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Identification of a Conserved Histidine as Critical for the Catalytic Mechanism and Functional Switching of the Multifunctional Proline Utilization A Protein†

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Abstract

Proline utilization A from *Escherichia coli* (*EcPutA*) is a multifunctional flavoenzyme that oxidizes proline to glutamate through proline dehydrogenase (PROD_H) and ¹-pyrroline-5-carboxylate dehydrogenase (P5CD_H) activities, while also switching roles as a DNA bound transcriptional repressor and a membrane bound catabolic enzyme. This phenomenon, referred to as functional switching, occurs through a redox-mediated mechanism in which flavin reduction triggers a conformational change that increases *EcPutA* membrane-binding affinity. Structural studies have shown that reduction of the FAD cofactor causes the ribityl moiety to undergo a crankshaft motion indicating that the orientation of the ribityl chain is a key element of PutA functional switching. Here, we test the role of a conserved histidine that bridges the FAD pyrophosphate to the backbone amide of a conserved leucine residue in the PROD_H active site. An *EcPutA* mutant (H487A) was characterized by steady-state and rapid-reaction kinetics, and cell-based reporter gene experiments. The catalytic activity of H487A is severely diminished (> 50-fold) with membrane vesicles as the electron acceptor, and H487A exhibits impaired lipid binding and *in vivo* transcriptional repressor activity. Rapid-reaction kinetic experiments demonstrate that H487A is 3-fold slower than wild-type *EcPutA* in a conformational change step following reduction of the FAD cofactor. Furthermore, the reduction potential (E_m) of H487A is about 40-mV more positive than wild-type *EcPutA* and H487A has an attenuated ability to catalyze the reverse PROD_H chemical step, i.e. re-oxidation by P5C. In this process, significant red semiquinone forms in contrast to the same reaction with wild-type *EcPutA*, in which facile 2-electron re-oxidation occurs without forming measurable semiquinone. These results indicate that His487 is critically important for the proline/P5C chemical step, conformational change kinetics, and functional switching in *EcPutA*.

Graphical Abstract

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PutA from *Escherichia coli* (*EcPutA*) is a multidomain trifunctional flavoenzyme (Figure 1A) capable of autogenous transcriptional repression as well as catalyzing the FAD dependent oxidation of proline to γ -pyrroline-5-carboxylate (P5C) followed by NAD⁺ dependent oxidation of glutamate- γ -semialdehyde (GSA) to glutamate (Scheme 1).^{1–4} The reactions catalyzed by PutA and its homologs have acquired growing significance due to a number of recent reports on proline related phenomenon including protection against oxidative and osmotic stress,^{5–7} redox signaling,^{5, 7} cancer biology,⁸ pathogenic virulence,⁹ and mental illness.^{10, 11} Therefore, the multifunctional properties of PutA and proline make them an intriguing combination for biochemical study.

The catalytic activities of *EcPutA* occur in spatially-separated proline dehydrogenase (PROD_H)^{2, 3, 12–15} and P5C dehydrogenase (P5CD_H) domains¹⁶, where the two active sites are connected by a tunnel through which the intermediate moves via substrate channeling.^{16–19} Recently, the kinetic framework of both PROD_H and P5CD_H active sites was combined elucidating a hysteric channeling mechanism, where *EcPutA* is activated after multiple catalytic turnovers.¹⁶

EcPutA also governs its own transcription through DNA^{1, 20, 21} and membrane binding activities.^{22–24} *EcPutA* represses transcription of the *put* operon that encodes the *putA* and *putP* (proline transporter) genes by blocking the transcriptional machinery when its FAD cofactor is oxidized. Upon proline reduction of the FAD cofactor, PutA undergoes a global conformational change^{22, 25–27} that increases its membrane binding affinity thereby relieving repression of the *put* operon and enabling electrons to pass from reduced FAD to ubiquinone^{2, 3, 28} in the cytoplasmic electron transport chain. *EcPutA*'s ability to switch from a DNA-bound repressor to a membrane-bound proline catabolic enzyme, or functional switching^{24, 27, 29}, is not completely understood. Although functional switching in *EcPutA* is known to occur through a redox-mediated mechanism²⁹, the molecular details of functional switching are limited.

Thus far, structures available for *EcPutA* are the separate N-terminal DNA binding ribbon-helix-helix (RHH)^{1, 20} and PROD_H domains.^{13, 15, 25} Insights into conformational changes in the FAD and the PROD_H active site have been generated from structures of the *EcPutA* PROD_H domain, which consists of a ($\beta\alpha$)₈ barrel-fold, captured in various ligand bound and

flavin redox states^{13, 15, 25}. In one case, the FAD in *EcPutA*-PRODH domain crystals was reduced with sodium dithionite.²⁵ In another case, *EcPutA*-PRODH was crystallized after inactivation with *N*-propargylglycine (PPG).¹⁵ Comparison of oxidized and reduced structures of the *EcPutA* PRODH domain have revealed that when the FAD cofactor is reduced, the ribityl 2'-OH group of FAD reorients to form a hydrogen bond (2.6 Å) with the FAD N(1) atom (Figure 1B), an interaction that is not observed with oxidized FAD. Other redox dependent molecular rearrangements include disruption of a hydrogen bond between Arg431 and the FAD N(5) and reorientation of Asp370 and Glu372 in the β 3- α 3 loop of the PRODH active site.^{15, 25, 30} Subsequent mutagenesis studies have shown that residues Arg431, Asp370 and Glu372 have critical roles in the functional switching mechanism of *EcPutA*.^{25, 30}

