM) and keeping proline constant (500 mM). For the DrPRODH mutants G63A (11 μM) and E64A (6.3 μM), the K_m for proline and k_{cat} were determined by varying proline (0 – 1000 mM) and holding CoQ1 constant (200 μM). The K_m for CoQ1 and k_{cat} for the DrPRODH mutants were determined by varying CoQ1 (10 – 450 μM) and holding proline constant (500 mM). These assays were performed in a total volume of 150 μl by mixing enzyme and substrate solutions using a Hi-Tech Scientific SF-61DX2 stopped flow instrument equipped with a 0.15 cm path length cell. Steady-state parameters were calculated by fitting initial rate data to the Michaelis-Menten equation and inhibition data were globally fit to a competitive inhibition model using Enzyme Kinetic Wizard (SigmaPlot 12.0).

RESULTS

Structure of DrPRODH_o-THFA

The crystal structure of oxidized DrPRODH (DrPRODH_o) complexed with the proline analog THFA was determined at 1.36 Å resolution (Table 1). It is the first structure of a

monofunctional PRODH complexed with a proline analog and the highest resolution structure of any PRODH or PutA.

The DrPRODH_o-THFA complex exhibits a distorted ($\beta\alpha$)₈ barrel fold in which the last helix, denoted as α 8 (residues 285-295), is located above the C-terminal ends of the strands rather than alongside β 8 as in the classic triosephosphate isomerase barrel (Figure 2A). This fold is also observed in the structures of TtPRODH (Figure 2B) and PutA and is considered to be a defining characteristic of the PRODH superfamily, which includes both monofunctional PRODHs and PutAs. The root mean square deviation between DrPRODH_o-THFA and TtPRODH is 1.2 Å; the variation from PutA PRODH domains is slightly higher at 1.5 – 1.9 Å.

THFA binds between the *si* face of the FAD and α 8 (Figure 2A). Arg291, Arg292, and Lys98 form ion pairs with the carboxylate of THFA, while Leu257, Tyr278, Tyr288 provide hydrophobic contacts with the pyrrolidine ring (Figure 3A). Arg291 and Arg292 are stabilized by ion pairs with Glu64 and Glu295, respectively. The plethora of residues clustered around THFA make the inhibitor and the *si* face of the flavin inaccessible to solvent. The oxidized flavin exhibits two conformations, which is unprecedented for PRODHs and PutAs (Figure 4A). Furthermore, we are unaware of another flavin oxidoreductase that exhibits multiple flavin conformations in the E-S complex.

The two conformations have occupancies of 0.56 (conformation A) and 0.44 (conformation B). Interestingly, the two FAD conformations do not correspond to THFA-bound and THFA-free states because the occupancy of THFA is clearly 1.0 (*B*-factor = 17.5 Å²). Rather, the enzyme is able to bind THFA using either of two different FAD conformations. The two FAD conformations differ in the orientations of the 2' and 3' hydroxyl groups of the ribityl chain (Figures 4A and S1). In conformation A (yellow in Figure 4A), the 2'-OH points toward the proline binding site, while the 3'-OH is oriented 180° away and interacts with the carbonyl of Gly191. Conformation B is related to conformation A by a crankshaft rotation around the C2'-C3' bond. In conformation B (pink in Figure 4A), the 2'-OH is tucked under the pyrimidine ring where it forms hydrogen bonds with the flavin N1 atom and N-H group of Gly191, while the 3'-OH interacts with Glu295 of α 8.

Ribityl conformation A appears to be unique among the PRODH superfamily. Although it resembles the ribityl of PutA86-669-THFA in that 2'-OH points into the proline site, the other two hydroxyl groups of conformation A are rotated 180° from the corresponding hydroxyls of PutA86-669-THFA (Figure 5A). Although the 4'-OH of DrPRODH overlaps the 3'-OH of PutA86-669-THFA (Figure 5A), the two hydroxyls are nonequivalent because they form different interactions. The 3'-OH of PutA86-669-THFA forms a hydrogen bond with conserved Glu559 of α 8 (Figure 3B), which corresponds to Glu295 of DrPRODH. Glu295, however, adopts a different conformation and is thus unable to form an analogous hydrogen bond with the 4'-OH (Figure 3B).

Ribityl B is more familiar. It is similar to the ribityl of ligand-free TtPRODH (Figure 5B). In both structures, the 2' and 3'-hydroxyls are below the pyrimidine ring, while the 4'-OH is below the dimethylbenzene ring. One notable difference is that the 4'-OH of DrPRODH ribityl B is located on the *si* face of the FAD, whereas that of TtPRODH is torsioned over to the *re* side. The *re* side location allows the hydroxyl to engage a structurally conserved water molecule that bridges the main chains of β 5 and β 6 in all PRODH and PutA structures (Figure 5B).

The flavins of DrPRODH and PutA also differ in the position of the adenosine group (Figure 5A). Because of a rotation around the pyrophosphate, the adenine ring positions of the two

structures differ by 13 Å. This difference was also noted in TtPRODH and is due to the presence of an extra α -helix in PutAs that is absent in monofunctional PRODHs.²¹

Structure of DrPRODH_r

A 1.75 Å resolution structure of DrPRODH_r was determined from a crystal of the DrPRODH_o-THFA complex that was soaked in sodium dithionite. Reduction causes large conformational changes in the flavin, which are observed in both molecules in the asymmetric unit. Large protein conformational changes are also observed in chain A of the asymmetric unit, and the electron density map is consistent with release of THFA. In the other chain, crystal contacts prevent large protein conformational changes, and the electron density map suggests that an acetate ion replaces THFA. We note that PutA86-669 crystallized in PEG 3350 also has an acetate ion bound in the active site.²⁸ The discussion below focuses on chain A, since it provides information about conformational changes in both the flavin and the protein.

