Steady-state kinetic mechanism of the proline:ubiquinone oxidoreductase activity of proline utilization A (PutA) from *Escherichia coli*

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**A B S T R A C T**

The multifunctional proline utilization A (PutA) flavoenzyme from *Escherichia coli* performs the oxidation of proline to glutamate in two catalytic steps using separate proline dehydrogenase (PRODH) and \( \Delta^1 \)-pyrroline-5-carboxylate (PSC) dehydrogenase domains. In the first reaction, the oxidation of proline is coupled to the reduction of ubiquinone (CoQ) by the PRODH domain, which has a \( \beta_8 \alpha_8 \)-barrel structure that is conserved in bacterial and eukaryotic PRODH enzymes. The structural requirements of the benzoquinone moiety were examined by steady-state kinetics using CoQ analogs. PutA displayed activity with all the analogs tested; the highest \( k_{cat}/K_m \) was obtained with CoQ. The kinetic mechanism of the PRODH reaction was investigated using a variety of steady-state approaches. Initial velocity patterns measured using proline and CoQ, combined with dead-end and product inhibition studies, suggested a two-site ping-pong mechanism for PutA. The kinetic parameters for PutA were not strongly influenced by solvent viscosity suggesting that diffusive steps do not significantly limit the overall reaction rate. In summary, the kinetic data reported here, along with analysis of the crystal structure data for the PRODH domain, suggest that the proline:ubiquinone oxidoreductase reaction of PutA occurs via a rapid equilibrium ping-pong mechanism with proline and ubiquinone binding at two distinct sites.

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**Introduction**

The oxidation of proline to glutamate is an energetically important pathway that is shared among prokaryotes and eukaryotes [1]. The first step in the pathway is coupled to reduction of the respiratory chain and is catalyzed by proline dehydrogenase (PRODH), a membrane-associated flavoenzyme, which is localized in the mitochondria. The importance of proline as a metabolic fuel is well documented in bacteria [2–5], yeast [6], parasites [7], insects [8,9], plants [10] and mammals [4,11,12]. For example, in bacterioids, increased proline oxidative flux is correlated with higher N\(_2\) fixation rates and seed yields [13]. Proline oxidative metabolism has also been shown to be critical for *Helicobacter pylori* colonization of the gut and in the closely related mouse pathogen *Helicobacter hepaticus* [14,15]. In fact, Nagata et al. have shown that patients infected with *H. pylori* have 10-fold higher proline levels than non-infected individuals, and that the pathogen uses \( L \)-proline as a preferred respiratory substrate in this biological niche [5].

In Gram-negative bacteria, proline oxidation is catalyzed by PutA, a bifunctional enzyme that combines PRODH and NAD*-dependent \( \Delta^1 \)-pyrroline-5-carboxylate (PSC) dehydrogenase (P5CDH) domains in the same polypeptide to catalyze the conversion of proline to glutamate (see Scheme 1A) [2,16–18]. In other organisms such as Gram-positive bacteria and eukaryotes, PRODH and P5CDH are separate enzymes.

Structural studies on PutA have shown that the PRODH domain has a core (\( \beta_8 \alpha_8 \) barrel structure that noncovalently binds the flavin adenine dinucleotide (FAD) cofactor (Fig. 1A). X-ray crystal structures of the PRODH domain of PutA have shown previously that the proline analog, \( L \)-tetrahydro-2-furoic acid (\( L \)-THFA), binds to the active site and mimics proline binding [19]. The carboxylate group of \( L \)-THFA is coordinated by two active site arginine residues (Arg555 and 556) that help position the proline analog near the surface of flavin (Fig. 1A). The (\( \beta_8 \alpha_8 \) barrel core is also found in monofunctional PRODHs from Gram-positive bacteria and is predicted to be the catalytic core in human PRODH [20].
Typical of flavoenzyme mechanisms, the redox steps catalyzed by the PutA/PRODH domain can be divided into reductive and oxidative half-reactions. In the reductive half-reaction, two electrons from proline are transferred to the flavin cofactor, whereas in the oxidative half-reaction, two electrons are transferred from reduced flavin to an electron acceptor such as ubiquinone (Scheme 1A). After P5C is hydrolyzed to γ-glutamate semialdehyde (GSA), the PutA/P5CDH domain catalyzes the oxidation of γ-GSA to yield glutamate [2,16,17].

In certain Gram-negative bacteria such as Escherichia coli, PutA also contains a N-terminal ribbon-helix-helix DNA binding domain that endows PutA with transcriptional regulatory activity [21–23]. The E. coli PutA polypeptide has 1320 residues with the DNA-binding domain localized at residues 1–52, 261–612, and 650–1130, respectively [1,22]. As a DNA-binding protein, E. coli PutA represses transcription of the putA and putP (Na+/proline transporter) genes when intracellular proline levels are low [23]. Increases in proline levels induce PutA to bind to the cytoplasmic membrane where it catalyzes the oxidation of proline to glutamate [23]. The mechanism by which PutA switches from a transcriptional repressor to a membrane-bound enzyme relies on conformational changes that dramatically enhance PutA-membrane interactions [18,24–28].