The PPG-inactivated structure of *EcPutA*-PRODH also reveals a hydrogen bond interaction between the imidazole ring of His487 and the O2 atom of the FAD pyrophosphate. A structural alignment (Figure 1B) of the oxidized L-tetrahydro-2-furoic acid (THFA) bound structure and the PPG inactivated structure of the *EcPutA* PRODH domain shows that the His487 imidazole N δ 1 atom is 3.7 Å from the FAD pyrophosphate moiety in the oxidized structure, whereas in the reduced state, the imidazole N δ 1 atom hydrogen bonds (2.8 Å) with the FAD pyrophosphate. The difference in interactions of His487 with the pyrophosphate in the two structures is due mainly to a $\sim 50^\circ$ rotation around the C β -C γ bond vector of His487 (χ 2). The structures imply that flavin reduction induces this rotation. His487 also hydrogen bonds via the imidazole N ϵ 2 atom to the amide backbone of Leu513 in the β 7- α 7 loop of the PRODH active site, an interaction that is observed in both the THFA-bound (3.0 Å) and PPG inactivated (2.9 Å) structures. Leu513 interacts with the methylene groups of the proline substrate and has been proposed to have a critical role in shaping the active site.¹³

The *EcPutA* active site residue His487 is conserved in all searched amino acid sequences of PutAs and PRODHs. Figure 1C shows a representative multiple sequence alignment of PutAs and PRODHs showing 100% conservation of His487. Structural analyses of PutAs from other organisms such as *Bradyrhizobium japonicum* (*BjPutA*, PDB:3HAZ)¹⁷, *Geobacter sulfurreducens* (*GsPutA*, PDBs:4NM9, 4NMD, 4NME)¹⁹, and *Sinorhizobium meliloti* (*SmPutA*, PDB:5KF7)³¹ provide additional insights into the structural role of His487 in *EcPutA*. These homologs all lack the DNA binding domain of *EcPutA* but share the bifunctional PRODH and P5CDH catalytic properties of *EcPutA*.^{17, 19, 31–33} *GsPutA* has yielded the most structural information of any PutA thus far with crystal structures of the full-length enzyme in oxidized, ligand bound, and reduced forms.¹⁹ All the PutA structures have a histidine residue that hydrogen bonds to the amide backbone of an active site leucine residue, analogous to His487 and Leu513 in *EcPutA* (Figure 1B). Oxidized structures of *BjPutA* and *SmPutA* show that the corresponding histidine residue is outside of hydrogen bonding distance to the FAD pyrophosphate (3.9 Å), similar to *EcPutA*. Interestingly, the analogous histidine residue in *GsPutA* is 3.0 Å from the O2 atom of the FAD pyrophosphate in both the oxidized and reduced forms, indicating that this interaction is not necessarily influenced by the FAD redox state. Because His487 is universally conserved in PutAs and its conformation and interactions may be influenced by the flavin redox state, we hypothesized

that His487 is likely to have an important role in the overall PRODH catalytic mechanism and *EcPutA* functional switching.

Recently, the kinetic mechanism for the PRODH domain of *EcPutA* was elucidated and shown to follow a two-site hybrid ping-pong mechanism.² The kinetic mechanism of the PRODH domain was further studied by stopped-flow spectroscopy, yielding microscopic rate constants for the proline reductive ($k_2 = 27.5 \text{ s}^{-1}$) and CoQ₁ oxidative ($k_6 = 5.4 \text{ s}^{-1}$) half-reaction.³ It was also shown that the proline reductive half-reaction is reversible ($k_{-2} = 1.6 \text{ s}^{-1}$) and that once the FAD cofactor is reduced, the enzyme undergoes a relatively slow conformational change ($k_3 = 2.2 \text{ s}^{-1}$).³ It was hypothesized that this kinetic step may be reporting on the FAD/protein dynamics involved in initiating functional switching as discussed above.³

Here we have collected redox equilibrium, steady-state/rapid reaction kinetic, and *in vivo* data for the *EcPutA* mutant H487A. These data provide evidence for the importance of His487 in *EcPutA* functional switching; a process of *EcPutA* transitioning from a DNA bound repressor to a membrane bound proline catabolic enzyme. Moreover, H487A was unexpectedly and significantly hindered in the reverse PRODH reaction (P5C to proline). Altogether, His487 appears to have an important role in the catalytic reversibility of the PRODH reaction and functional switching of *EcPutA*.

EXPERIMENTAL PROCEDURES

Materials

All chemicals and buffers were purchased from Fisher Scientific and Sigma-Aldrich, unless otherwise noted. *E. coli* strains XL-Blue and BL21(DE3) pLysS were purchased from Novagen. Because of the lack of commercial availability, DL-P5C was synthesized according to the method of Williams and Frank and stored in 1 M HCl at 4 °C.³⁴ P5C concentrations were measured by adding *o*-aminobenzaldehyde (*o*-AB) and monitoring the absorbance at 443 nm ($\epsilon_{443 \text{ nm}} = 2590 \text{ M}^{-1} \text{ cm}^{-1}$).³⁴ *EcPutA* mutant H487A was generated with the site directed mutagenesis kit from Novagen with the forward primer (5'-CGCAGTTCGCGACGGCCAACGCCCATACG-3') and its reverse complement purchased from Integrated DNA technologies. *EcPutA* wild-type (UniProt Accession ID:P09546) and mutant H487A were expressed and purified by Ni-NTA Superflow affinity (Qiagen) chromatography using a N-terminal 6xHis-tag as previously described.²⁵ The N-terminal His-tag was retained for *EcPutA* wild-type and mutant H487A in subsequent experiments. After the Ni-NTA column, each protein was subjected to an anion exchange column (HiTrap Q HP, GE Healthcare) using a 0–1 M NaCl gradient in 50 mM Tris (pH 7.5), 0.5 mM EDTA, and 5% glycerol. Purified *EcPutA* proteins were dialyzed into 50 mM potassium phosphate buffer (pH 7.4) containing 5% glycerol, 50 mM NaCl, and stored at –80 °C. The concentration of *EcPutA* wild-type and mutant H487A were determined spectrophotometrically using a molar extinction coefficient of $12700 \text{ M}^{-1} \text{ cm}^{-1}$ at 451 nm, and also using the BCA method (Pierce).³⁵ All experiments used Nanopure water.