Reduction by dithionite induces substantial conformational changes in the flavin. The reduced flavin exhibits a 20° butterfly bend in the isoalloxazine (Figure 4C), which contrasts the planar isoalloxazine of the oxidized enzyme. Severe butterfly bending (20°–30°) has also been observed in the dithionite-reduced flavin of PutA86-669²⁸ and the covalently modified, reduced flavins of N-propargylglycine-inactivated PRODH²⁹ and PutA.³⁰ The bending of the flavin pushes the N5-N10 axis toward the proline binding site, which results in the *si* face being convex. The reduced flavin of DrPRODH_r exhibits just one ribityl conformation (Figure 4B), which is nearly identical to the ribityl chains of the oxidized FAD in TtPRODH and the reduced flavin in the N-propargylglycine-inactivated *E. coli* PutA PRODH domain³⁰ (Figure 5C).

Flavin reduction also dramatically changes the protein conformation. Upon flavin reduction, α 8 shifts away from the isoalloxazine by 1.7 Å, and the β 1- α 1 loop (residues 62 - 69) withdraws from the active site by over 5 Å (Figure 6A). One major consequence of these movements is the rupture of the Arg291-Glu64 ion pair, which links α 8 and the β 1- α 1 loop in the THFA complex. The electron density for the ion pair in the THFA complex is exceptional, indicating that this interaction is quite strong (Figure 3A). Its rupture is thus a significant event. Breaking of the ion pair causes Glu64 and Arg291 to separate from each other. Glu64 is driven to the protein surface along with the rest of the β 1- α 1 loop (Figures 6A and 2B), although we note that electron density for the β 1- α 1 loop is relatively weak in DrPRODH_r, implying high mobility. In fact, density for the side chain of Glu64 in DrPRODH_r is absent, indicating that the side chain is disordered. Electron density for the side chain of Arg291 is likewise weak, but suggests that the guanidinium group turns away from the proline binding site (Figure 6A). Flavin reduction also induces disorder in another key side chain of α 8, Glu295, which hydrogen bonds with the FAD adenine and ribityl groups in the THFA complex.

The large movements of α 8 and the β 1- α 1 loop dismantle the proline binding site and profoundly change the solvent accessibility of the flavin. In the THFA complex, the isoalloxazine and THFA are buried beneath the Arg291-Glu64 ion pair (Figure 6B), and the interaction between Glu295 and the ribityl seals the bottom of the active site (Figure 6B, inset). In the reduced enzyme, the separation of Arg291 from Glu64 creates a large, solvent exposed cavity in the upper part of the active site, which contains the isoalloxazine (Figure 6C). Furthermore, the combination of disorder in Glu295 and change in the ribityl conformation creates a hole in the bottom of the active site of the reduced enzyme (Figure 6C, inset). Thus, active site of the reduced, ligand-free enzyme is open and the flavin is highly solvent exposed.

Kinetic Parameters of DrPROD_H and DrPROD_H β 1- α 1 Loop Mutants

The observation of large conformational changes in the β 1- α 1 loop induced by flavin reduction and release of THFA prompted investigation of this loop using site-directed mutagenesis. As described by White, *et al.*, the β 1- α 1 loop harbors one of 9 conserved sequence motifs that define the PROD_H superfamily.²¹ This motif includes a Gly-Glu pair that is present in all monofunctional PROD_Hs and PutAs and corresponds to Gly63-Glu64 of DrPROD_H. These residues were therefore investigated by creating the site-directed mutant enzymes G63A and E64A.

The kinetic parameters for wild-type DrPROD_H were first determined (Table 2). The values of k_{cat} and K_{m} using proline as the variable substrate with fixed CoQ1 are 8.7 s^{-1} and 290 mM, respectively, resulting in a $k_{\text{cat}}/K_{\text{m}}$ of $30 \text{ s}^{-1}\text{M}^{-1}$. For comparison, the corresponding parameters of the closely related TtPROD_H (47 % identical to DrPROD_H) are $k_{\text{cat}} = 13 \text{ s}^{-1}$, $K_{\text{m}} = 27 \text{ mM}$.²¹ Those of the *E. coli* PutA are $k_{\text{cat}} = 5.2 \text{ s}^{-1}$, $K_{\text{m}} = 42 \text{ mM}$.²⁷ Thus, the K_{m} for proline of DrPROD_H is higher than expected. The kinetic parameters were also determined using CoQ1 as the variable substrate at fixed proline concentration. These values are $k_{\text{cat}} = 14 \text{ s}^{-1}$, $K_{\text{m}} = 155 \text{ }\mu\text{M}$. The corresponding values for TtPROD_H are not available. Those of the *E. coli* PutA are $k_{\text{cat}} = 3.4 \text{ s}^{-1}$, $K_{\text{m}} = 110 \text{ }\mu\text{M}$.²⁷ Finally, THFA was found to inhibit DrPROD_H competitively with proline. The estimated K_{I} value of 38 mM is over 10 times higher than those of TtPROD_H (1 mM²¹) and PutA (1.6 mM²⁷). These results suggest that the affinity of DrPROD_H for proline is atypically low.

Catalytic activity is severely impaired in the mutants G63A and E64A (Table 2). Using proline as the varying substrate, k_{cat} values of 0.08 s^{-1} and 0.055 s^{-1} were obtained for G63A and E64A, respectively, which are over 100 times lower than that of DrPROD_H. The catalytic efficiencies of G63A and E64A for proline are 140-fold and 27-fold lower than that of DrPROD_H. Using CoQ1 as the varying substrate, the k_{cat} values are 0.043 s^{-1} and 0.046 s^{-1} for G63A and E64A, respectively, which are 300-fold lower than that of DrPROD_H. The efficiencies of the mutants for CoQ1 are 60-fold lower relative to DrPROD_H. In summary, the kinetic analysis of G63A and E64A confirms the significance of the conserved sequence motif and is consistent with the structures, which suggest that Gly63 is important for the flexibility of the β 1- α 1 loop, and Glu64 is important for stabilizing the closed active site.

DISCUSSION

The DrPROD_H structures provide new information about the conformations populated by monofunctional PROD_Hs during the reductive half-reaction (Figure 1B). In particular, the DrPROD_H-THFA structure represents the E-S complex (state II, Figure 1B). The active site of the E-S complex is closed, and the substrate is buried. The tight packing within the complex is also observed in PutA86-669 (Figure 3B) and presumably helps align proline with the flavin for hydride transfer. A water molecule is present in the E-S complex, which bridges conserved Tyr193 and the THFA heteroatom (N atom of proline). An analogous water molecule is also present in the PutA86-669-THFA complex (Figure 3B), suggesting that it is conserved in the PROD_H superfamily. It is tempting to speculate that this water molecule hydrolyzes P5C to GSA. If so, the hydrolysis reaction occurs within the active site rather than in solution as implied in Figure 1A and most Biochemistry textbooks and literature articles on proline catabolism.

The E-S complexes of DrPROD_H and PutA86-669 are very similar (Figure 3B). In both cases, the inhibitor is completely buried between the *si* face of the FAD and α 8. The enzyme-inhibitor interactions and ion pair gate conformation are virtually identical in the two structures. Thus, substrate recognition is highly conserved in the PROD_H superfamily. The only notable difference involves the conserved glutamate of α 8 (Glu295 of DrPROD_H

and Glu559 of PutA). The different orientations of this side chain seem to be related to the different conformations of the FAD adenosine groups. In DrPROD_H, the adenine ring packs against α 8 and forms a hydrogen bond with Glu295 (Figure 3B). This interaction is not possible in PutA because of the markedly different location of the adenosine (Figure 5A), which is due to the presence of an extra α -helix in PutAs that is absent in monofunctional PROD_Hs.²¹

Given the sequence and structural conservation of the proline binding site, it is surprising that DrPROD_H has markedly lower substrate affinity than PutA and TtPROD_H. One possibility is that the FAD ribityl plays a role in tuning substrate affinity. DrPROD_H is unique in that two conformations of the ribityl are observed in the E-S complex, neither of which corresponds to that of the high affinity PutA86-669-THFA complex, although conformation A is reminiscent of the flavin in PutA86-669-THFA. It is possible that conformation B represents an inactive state and that a conformational change is required to transform it to conformation A before hydride transfer occurs. If so, Glu295 may play a role in this transformation since it hydrogen bonds with the 3'-OH of conformation B (Figure 4A). Additional structures of PROD_H-THFA complexes would shed light on this issue.

Comparison of the DrPROD_H_o-THFA and DrPROD_H_r structures provides insight into conformational changes that occur upon flavin reduction and product release (state II to state IV in Figure 1B). These include severe bending of the isoalloxazine, dihedral rotation of the ribityl chain, shifting of α 8, reconfiguration of the β 1- α 1 loop, rupture of the conserved Arg291-Glu64 ion pair, and disordering of Glu295.

The structures suggest a scenario for how the enzyme moves from state II to state IV of the reductive half-reaction. The butterfly bending of the isoalloxazine appears to initiate a cascade of events that leads to product release. Bending of the ring system pushes the N5-N10 axis of the flavin toward the newly formed product, creating steric clash in the highly crowded active site. The shifting of α 8 and rupture of the Arg291-Glu64 ion pair alleviate this clash, creating a large cavity that presumably allows facile product dissociation.

The Arg291-Glu64 ion pair is a central player in the conformational changes observed in DrPROD_H. Because it is formed in the closed E-S complex and broken in the ligand-free state, the ion pair appears to function as the active site gate. Since the ion pairing residues are identically conserved in monofunctional PROD_Hs and PutAs, we suggest that the gate is a universal aspect of PROD_H catalysis.

Whereas the ion pair gate opens to the bulk medium in DrPROD_H, the gate of PutA separates the proline binding site from a large, internal cavity that traverses 41 Å to the P5CDH active site.²⁰ This cavity serves as a conduit through which P5C/GSA is channeled to the P5CDH active site. In the structure of full-length *Bradyrhizobium japonicum* PutA, the gate is closed and a sulfate ion is bound in the PROD_H active site. A structure of this enzyme with the proline site empty is not available, but based on the DrPROD_H structures we predict that reduction of the FAD in PutA opens the gate allowing P5C/GSA to enter the substrate-channeling cavity. Curiously, dithionite-reduction of crystalline PutA86-669-THFA does not break the ion pair, although the flavin conformation is altered as in DrPROD_H_r.²⁸ It is possible that crystal contacts inhibit rupture of the gate in crystalline PutA86-669. Clearly, new structures of full-length PutAs are needed to understand how the ion pair gate functions in PutA.

The structural changes observed in DrPROD_H perhaps also provide insight into functional switching of trifunctional PutAs. Trifunctional PutA is a type of flavin switch protein that serves as a transcriptional repressor of the *put* regulon in addition to having PROD_H and P5CDH catalytic activities.³¹ How trifunctional PutA switches from being a DNA-bound

transcriptional repressor to membrane-associated bifunctional enzyme (aka functional switching) is a major question in PutA biochemistry. Recent rapid reaction kinetic measurements of the paradigmatic trifunctional PutA from *E. coli* revealed an isomerization step that occurs after reduction of FAD, and this step was proposed to report on flavin-dependent, global conformational changes that drive functional switching.³² Since rupture of the ion pair gate and movement of $\alpha 8$ occur after flavin reduction in DrPROD_H, it is possible that analogous changes are also part of functional switching. Because $\alpha 8$ and the $\beta 1$ - $\alpha 1$ loop contact other structural elements in PutA that are absent in monofunctional PROD_Hs, rupture of the ion pair and shifting of $\alpha 8$ in trifunctional PutA could help initiate a cascade of events that eventually leads to a larger, more global isomerization of the protein that unveils a high affinity membrane-association domain.