Steady-State Kinetics of H487A

Proline:CoQ₁ oxidoreductase activity was monitored by absorbance at 278 nm, which presumably reports on the reduction of CoQ₁ (CoQ₁, $\epsilon_{278\text{ nm}} = 14.5\text{ mM}^{-1}\text{ cm}^{-1}$; using a path length of either 1 or 0.15 cm). These assays were performed in 50 mM HEPES (pH 7.4) at 20 °C. Steady-state kinetic parameters for CoQ₁ and proline were determined by varying CoQ₁ (10–300 μM) with fixed 400 mM proline and by varying proline (10–300 mM) with fixed 300 μM CoQ₁, respectively, and then fitting initial rates to the Michaelis-Menten equation using Sigma Plot 12 software (SYSTAT).

Steady-state kinetic parameters for proline with inverted membrane vesicles prepared from *E. coli* JT31 PutA[−] cells³⁶ were obtained by continuously monitoring the reaction of P5C with α -AB ($\epsilon_{443\text{ nm}} = 2590\text{ M}^{-1}\text{ cm}^{-1}$) in 20 mM MOPS (pH 7.5) containing 10 mM MgCl₂, 4 mM α -AB, 0.25 μM enzyme and 80 $\mu\text{g/mL}$ of vesicles. Initial rates were obtained and fitted to the Michaelis-Menten equation.

Lipid Pull-Down Assays

Physical lipid binding assays were explained in detail previously.¹⁵ Assays were performed anaerobically under a nitrogen atmosphere in a anaerobic chamber (Belle Technology Glovebox) with vesicles prepared from *E. coli* polar lipids (Avanti Polar Lipids).¹⁵ PutA proteins were incubated with *E. coli* polar lipid vesicles (0.8 mg/mL) in 10 mM HEPES (pH 7.4) with 150 mM NaCl with or without 5 mM proline for 1 h and then centrifuged.¹⁵ The soluble and lipid fractions were then analyzed by SDS-PAGE (10% gels) and stained with Coomassie Blue G-250.

Anaerobic Conditions for Stopped-Flow Experiments

All stopped-flow experiments were conducted under strictly anaerobic conditions to avoid the slow re-oxidation of FADH₂ by dissolved O₂ (as described previously³), which would have potentially altered the experimental kinetic traces. To maintain anaerobic conditions, protocatechuate dioxygenase (PCD) and protocatechuic acid (PCA) were added to the reagents and enzyme solutions at final concentrations of 0.05 U/mL and 100 μM , respectively.³⁷ Prior to performing the stopped-flow experiments, the stopped-flow mixing chambers were thoroughly washed with anaerobic buffer and incubated overnight with buffer containing PCD/PCA. All stopped-flow experiments were conducted on a Hi-Tech Scientific SF-61DX2 stopped-flow instrument equipped with a photodiode array detector and KinetAsyst software. The temperature of the mixing chamber for all experiments was maintained at 20 °C with a circulating water bath.

Anaerobic Reductive Half-Reaction of H487A with Proline

The reductive half-reaction was examined by rapidly mixing H487A (10 μM after mixing) with different initial concentrations of proline (0.025, 0.1, 0.4, 0.8, 1.2, and 1.5 M, after mixing) in 50 mM potassium phosphate (pH 7.4, 50 mM NaCl), as described previously for wild-type EcPutA.³ The reaction was monitored in the stopped-flow by multiwavelength absorption in the range of 300–700 nm.

Anaerobic Oxidative Half-Reaction of Reduced H487A with P5C and CoQ₁

Anaerobic oxidized H487A (23.6 μ M) was titrated with proline in an anaerobic chamber. Complete reduction of the flavin was achieved at a final concentration of 40 μ M proline. Proline reduced H487A was then loaded into an anaerobic stopped-flow syringe and attached to the stopped-flow mixing unit. For the reaction of reduced H487A with P5C, P5C stock solutions (50/50 mixtures of DL-P5C) were neutralized the day of the experiment on ice before being degassed as described previously.³ Reduced H487A (11.8 μ M after mixing) was shot against 6.6 mM DL-P5C (3.3 mM L-P5C after mixing) and followed by multiwavelength absorption (300–700 nm). For reactions with CoQ₁, proline reduced H487A (11.8 μ M after mixing) was rapidly mixed with varying concentrations of CoQ₁ (0–300 μ M, after mixing) and the reaction was monitored by multiwavelength absorption in the range of 300–700 nm or by single wavelength data collection at 451 nm.

Analysis of the H487A Kinetic Mechanism

Stopped-flow data were analyzed by fitting the data to simulated mechanisms using KinTek Explorer software (KinTek Corp., Austin, TX).^{38–40} The average of at least three kinetic traces was obtained at each substrate concentration and was used for the global fitting analysis. The mechanism described previously for wild-type *EcPutA* was used to fit the H487A stopped-flow data.³ For fitting the reductive and oxidative half-reaction with proline and CoQ₁, respectively, initial values for the association and dissociation rate constants were used from wild-type data to start the fitting process.³ The confidence intervals of the parameters were determined for each fitted mechanism and are shown as two-dimensional plots of two fitted parameters and scaled according to the chi squared value (χ^2 , error between the model and the data).³⁹ All fitted microscopic rate constants and confidence intervals can be found in Table 2.

Cell-based Gene Expression Assay

E. coli strain JT31 *putA*[−] *lacZ*[−] containing *putC::lacZ* (pUT03)¹ and *putA* gene constructs (pUC18-*EcPutA* wild-type and pUC18-*EcPutA* H487A) were grown at 37 °C in minimal medium containing ampicillin (50 μ g/mL) and chloramphenicol (30 μ g/mL) to OD at 600 nm (OD₆₀₀) ~0.8. Cells were then pelleted and immediately re-suspended in ice-cold buffer Z (0.1 M sodium phosphate, 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol, pH 7). β -galactosidase activity assays were then performed as previously described³⁰ with activity calculated in Miller units according to the method of Miller.⁴¹ The results reported here are the average values from ten independent experiments.