Finally, although it was not possible to crystallize DrPROD_H in the absence of THFA, presumably the active site of the oxidized enzyme is open prior to substrate binding (state I in Figure 1B). Indeed, this is true for TtPROD_H. The structure of oxidized, ligand-free TtPROD_H resembles DrPROD_H_r in that the ion pair gate is open and $\alpha 8$ is shifted away from the isoalloxazine (Figure 2B). In fact, the shift of $\alpha 8$ in TtPROD_H is 1.3 Å larger than that of DrPROD_H_r (Figure 2B). Whether substrate binding to the open enzyme induces closure of the gate (induced fit) or the substrate binds to closed-like conformations that arise from thermal fluctuations (conformational selection) remains to be determined. However, the large conformational changes that are required to form the closed E-S complex seem to favor conformational selection as a plausible mechanism for substrate binding.³³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

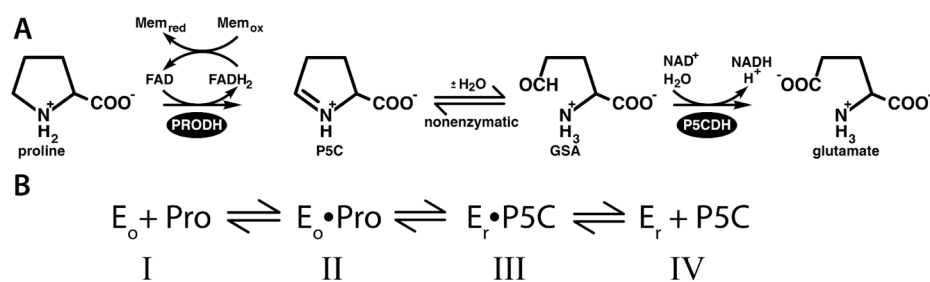
PROD_H	proline dehydrogenase
P5C	1-pyrroline-5-carboxylate
GSA	glutamate semialdehyde
P5CDH	1-pyrroline-5-carboxylate dehydrogenase
POX	human proline oxidase
TtPROD_H	proline dehydrogenase from <i>Thermus thermophilus</i>
PutA	proline utilization A
DrPROD_H	proline dehydrogenase from <i>Deinococcus radiodurans</i> R1

DrPRODH_o	oxidized proline dehydrogenase from <i>Deinococcus radiodurans</i> R1
DrPRODH_r	reduced proline dehydrogenase from <i>Deinococcus radiodurans</i> R1
THFA	L-tetrahydrofuroic acid
CoQ1	Coenzyme Q1
TEVP	Tobacco Etch Virus protease
PutA86-669	PRODH construct of <i>E. coli</i> PutA containing residues 86-669

References

- Adams E, Frank L. Metabolism of proline and the hydroxyprolines. *Annu Rev Biochem.* 1980; 49:1005–1061. [PubMed: 6250440]
- Tanner JJ. Structural biology of proline catabolism. *Amino Acids.* 2008; 35:719–730. [PubMed: 18369526]
- Singh RK, Tanner JJ. Unique Structural Features and Sequence Motifs of Proline Utilization A (PutA). *Front Biosci.* 2012; 17:556–568. [PubMed: 22201760]
- Phang JM. The regulatory functions of proline and pyrroline-5-carboxylic acid. *Curr Top Cell Reg.* 1985; 25:92–132.
- Phang JM, Donald SP, Pandhare J, Liu Y. The metabolism of proline, a stress substrate, modulates carcinogenic pathways. *Amino Acids.* 2008; 35:681–690. [PubMed: 18401543]
- Donald SP, Sun XY, Hu CA, Yu J, Mei JM, Valle D, Phang JM. Proline oxidase, encoded by p53-induced gene-6, catalyzes the generation of proline-dependent reactive oxygen species. *Cancer Res.* 2001; 61:1810–1815. [PubMed: 11280728]
- Hu CA, Donald SP, Yu J, Lin WW, Liu Z, Steel G, Obie C, Valle D, Phang JM. Overexpression of proline oxidase induces proline-dependent and mitochondria-mediated apoptosis. *Mol Cell Biochem.* 2007; 295:85–92. [PubMed: 16874462]
- Liu Y, Borchert GL, Surazynski A, Hu CA, Phang JM. Proline oxidase activates both intrinsic and extrinsic pathways for apoptosis: the role of ROS/superoxides, NFAT and MEK/ERK signaling. *Oncogene.* 2006; 25:5640–5647. [PubMed: 16619034]
- Pandhare J, Cooper SK, Phang JM. Proline oxidase, a proapoptotic gene, is induced by troglitazone: evidence for both peroxisome proliferator-activated receptor gamma-dependent and -independent mechanisms. *J Biol Chem.* 2006; 281:2044–2052. [PubMed: 16303758]
- Cooper SK, Pandhare J, Donald SP, Phang JM. A novel function for hydroxyproline oxidase in apoptosis through generation of reactive oxygen species. *J Biol Chem.* 2008; 283:10485–10492. [PubMed: 18287100]
- Phang JM, Pandhare J, Liu Y. The metabolism of proline as microenvironmental stress substrate. *J Nutr.* 2008; 138:2008S–2015S. [PubMed: 18806116]
- Phang JM, Pandhare J, Zabinnyk O, Liu Y. PPARgamma and Proline Oxidase in Cancer. *PPAR Res.* 2008; 2008:542694. [PubMed: 18670615]
- Liu Y, Borchert GL, Donald SP, Diwan BA, Anver M, Phang JM. Proline oxidase functions as a mitochondrial tumor suppressor in human cancers. *Cancer Res.* 2009; 69:6414–6422. [PubMed: 19654292]
- Liu Y, Borchert GL, Donald SP, Surazynski A, Hu CA, Weydert CJ, Oberley LW, Phang JM. MnSOD inhibits proline oxidase-induced apoptosis in colorectal cancer cells. *Carcinogenesis.* 2005; 26:1335–1342. [PubMed: 15817612]
- Phang JM, Hu CA, Valle D. Disorders of proline and hydroxyproline metabolism. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D., editors. *Metabolic and molecular basis of inherited disease.* McGraw Hill; New York: 2001. p. 1821–1838.
- Willis A, Bender HU, Steel G, Valle D. PRODH variants and risk for schizophrenia. *Amino Acids.* 2008; 35:673–679. [PubMed: 18528746]

17. Lee YH, Nadaraia S, Gu D, Becker DF, Tanner JJ. Structure of the proline dehydrogenase domain of the multifunctional PutA flavoprotein. *Nat Struct Biol.* 2003; 10:109–114. [PubMed: 12514740]
18. Zhang M, White TA, Schuermann JP, Baban BA, Becker DF, Tanner JJ. Structures of the *Escherichia coli* PutA proline dehydrogenase domain in complex with competitive inhibitors. *Biochemistry.* 2004; 43:12539–12548. [PubMed: 15449943]
19. Ostrander EL, Larson JD, Schuermann JP, Tanner JJ. A conserved active site tyrosine residue of proline dehydrogenase helps enforce the preference for proline over hydroxyproline as the substrate. *Biochemistry.* 2009; 48:951–959. [PubMed: 19140736]
20. Srivastava D, Schuermann JP, White TA, Krishnan N, Sanyal N, Hura GL, Tan A, Henzl MT, Becker DF, Tanner JJ. Crystal structure of the bifunctional proline utilization A flavoenzyme from *Bradyrhizobium japonicum*. *Proc Natl Acad Sci U S A.* 2010; 107:2878–2883. [PubMed: 20133651]
21. White TA, Krishnan N, Becker DF, Tanner JJ. Structure and kinetics of monofunctional proline dehydrogenase from *Thermus thermophilus*. *J Biol Chem.* 2007; 282:14316–14327. [PubMed: 17344208]
22. Kabsch W. XDS. *Acta Crystallogr D Biol Crystallogr.* 2010; 66:125–132. [PubMed: 20124692]
23. Evans P. Scaling and assessment of data quality. *Acta Cryst.* 2006; D62:72–82.
24. Vagin A, Teplyakov A. MOLREP: an automated program for molecular replacement. *J Appl Cryst.* 1997; 30:1022–1025.
25. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Cryst.* 2004; D60:2126–2132.
26. Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr, Sect D.* 2010; 66:213–221. [PubMed: 20124702]
27. Moxley MA, Tanner JJ, Becker DF. Steady-state kinetic mechanism of the proline:ubiquinone oxidoreductase activity of proline utilization A (PutA) from *Escherichia coli*. *Arch Biochem Biophys.* 2011; 516:113–120. [PubMed: 22040654]
28. Zhang W, Zhang M, Zhu W, Zhou Y, Wanduragala S, Rewinkel D, Tanner JJ, Becker DF. Redox-induced changes in flavin structure and roles of flavin N(5) and the ribityl 2'-OH group in regulating PutA-membrane binding. *Biochemistry.* 2007; 46:483–491. [PubMed: 17209558]
29. White TA, Johnson WH Jr, Whitman CP, Tanner JJ. Structural basis for the inactivation of *Thermus thermophilus* proline dehydrogenase by N-propargylglycine. *Biochemistry.* 2008; 47:5573–5580. [PubMed: 18426222]
30. Srivastava D, Zhu W, Johnson WH Jr, Whitman CP, Becker DF, Tanner JJ. The structure of the proline utilization a proline dehydrogenase domain inactivated by N-propargylglycine provides insight into conformational changes induced by substrate binding and flavin reduction. *Biochemistry.* 2010; 49:560–569. [PubMed: 19994913]
31. Becker DF, Zhu W, Moxley MA. Flavin redox switching of protein functions. *Antioxid Redox Signal.* 2011; 14:1079–1091. [PubMed: 21028987]
32. Moxley MA, Becker DF. Rapid reaction kinetics of proline dehydrogenase in the multifunctional proline utilization A protein. *Biochemistry.* 2012; 51:511–520. [PubMed: 22148640]
33. Grant BJ, Gorfe AA, McCammon JA. Large conformational changes in proteins: signaling and other functions. *Curr Opin Struct Bio.* 2010; 20:142–147. [PubMed: 20060708]
34. Engh RA, Huber R. Accurate bond and angle parameters for x-ray protein structure refinement. *Acta Cryst.* 1991; A47:392–400.
35. Lovell SC, Davis IW, Arendall WB 3rd, de Bakker PI, Word JM, Prisant MG, Richardson JS, Richardson DC. Structure validation by C α geometry: phi,psi and C β deviation. *Proteins.* 2003; 50:437–450. [PubMed: 12557186]
36. DeLano, WL. The PyMOL User's Manual. DeLano Scientific; Palo Alto, CA, USA: 2002.

**Figure 1.**

(A) Reactions catalyzed by PRODH and P5CDH. (B) Scheme showing the enzyme states that occur during the PRODH reductive half-reaction.