Expression of *EcPutA* wild-type and H487A proteins in JT31 *putA*[−] *lacZ*[−] were confirmed by Western blot analysis using an antibody against a polypeptide containing PutA residues 1–47.¹ Cell pellets from 5 mL culture grown in LB Broth overnight were resuspended in 50 μ L of SDS sample buffer and boiled for 10 min. The proteins were then separated by SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad) using an EBU-4000 semi-dry electrophoretic blotting system. After treatment with anti-PutA47 and anti-rabbit antibodies, immunoreactive bands were detected using Enhanced Chemiluminescence Western blotting reagents (Amersham Inc.).

DNA Binding Assay

Gel-shift assays were performed with *E. coli put* intergenic DNA PCR amplified from the pUTC01 vector²¹ using M13 reverse and M13 forward primers with 5' end-labeled IRdye-700 (LI-COR, Inc.). The resulting IRdye-700 labeled *put* intergenic DNA (420 bp) was purified and quantified by measuring the nucleic acid concentration at 260 nm and the absorbance of the IRdye-700 at 685 nm using an extinction coefficient of 170 mM⁻¹ cm⁻¹. The purified *putA* intergenic DNA (2 nM) was then incubated with *EcPutA* wild-type and mutant H487A (0, 200, 500 nM) in for 20 min at 20 °C prior to electrophoresis in 50 mM Tris (pH 7.5) containing 10% glycerol. Calf thymus competitor DNA (100 µg/mL) was added to the binding mixtures to prevent nonspecific protein-DNA interactions. The protein-DNA complexes were separated using a native polyacrylamide gel (4 %) at 4 °C at 8 volts/cm. The gels were visualized using a LI-COR Odyssey Imager.

Circular Dichroism (CD) Spectroscopy

FAR-UV-CD spectra of *EcPutA* wild-type (1 µM) and mutant H487A (0.9 µM) were recorded in 50 mM potassium phosphate buffer (pH 7.4) at 20 °C from 260–190 nm using a JASCO CD spectrometer (Model J815). Spectra were normalized with protein concentration.

Redox Potential Measurements

The redox potential (E_m) determination of H487A was performed according to the xanthine/xanthine oxidase method of Massey⁴² and as previously described for wild-type *EcPutA*.³⁰ H487A (15 µM) was mixed with 200 µM xanthine, 100 µM methyl viologen (mediator dye), and 5 µM resorufin ($E_m = -79$ mV, pH 7.5) in 50 mM potassium phosphate buffer (10 % glycerol, 50 mM NaCl, pH 7.5). The H487A solution was degassed with nitrogen and experiments were performed at 23 °C under a nitrogen atmosphere in an anaerobic chamber (Bell Technology Glovebox). Reduction of H487A was initiated by the addition of 10 nM xanthine oxidase. UV-visible spectra were recorded every 2 min for 3 h using a Cary 100 UV-vis spectrophotometer.

RESULTS

Steady-state Kinetics of H487A

Steady-state parameters for the PutA mutant H487A were determined with proline and CoQ₁, a soluble ubiquinone analog used previously with wild-type *EcPutA*.² The fitted steady-state parameters (Table 1) for proline in the presence of CoQ₁ for H487A, k_{cat} and k_{cat}/K_m , were about 1.8- and 5.8-fold less than those of wild-type, while the k_{cat}/K_m for CoQ₁ was 3.9-fold lower than wild-type. These data suggest a moderate catalytic deficiency for H487A with a decline in k_{cat} and a likely decrease in proline affinity compared to wild-type *EcPutA*. Interestingly, the k_{cat}/K_m of the proline:ubiquinone oxidoreductase activity with *E. coli* JT31 (PutA⁻) inverted membrane vesicles³⁶ was about 52-fold less than that of wild-type enzyme.

Lipid Binding of H487A

Because membrane binding is a prerequisite for proline:ubiquinone oxidoreductase activity with membrane vesicles, these results suggested that the membrane binding function of H487A may be disrupted. To test this possibility, physical lipid binding assays with H487A and wild-type *EcPutA* were performed by incubating the enzymes with *E. coli* polar lipids with or without proline, followed by centrifugation to separate soluble and membrane bound protein. Figure 2A shows that in the absence of proline *EcPutA* wild-type is mostly in the supernatant (S) but switches to the membrane pellet (P) fraction in the presence of 5 mM proline. These results are consistent with the activation of *EcPutA* membrane binding in the presence of proline as described previously.^{22,30} In contrast to wild-type enzyme, H487A appeared to exhibit no change in membrane binding upon addition of proline (5 mM). H487A is fully reduced by proline (Figure 4A), which demonstrates that incomplete reduction of the FAD cofactor is not a factor in the lack of H487A response to proline. The inability of proline to activate H487A membrane binding is consistent with the low proline:oxidoreductase activity of H487A associated with membrane vesicles.

The ability of H487A to function as a proline sensitive transcriptional repressor was also tested using DNA-binding and cell-based transcription assays. Figure 2B shows that H487A binds DNA to form a complex that is like that of wild-type *EcPutA*. H487A was next tested in cell-based assays in which β -galactosidase activity is monitored in response to proline with *EcPutA* repression of the *lacZ* reporter gene relieved in the presence of proline. Figure 2C shows that in cells with wild-type *EcPutA*, proline induces a 7-fold increase in β -galactosidase activity indicating functional switching of *EcPutA*. In cells with H487A, repression of the *lacZ* reporter gene is weaker without proline and only a 2-fold increase in β -galactosidase activity is observed with proline (Figure 2C). With empty vector alone, no change is observed in β -galactosidase activity with proline. The results of the cell-based assays indicate that the H487A mutation impairs the proline-responsive transcriptional function of *EcPutA*. Analysis of the overall secondary structure of H487A by circular dichroism (CD) spectroscopy showed no significant difference between *EcPutA* wild-type and the H487A mutant (Figure 2D). Thus, the inability of H487A to fully respond to proline in membrane binding and transcriptional regulation does not appear to be due to a global structural change.