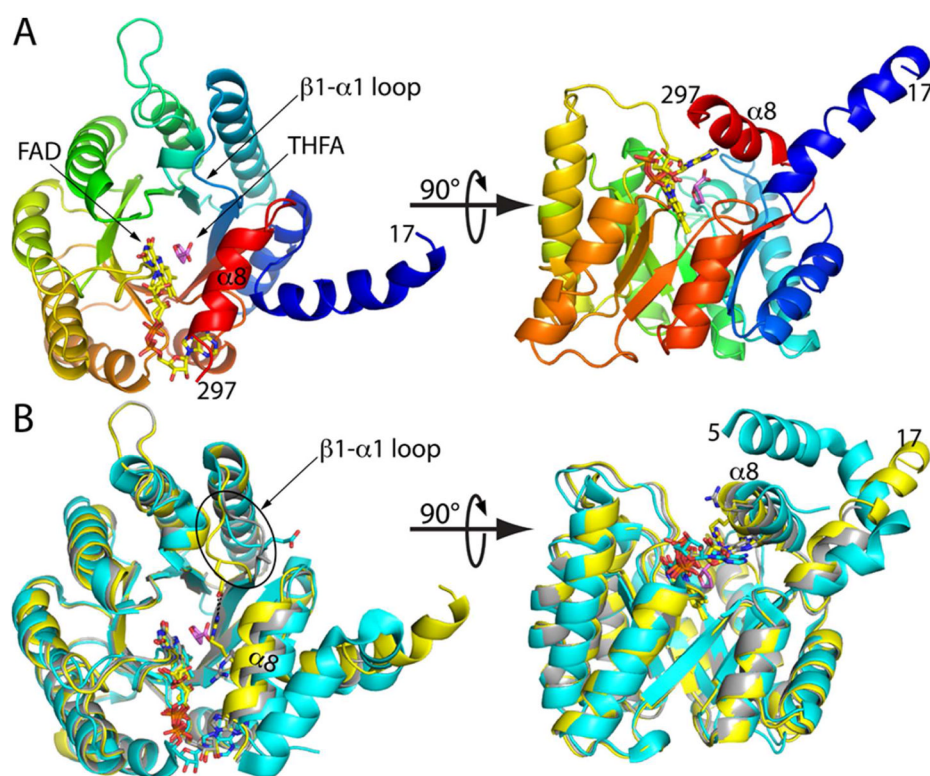


Figure 2. Overall fold of PRODH. (A) Two views of the DrPRODH_O-THFA structure. The backbone is colored according to a rainbow scheme, with blue at the N-terminus and red at the C-terminus. FAD is colored yellow. THFA is colored pink. Residue numbers of the N- and C-termini are noted. (B) Superposition of DrPRODH_O-THFA (yellow), DrPRODH_I (gray), and TtPRODH (cyan, PDB code 2g37). The R291-E64 ion pair is observed only in the DrPRODH_O-THFA structure. This figure and others were prepared with PyMOL.³⁶

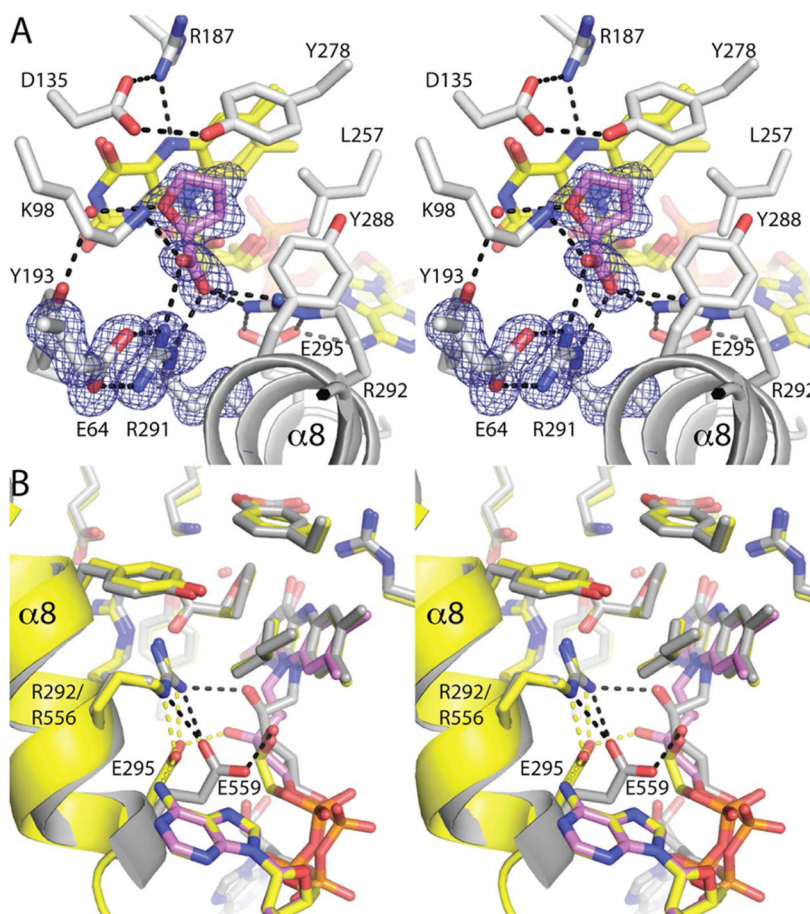


Figure 3.

The active site of DrPRODHD₀-THFA (relaxed stereographic views). (A) Electron density map for DrPRODHD₀-THFA. The cage represents a simulated annealing s_A -weighted $F_o - F_c$ omit map (3.0 s). (B) Superposition of DrPRODHD₀-THFA (yellow) and PutA86-669-THFA (gray, PDB code 1tiw), highlighting differences in the orientation of the conserved glutamate of $\alpha 8$ (Glu295 in DrPRODHD, Glu559 in PutA). Yellow and black dashes denote the unique electrostatic interactions of DrPRODHD and PutA86-669, respectively. Conformations A and B of the FAD in DrPRODHD₀-THFA are colored yellow and pink, respectively.

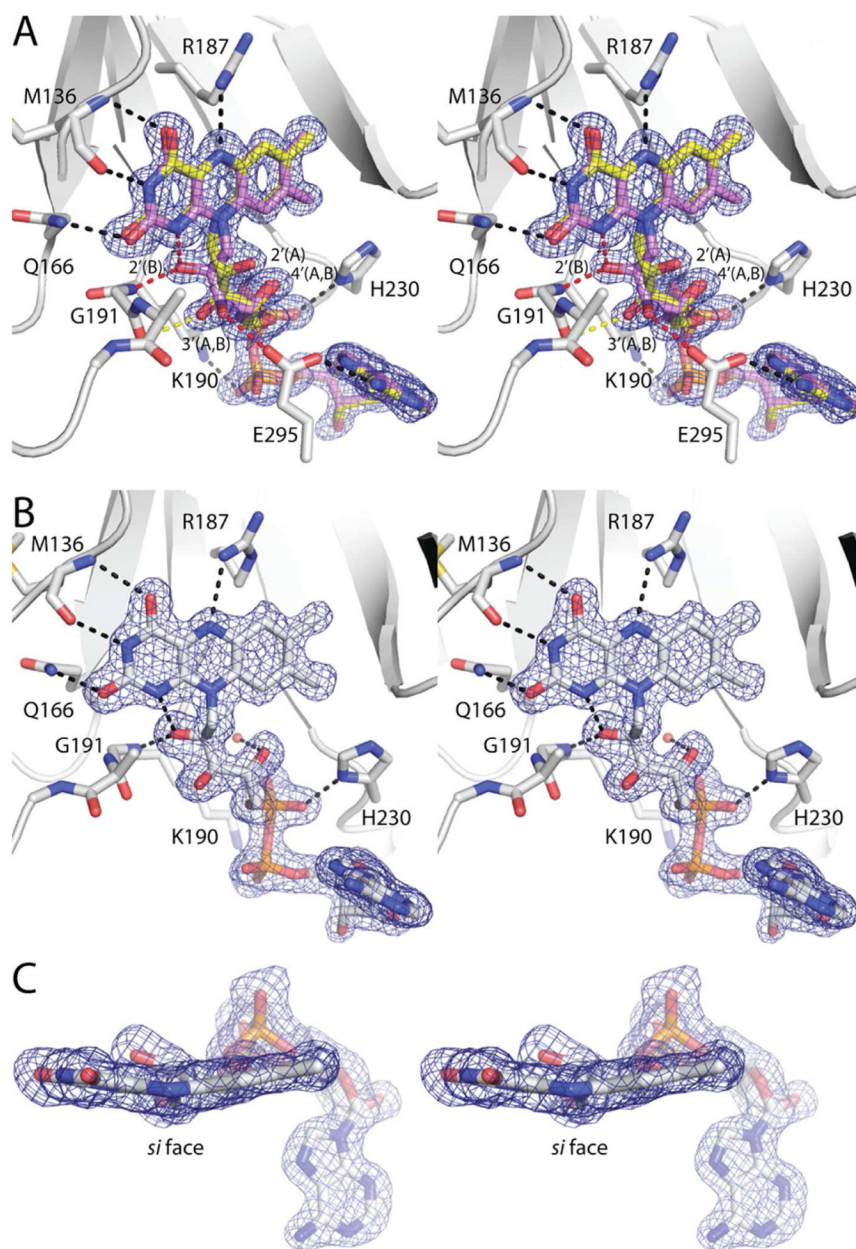


Figure 4.

Conformations of the flavin in DrPRODHD (relaxed stereographic views). (A) The dual FAD conformations of DrPRODHD₀-THFA. Conformations A (q = 0.56) and B (q = 0.44) are colored yellow and pink, respectively. Yellow and red dashes denote the unique hydrogen bonds of conformations A and B, respectively. Note that the two conformations differ mainly in the orientations of the 2'-OH and 3'-OH groups of the ribityl. (B and C) Two views of the dithionite-reduced flavin of DrPRODHD_r. In all three panels, the cage represents a simulated annealing s_A -weighted $F_0 - F_c$ omit map (3.0 s).

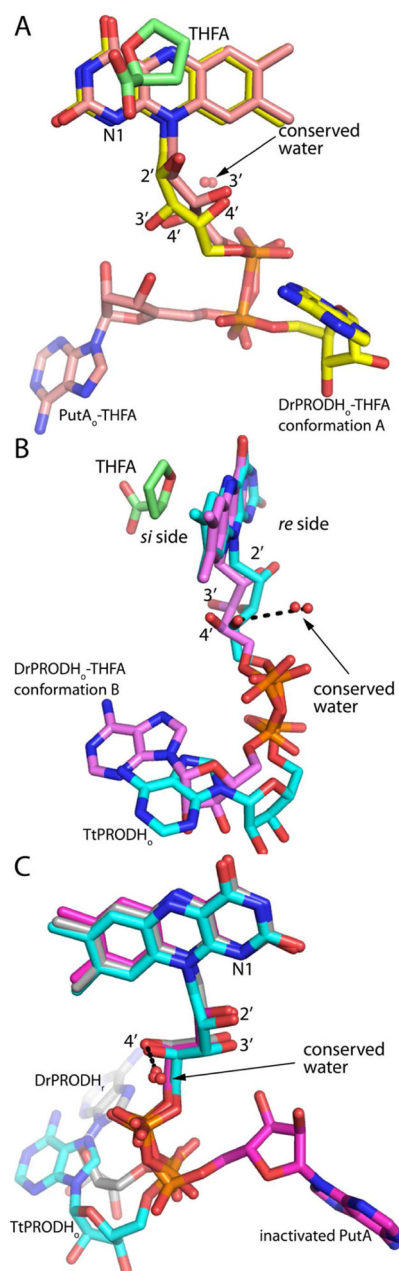


Figure 5. Comparison of flavin conformations in PRODhs and PutAs. (A) Superposition FAD conformation A of DrPROD_h-THFA (yellow) and the FAD of PutA₈₆₋₆₆₉-THFA (salmon, PDB code 1tiw). (B) Superposition of FAD conformation B of DrPROD_h-THFA (pink) and the oxidized flavin of TtPROD_h (cyan, PDB code 2g37). (C) Superposition of the reduced flavin of DrPROD_h (gray), the oxidized FAD of TtPROD_h (cyan), and the inactivated flavin of the PutA PROD_h domain (magenta, PDB code 3ITG).