H487A Reductive Half-reaction

Recently, stopped-flow studies of wild-type *EcPutA* with proline revealed a second kinetic phase; this was assigned to a conformational change occurring in the active site of the enzyme in accordance with previous results.³ Given that H487A seemed to be altered in the ability to switch from a soluble protein to a membrane-bound protein, we used stopped-flow spectroscopy to pinpoint differences in the kinetic mechanism and individual rate constants from those of wild-type *EcPutA*.

Oxidized H487A was rapidly mixed with different concentrations of proline under anaerobic conditions and followed by multiwavelength (Figure 3A) and single wavelength detection (Figure 3B). Upon mixing H487A with 100 mM proline (Figure 3A), no flavin semiquinone was observed.⁴³ This observation is consistent with wild-type *EcPutA*.³

The concentration dependence of the reductive half-reaction with proline (Figure 3B) was also determined, where single wavelength traces were fitted to the kinetic mechanism shown in Scheme 1 (reductive half-reaction) using global fitting.³⁸ These averaged H487A single wavelength traces at 451 nm were fitted to the same kinetic mechanism fitted previously with wild-type *EcPutA*³, which included proline binding to the oxidized enzyme (E, Scheme 1), reduction of the FAD cofactor (F, Scheme 1), followed by an isomerization step (f, Scheme 1). Table 2 shows the best-fit parameters for H487A, which relative to wild-type *EcPutA*, were similar for proline binding and reduction of the FAD cofactor (k_2) but differed in the reverse chemical step (k_{-2}) and the forward step assigned to an FAD dependent conformational change (k_3) (Figure 3C).

Using Kintek³⁹ software, the best-fit parameters were perturbed to obtain confidence intervals, or a best-fit range for the rate constants. This analysis showed that the forward chemical step (k_2 , Scheme 1) is well constrained, or has a small best-fit range (Table 2, see k_2 boundaries), but the reverse chemical step (k_{-2}) varies within a 10X range.⁴⁰ Despite this variability, the best-fit value for the reverse chemical step is about 10X less than that of *EcPutA* wild-type, and confidence intervals for H487A and wild-type *EcPutA* do not overlap (see Table 2). The rate constant for the isomerization step in the mechanism was well determined and 3–10 fold slower than wild-type *EcPutA*, suggesting that the H487A mutation has a deleterious effect on the conformational change step following proline reduction of the FAD cofactor.³

Reaction of Reduced H487A with P5C

To further explore the possibility that H487A has an attenuated ability to catalyze the reverse PRODH chemical step, anaerobic reduced H487A (11.8 μ M after mixing) was rapidly mixed with 3.3 mM DL-P5C (after mixing) and monitored by a multiwavelength detector (Figure 4A). The observed spectrum was drastically different from that of wild-type *EcPutA* (Figure 4A, inset).³ Reduced wild-type *EcPutA* displays a typical two-electron oxidation of the FAD spectrum upon mixing with P5C, with an isosbestic point near 350 nm indicating only two species present.³

The multiwavelength data shown in Figure 4A were deconvoluted by singular value decomposition (SVD) using Global Kinetic Explorer software (Kintek) (Figure 4B). This analysis showed many components comprising the observed spectra. Most notably, one of the components was red anionic semiquinone (one-electron reduced flavin intermediate, SV = 0.64) with a peak at 390 nm.⁴³ The two-electron reduced species (SV = 13) (Figure 4B, dashed line) and oxidized flavin (Figure 4B, solid line) (SV = 0.067) were also detected. Even after monitoring the reaction for 60 min, the oxidized form did not accumulate appreciably (Figure 4A; red line). Kinetic traces at 451 and 390 nm, from (A), were extracted and were well fitted to a double exponential equation (Figure 4C and D). These fits yielded observed rate constants (k_{obs}) of 0.70 and 0.031 min^{-1} at 390 nm, and 0.81 and 0.057 min^{-1} at 451 nm.

Oxidation of Reduced H487A by CoQ₁

The oxidation of H487A, which had been reduced by proline, by CoQ₁ was studied anaerobically by stopped-flow spectroscopy (Figure 5). Reduced H487A (11.8 μ M, after mixing) was rapidly mixed with 60 μ M CoQ₁ (after mixing) and followed by multiwavelength detection (Figure 5A). The isosbestic point near 350 nm is indicative of two species in solution, suggesting at least no observable intermediate like wild-type.

Concentration-dependent CoQ₁ single wavelength traces at 451 nm for H487A were globally fitted (Figure 5B) to the same mechanism as wild-type *EcPutA*³, which is defined in Scheme 1 (bottom). This mechanism includes binding (k_5) and chemical (k_6) steps, and a conformational change (k_7) step assigned to the reversal of the membrane binding conformation.³ The best-fit forward rate constant for the chemical step (k_6) of the oxidative half-reaction of H487A (1.2 s⁻¹) was 4.5-fold lower than wild-type (5.4 s⁻¹). Interestingly, the reverse chemical step (k_{-6}) with a best-fit value of 0.026 s⁻¹ was measurable in contrast to wild-type *EcPutA* for which this parameter was pushed to zero in the fitting.³ Importantly, this rate constant (k_{-6}) was well determined (Figure 5D; Table 2).³⁹ The best-fit value for the rate constant assigned to the reversal of the membrane binding conformational change ($k_7 = 1.2$ s⁻¹) was decreased relative to wild-type (4.7 s⁻¹). Confidence intervals of k_7 for H487A were tighter compared to wild-type *EcPutA*³, and shows that the rate constant for the reverse conformational change may range 3- to 10-fold slower for H487A than that of wild-type *EcPutA* (Figure 5C; Table 2).