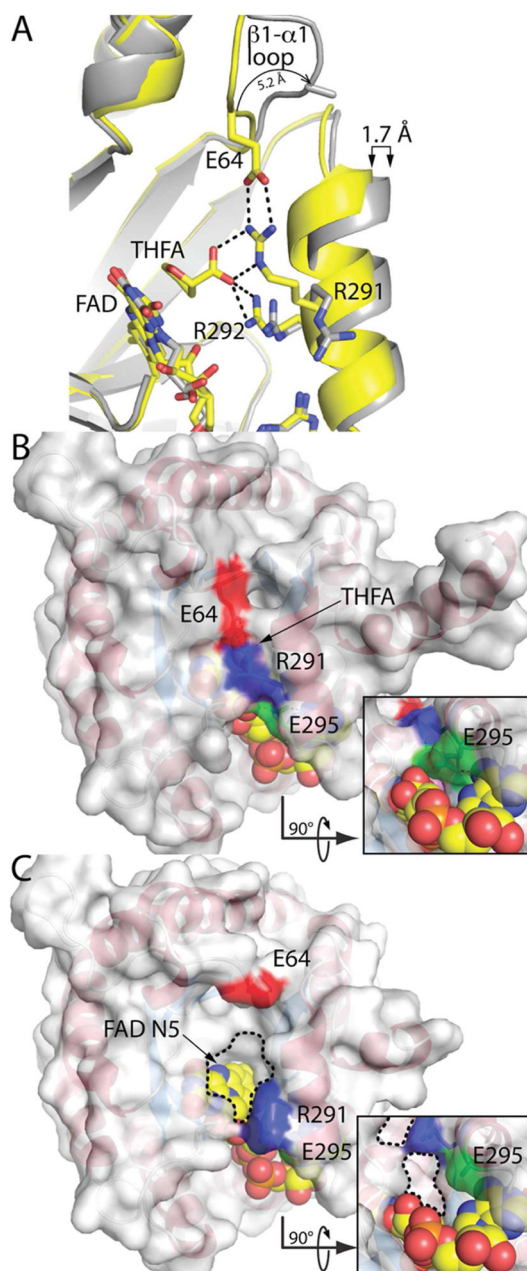


Figure 6.

Conformational changes induced by flavin reduction and product release. (A) Superposition of the active sites of DrPRODHo-THFA (yellow) and DrPRODHr (gray) highlighting the shift of $\alpha 8$, reconfiguration of the $\beta 1$ - $\alpha 1$ loop, and breaking of the Arg291-Glu64 ion pair. Black dashes indicate electrostatic interactions in DrPRODHo-THFA. (B) Surface representation of DrPRODHo-THFA. FAD and THFA are drawn as spheres in yellow and pink, respectively. Glu64 and Arg291 are colored red and blue, respectively. Glu295 is colored green. The inset shows a view from the bottom of the active site. (C) Surface representation of DrPRODHr. The coloring is the same as in panel B. The dashes outline the openings to the active site.

TABLE 1

X-ray Diffraction Data Collection and Refinement^a

	DrPRODH _o -THFA	DrPRODH _r
FAD redox state	oxidized	reduced
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit cell parameters (Å)	<i>a</i> = 44.5, <i>b</i> = 95.5, <i>c</i> = 136.4	<i>a</i> = 43.4, <i>b</i> = 95.7, <i>c</i> = 136.0
Wavelength	0.979	1.00
Resolution (Å)	95.5 – 1.36 (1.43 – 1.36)	47.8 – 1.75 (1.84 – 1.75)
Observations	450935	415890
Unique reflections	124272	58089
<i>R</i> _{merge} (<i>I</i>)	0.032 (0.431)	0.065 (0.837)
<i>R</i> _{meas} (<i>I</i>)	0.043 (0.576)	0.071 (0.905)
<i>R</i> _{pim} (<i>I</i>)	0.021 (0.294)	0.026 (0.341)
Mean <i>I</i> /σ	17.1 (2.4)	22.1 (2.5)
Completeness (%)	98.9 (98.5)	100.0 (100.0)
Multiplicity	3.6 (3.7)	7.2 (7.0)
No. of protein residues	562	550
No. of atoms	5091	4557
No. of FAD atoms	212	106
No. of THFA atoms	16	0
No. of water molecules	475	297
<i>R</i> _{cryst}	0.148 (0.239)	0.182 (0.276)
<i>R</i> _{free} ^b	0.181 (0.279)	0.213 (0.323)
rmsd bond lengths (Å) ^c	0.015	0.013
rmsd bond angles (°)	1.75	1.45
Ramachandran plot ^d		
Favored (%)	99.3	99.3
Allowed (%)	0.7	0.7
Outliers (%)	0.0	0.0
Average B (Å ²)		
Protein	21.4	28.6
FAD	17.7	23.7
THFA	17.5	n/a
Acetate	n/a	26.2
Water	31.5	31.4
Coordinate error (Å) ^e	0.14	0.19
PDB code	4H6Q	4H6R

^aValues for the outer resolution shell of data are given in parenthesis.

^bA common test set (5 %) was used for refinement of both structures.

^cCompared to the parameters of Engh and Huber.³⁴

^dThe Ramachandran plot was generated with RAMPAGE.³⁵

^eMaximum likelihood-based coordinate error estimate.

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TABLE 2Kinetic Parameters for DrPROD_H and DrPROD_H Mutant Enzymes Using Proline and CoQ1 as the Substrates

	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ M ⁻¹)	Variational Effect ($k_{\text{cat}}/K_{\text{m}}$ of mutant)/($k_{\text{cat}}/K_{\text{m}}$ of DrPROD _H)
Proline as the variable substrate ^a				
DrPROD _H	8.7 ± 0.58	290 ± 39	30 ± 4.5	
G63A	0.080 ± 0.006	384 ± 68	0.21 ± 0.04	0.007 ± 0.002
E64A	0.055 ± 0.001	50 ± 6	1.1 ± 0.13	0.037 ± 0.007
CoQ1 as the variable substrate ^b				
DrPROD _H	14 ± 1	0.155 ± 0.04	90323 ± 24185	
G63A	0.043 ± 0.003	0.028 ± 0.01	1535 ± 559	0.017 ± 0.008
E64A	0.046 ± 0.003	0.032 ± 0.01	1438 ± 458	0.016 ± 0.007

^a[CoQ1] was fixed at 200 μM.^b[Proline] was fixed at 500 mM.