Redox Potential of H487A

A change in the redox potential of the bound flavin in H487A could contribute to the different kinetic and spectral properties of the mutant enzyme relative to wild-type. The flavin redox potential (E_m) of H487A was determined using the xanthine oxidase/dye method as shown in Figure 6. A redox potential of - 65 mV was determined for H487A, which is about 40 mV more positive than that of wild-type PutA (-106 mV, pH 7.5).³⁰ The redox potential of the proline/P5C couple is -123 mV³⁵, thus, proline reduction of the bound flavin remains favorable in H487A. However, the reaction of reduced H487A with P5C would be less thermodynamically favored consistent with the kinetic data in Figure 4.

DISCUSSION

Evidence for His487 having an important role in *EcPutA* functional switching was generated here by characterizing the kinetic and physical lipid binding properties of *EcPutA* mutant H487A. Our results show H487A has a 52-fold lower k_{cat}/K_m (proline) relative to the wild-type enzyme using membrane vesicles as the electron acceptor, whereas in assays using soluble CoQ₁, H487A exhibits only a 6-fold lower k_{cat}/K_m (proline) relative to the wild-type enzyme. The significantly lower activity of H487A with membrane vesicles compared to soluble CoQ₁ indicates H487A is impaired in membrane binding. The physical lipid binding assays show that wild-type *EcPutA* partitions between soluble and lipid bound fractions in a proline dependent manner with the lipid-bound form predominating in the presence of proline. However, H487A remains in the soluble fraction upon the addition of proline, indicating that H487A is less effective at transitioning into the lipid fraction than wild-type

EcPutA. The attenuated ability of H487A to switch from a soluble to a lipid bound protein further supports the hypothesis that H487 is important for maintaining the FAD conformation and the PRODH active site environment. Furthermore, *lacZ* reporter assays showed that cells with H487A had weaker repression and response to proline relative to cells expressing *EcPutA* wild-type. These results indicate that H487A is less capable as a transcriptional regulator than wild-type *EcPutA* *in vivo* despite exhibiting *in vitro* DNA-binding activity.

Rapid-reaction kinetics of H487A provided further insights into the functional deficiency of H487A by revealing changes in microscopic rate constants relative to wild-type *EcPutA*. Although the H487A reductive half-reaction with proline is similar to *EcPutA* wild-type³, we found that the reverse rate constant (k_{-2}) of the chemical step is decreased by ~10-fold in H487A (0.116 s^{-1}) relative to wild-type *EcPutA* (1.6 s^{-1}) (Table 2). The reverse chemical step (k_{-2}) was further investigated by reacting reduced H487A with P5C, the results of which confirmed a significant difference in the reverse step for H487A versus wild-type *EcPutA*.³ Proline reduced wild-type *EcPutA* displays a typical oxidized spectrum upon mixing with P5C (Figure 4A; inset), whereas, proline reduced H487A displays spectra characteristic of a red semiquinone FAD intermediate with a peak at 390 nm (Figure 4A; main). It is also apparent that wild-type *EcPutA* reacts with P5C on a much faster timescale compared to H487A (Figure 4). At the highest P5C concentration used (3.3 mM), proline reduced wild-type *EcPutA* is fully re-oxidized within 5 min (See Figure 4B in³); however, proline reduced H487A continues to slowly react over the course of one hour (Figure 4A; main (red line)), never reaching the fully oxidized form. Spectral deconvolution of the multiwavelength data from the reaction of proline reduced H487A with P5C⁴⁴ clearly shows four different spectra are generated with the proline reduced species dominating followed by flavin red semiquinone as the second most predominant species. The reaction of reduced H487A with CoQ₁, however, did not reveal any spectral differences compared to wild-type (Figure 5A) as no flavin semiquinone intermediates were observed. Thus, H487A mainly appears to impact the reverse step of the proline reductive half-reaction. The 40-mV positive shift in the redox potential of H487A relative to wild-type *EcPutA*, is expected to make the reverse PRODH reaction less favorable thermodynamically, which is consistent with the kinetic data for H487A. In accordance with wild-type *EcPutA*, our kinetic analysis of H487A also revealed that the overall proline:CoQ₁ oxidoreductase activity is rate limited by the oxidative half-reaction with CoQ₁.

Additionally, the forward rate constant (k_3), which was previously assigned to a conformational change required for membrane binding,³ was found to be 3-fold smaller in H487A relative to wild-type *EcPutA* (Table 2). Based on the conserved structural role of His487 in the PRODH active site, it is quite plausible that removal of the imidazole side chain would negatively impact catalytic steps, k_{-2} and k_3 . Potential disruption of interactions in the PRODH active site in the H487A mutant are also expected to impede the subsequent conformational change in *EcPutA* as reflected by a decrease in k_3 . Thus, the kinetic data reveal a specific effect of the H487A mutation on the reversibility of the reductive chemical step (k_{-2}) and subsequent conformational step (k_3), which is consistent with His487 having an important structural role in the PRODH active site as postulated from the X-ray structure

of the *EcPutA*-PROD_H domain and PutA homologs. The slower k_3 for H487A also corroborates the diminished membrane-binding activity of H487A relative to wild-type.

In summary, His487 is postulated to play an important role in the functional switching of *EcPutA* and catalytic mechanism of PROD_H based on its high conservation, direct interaction with the reduced FAD cofactor, and hydrogen bonding to the amide backbone of Leu513. Loss of His487 in H487A results in a significant decrease in the reversibility of the proline reductive half-reaction (k_{-2}) and appearance of red anionic flavin semiquinone, 40-mV positive shift in the flavin redox potential, slower conformational change kinetics observed by a decreased k_3 rate constant, a > 50-fold drop in catalytic efficiency with inverted membrane vesicles as an electron acceptor, deficiency in lipid binding, and inefficient functioning as a transcriptional regulator. Based on its high conservation, we propose His487 will have similar importance in the catalytic mechanisms of PROD_H homologs and subsequently in the functional switching mechanism of other trifunctional PutAs.

ABBREVIATIONS

BjPutA	proline utilization A from <i>Bradyrhizobium japonicum</i>
CD	circular dichroism
CTD	C-terminal domain
EcPutA	proline utilization A from <i>Escherichia coli</i>
FAD	flavin adenine dinucleotide
GSA	glutamate- γ -semialdehyde
GsPutA	proline utilization A from <i>Geobacter sulfurreducens</i>
M	molecular mass
NAD⁺	nicotinamide adenine dinucleotide
<i>o</i>-AB	<i>o</i> -aminobenzaldehyde
PPG	<i>N</i> -propargylglycine
PROD_H	proline dehydrogenase
PCD	protocatechuate dioxygenase
PCA	protocatechuic acid
P5C	¹ -pyrroline-5-carboxylate
P5CDH	P5C dehydrogenase
PutA	proline utilization A
RHH	ribbon-helix-helix

SmPutA	proline utilization A from <i>Sinorhizobium meliloti</i>
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SVD	singular value decomposition
THFA	L-tetrahydro-2-furoic acid

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Lys329 resulting from inactivation with PPG is shown in grey. Previously, the PPG inactivated form of *EcPutA* was shown to mimic the dithionite- and proline-reduced forms of *EcPutA*.¹⁵ Potential hydrogen bonding interactions are shown with dashed lines for the oxidized structure (yellow) and PPG inactivated structure (blue). The adjacent threonine residue (Thr486) is also shown hydrogen bonding to the O2 atom of the phosphate. This hydrogen bond is conserved in all PutA structures known thus far. The main conformational differences in FAD between the overlaid structures are the reorientation of the ribityl chain, which enables the ribityl 2'-OH to hydrogen bond to the FAD N(1) atom, and distinct butterfly bending of the isoalloxazine ring in the reduced structure. These conformational changes have been observed in all structures of reduced PutAs.^{19, 25, 30} (C) Multiple sequence alignment of various PutA/PRODH homologs. The alignment was computed using COBALT on the NCBI server. The *EcPutA* residue His487 is highlighted with an asterisk. Aligned sequences (GenBank Accession ID) shown are *E. coli* PutA (AAB59985), *Pseudomonas putida* PutA (ADR62288), *Bradyrhizobium japonicum* PutA (AHY54390), *Geobacter sulfurreducens* PutA (AAR36785), *Thermus thermophilus* PRODH (WP_014629136), *Homo sapiens* PRODH1 (O43272), *Saccharomyces cerevisiae* Put1p (EDN59681), *Mus musculus* PRODH (EDK97501), *Arabidopsis thaliana* PRODH (OAP05800), and *Drosophila melanogaster* PRODH (AAA02748).

Figure 2.

Functional characterization of *EcPutA* H487A. (A) Lipid pull-down assays with *EcPutA* wild-type and mutant H487A. PutA proteins (0.3 mg/mL) were incubated with *E. coli* polar lipids (0.8 mg/mL) in the absence and presence of 5 mM proline. From the left, lanes 1–4, supernatant (S) and pellet (P) fractions of wild-type *EcPutA* (lanes 1–4) after separation by Air-fuge ultracentrifugation. Lanes 5–8, the supernatant (S) and pellet (P) fractions of the *EcPutA* mutant H487A after separation by Air-fuge ultracentrifugation. (B) Gel mobility shift assays of *EcPutA* wild-type (0, 200, and 500 nM) and H487A mutant (0, 200, and 500 nM) added to binding mixtures containing 2 nM of fluorescent labeled (IRDye-700 label) *put* control DNA (420 bp) and 100 µg/mL of nonspecific calf thymus DNA. Free DNA and PutA-DNA complexes were separated on a nondenaturing 4% polyacrylamide gel. (C) Cell-based transcriptional repressor assays in *E. coli* strain JT31 *putA*⁻ *lacZ*⁻ cells containing the *putC:lacZ* reporter gene plasmid and *EcPutA* wild-type, *EcPutA* mutant H487A, and the pUC18 empty vector (EV). Cells were grown at 37 °C in minimal media supplemented with 0 and 15 mM proline. β-galactosidase activity is reported in Miller units⁴¹ as the average of 10 independent experiments ± standard errors. (D) FAR-UV-CD spectra overlay of *EcPutA* wild-type (red) and H487A (black). Spectra were recorded in 50 mM potassium phosphate buffer (pH 7.4) at 20 °C.

Figure 3.

Rapid reaction kinetics of the H487A proline reductive half-reaction. (A) Oxidized H487A (11.8 μ M) was mixed with 100 mM proline and followed with a photo diode array detector for 7.4 sec. Reduction of the FAD spectrum is shown at time points from 0.001–7.4 sec. (B) Averaged single wavelength traces at 451 nm from rapid mixing of oxidized H487A (10 μ M) with 0.025 M (circles), 0.1 M (squares), 0.4 M (diamonds), 0.8 M (crosses), 1.2 M (omitted for clarity), and 1.5 M (stars) proline were fitted (solid lines) to the simulated proline reductive half-reaction mechanism shown in Scheme 1. (C) Plots showing the normalized error ($\chi^2/\chi^2_{\min}$) between the model simulation and the data, while the rate constants are varied. Dark red shading indicates rate constant values that give the best-fit, while dark blue shading indicates rate constant values that produce poorer fitness.

Figure 4.

Oxidative half-reaction of proline reduced H487A with P5C. (A) (Main panel) After H487A (11.8 μ M after mixing) was anaerobically reduced via proline titration, it was mixed with 3.3 mM DL-P5C (after mixing) and followed with a photodiode array detector for up to 60 min. Shown are spectra at 1 msec, 7.5 min, 24 min, and 60 min. (Inset) Spectral traces of the reaction of proline reduced *EcPutA* wild-type with DL-P5C (3.3 mM final concentration) replotted from a previous study.³ (B) Multiwavelength data in (A) were deconvoluted by singular value decomposition (SVD) using KGE software with singular values assigned as follows: 13 (dashed line), 0.64 (red line), 0.22 (dotted line), and 0.078 (solid line). (C) Single wavelength data at 390 nm were extracted from (A) and fitted to a double exponential equation yielding a $k_{\text{obs}}(1)$ of 0.70 ± 0.05 and a $k_{\text{obs}}(2)$ of $0.031 \pm 0.002 \text{ min}^{-1}$. Data are shown as black circles and the fit is a red line. (D) Single wavelength data at 451 nm was extracted from (A) and fitted to a double exponential equation yielding a $k_{\text{obs}}(1)$ of 0.81 ± 0.06 and a $k_{\text{obs}}(2)$ of $0.057 \pm 0.005 \text{ min}^{-1}$. Data are shown as black circles and the fit is a red line.

Figure 5.

H487A oxidative half-reaction with CoQ₁. (A) After H487A (11.8 μ M after mixing) was anaerobically reduced via proline titration, it was mixed with 60 μ M CoQ₁ (after mixing) and monitored by a photodiode array detector (spectral scans progress from 0.0025 to 25 sec). (B) Single wavelength traces at 451 nm were fitted to the simulated CoQ₁ oxidative half-reaction mechanism shown in Scheme 1, using CoQ₁ concentrations of 50 (circles), 125 (squares), 175 (diamonds), 200 (crosses), and 300 μ M (omitted for clarity). (C) Plots showing the normalized error ($\chi^2/\chi^2_{\min}$) between the model simulation and the data, while the rate constants are varied. Dark red shading indicates rate constant values that give the best-fit, while dark blue shading indicates rate constant values that produce poorer fitness.

Figure 6.

Redox potential determination of H487A. H487A (15 μM) was mixed with 200 μM xanthine, 100 μM methyl viologen (mediator dye), and 5 μM resorufin (indicator dye) in 50 mM potassium phosphate buffer (10 % glycerol, 50 mM NaCl, pH 7.5). Xanthine oxidase was added to initiate reduction of H487A and dye. Spectra were recorded every 2 min over 3 h. (Inset) Nernst plot of oxidized/reduced flavin (H487A) versus oxidized/reduced resorufin from which a redox potential of -67 mV was determined (-0.9 unit slope). The reported redox potential of -65 ± 2 mV is the mean $\pm$ S.D. from three individual experiments.

PutA Overall Reaction **Kinetic Model for PRODH Reaction****Proline reductive half-reaction** **CoQ oxidative half-reaction** **Scheme 1.**

Reactions catalyzed by PutAs and the kinetic mechanism used to describe the proline reductive and CoQ₁ oxidative half-reactions of the *Ec*PutA mutant H487A. Enzyme forms E and F, represent oxidized and 2e⁻ reduced FAD in the soluble conformation, and the oxidized (e) and 2e⁻ reduced FAD (f) in the membrane binding conformation, respectively.³ Note that product release steps were not included in the fitting since they had little to no effect on the simulation but are shown here for reference.

Table 1^a Steady-state Kinetic Constants for *EcPutA* ^b wild-type and H487A

	k_{cat} (s ⁻¹)	K_{m}	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
Proline (CoQ ₁)			
wild-type	3.3 ± 0.1	48 ± 3 mM	70 ± 4
H487A	1.8 ± 0.2	150 ± 29 mM	12 ± 3
CoQ ₁ (proline)			
wild-type	3.4 ± 0.2	110 ± 15 μM	3.1 × 10 ⁴ ± (0.5 × 10 ⁴)
H487A	1.7 ± 0.1	212 ± 32 μM	8 × 10 ³ ± (1 × 10 ³)
Proline (membrane vesicles)			
wild-type	0.43 ± 0.02	1.5 ± 0.3 mM	287 ± 59
H487A	0.083 ± 0.003	15 ± 2 mM	5.5 ± 0.7

^aThe substrate shown in parentheses was held fixed at a saturating concentration while the other substrate was varied.^bData were taken from Moxley et al.²

Table 2Rate Constants Obtained from Global Fitting for *EcPutA* mutant H487A

rate constant	best-fit value	lower bound	upper bound
reductive half-reaction			
k_1	$b_{550} \text{ M}^{-1}\text{s}^{-1} (506)^a$		
k_{-1}	$b_{655} \text{ s}^{-1} (255)$		
k_2	$20.1 \text{ s}^{-1} (27.5)$	$19.6 \text{ s}^{-1} (26)$	$20.8 \text{ s}^{-1} (30)$
k_{-2}	$0.116 \text{ s}^{-1} (1.6)$	$0.0224 \text{ s}^{-1} (1.1)$	$0.296 \text{ s}^{-1} (2.7)$
k_3	$0.668 \text{ s}^{-1} (2.2)$	$0.337 \text{ s}^{-1} (1.5)$	$1.23 \text{ s}^{-1} (3.7)$
k_{-3}	ND		
oxidative half-reaction			
k_5	$b_{107} \text{ mM}^{-1}\text{s}^{-1} (21)$		
k_{-5}	$b_{6.06} \text{ s}^{-1} (2.9)$		
k_6	$1.29 \text{ s}^{-1} (5.4)$	$1.21 \text{ s}^{-1} (4.4)$	$1.41 \text{ s}^{-1} (7.6)$
k_{-6}	$0.026 \text{ s}^{-1} (\text{ND}^c)$	0.013 s^{-1}	0.0605 s^{-1}
k_7	$1.2 \text{ s}^{-1} (4.7)$	$1.07 \text{ s}^{-1} (3)$	$1.41 \text{ s}^{-1} (18)$
k_{-7}	$0.0252 \text{ s}^{-1} (\text{ND}^c)$	No boundary	0.105 s^{-1}

^aValues in parentheses are from the global fitting analysis of wild-type *EcPutA* conducted previously.³^bValues held fixed for the final fitting.^cThis rate constant was pushed to zero for wild-type. ND; Not determined.