Although the importance of proline oxidation in bioenergetics is well known, the catalytic mechanism of PutA/PRODH has not been examined in detail. Therefore, we have investigated the PRODH activity in PutA using a variety of steady-state kinetic approaches. This study is the first detailed report on the kinetic mechanism of PutA/PRODH activity and will be helpful for understanding the kinetic properties of other PutAs as well as monofunctional PRODHs.

Materials and methods

Materials

All chemicals and buffers were purchased from Fisher Scientific and Sigma–Aldrich, unless otherwise noted. Atpenin A5 (3-(2S,4S,5R)-5,6-dichloro-2,4-dimethyl-1-oxohexyl)-4-hydroxy-5,6-dimethoxy-2(1H)-pyridinone) was purchased from Enzo life sciences. E. coli strains XL-Blue and BL21(DE3)pLysS were purchased from Novagen. Inverted membrane vesicles were prepared from E. coli strain JT31 putA–29 [29]. 2-oxo-P5C, which is not commercially available, was synthesized according to the method of Williams and Frank and stored in 1 M HCl at 4 °C [30]. PSC concentrations were determined by measuring the formation of the covalent complex with o-aminobenzaldehyde (o-AB) at 443 nm (ε443nm = 2590 M⁻¹ cm⁻¹) [30]. PutA was expressed and purified as previously described [26,27]. PutA was further purified by anion exchange chromatography and eluted from the column (HiTrap Q HP, GE Healthcare) using a 0–1 M NaCl gradient (1 L) in 50 mM Tris (pH 7.5), 0.5 mM EDTA, and 5% glycerol. Purified PutA was dialyzed into 50 mM potassium phosphate buffer (pH 7.4) containing 10% glycerol, 50 mM NaCl, and stored at −80 °C. All experiments used Nano-pure water.

Alternative substrate and initial velocity pattern

The concentration of wild-type PutA was determined spectrophotometrically using a molar extinction coefficient of 12,700 M⁻¹ cm⁻¹ at 451 nm [31]. Steady-state kinetic parameters for duroquinone (tetramethyl-p-benzoquinone), menadione (2-methyl-1,4-naphthoquinone), and ubiquinone analogs, CoQ1, CoQ2, and CoQ4 were measured by the decrease in quinone absorbance in the UV range using the following molar extinction coefficients for the quinones: duroquinone (ε271nm = 18.5 mM⁻¹ cm⁻¹), menadione (ε262nm = 14 mM⁻¹ cm⁻¹), CoQ1 (ε278nm = 14.5 mM⁻¹ cm⁻¹), CoQ2 (ε282nm = 8 mM⁻¹ cm⁻¹), and CoQ4 in 468 μM Triton X-100 (ε282nm = 16.6 mM⁻¹ cm⁻¹). Assays were performed in 50 mM HEPES (pH 7.4) at 20 °C with 200 mM proline, 0.5 μM PutA enzyme, and varying concentrations of quinone. Triton X-100 (468 μM) was included in assays using CoQ4. Stock solutions of the quinones were made in ethanol. The final ethanol concentration in the assays was ≤2%. Assays to obtain steady-state kinetic parameters for proline using different quinones were performed in 50 mM HEPES (pH 7.4) with 0.5 μM PutA, with the product P5C monitored.

![Scheme 1. Overall reaction catalyzed by PutA. (A) (Top) The PRODH domain of PutA catalyzes the proline:ubiquinone oxidoreductase reaction which involves the oxidation of proline and subsequent reduction of CoQ. (Bottom) The P5CDH domain of PutA catalyzes the oxidation of glutamate semialdehyde to glutamate with the concomitant reduction of NAD⁺. (B) Chemical structures of CoQs, ubiquinone analogs, and THFA.](image-url)
using the o-AB reaction [30]. Assays were performed using quinone concentrations at least 3 times higher than the respective Km value for each quinone. Steady-state kinetic parameters for proline obtained with inverted E. coli JT31 membrane vesicles were performed in 20 mM MOPS (pH 7.5) containing 10 mM MgCl2, 4 mM o-AB, 0.25 μM enzyme and 80 μg/ml of vesicles. The kinetic parameters Km and kcat were estimated by nonlinear regression analysis of the initial reaction velocity versus proline or quinone concentration using the Michaelis–Menten equation.

To determine the order of substrate binding and product release, steady-state kinetic parameters were determined by varying the proline concentration (5–200 mM), constant CoQ1 (200 μM), and different fixed concentrations (0.2, 2.6, and 10 mM) of the inhibitor, L-THFA, were fitted to the nonlinear form of the competitive inhibition equation, Eq. (2), where [S] is the varied initial proline concentration, Km is the Michaelis–Menten constant for proline, [I] is the l-THFA concentration, and KI is the competitive inhibition constant for l-THFA.

\[
\frac{v}{[E_T]} = \frac{k_{cat}[A]}{K_{m_a} + [A]\left(1 + \frac{K_{m_b}}{[B]}\right)}
\]

(1)

**Dead-end inhibition**

Dead-end inhibition initial velocity experiments were conducted by varying substrate concentration at different fixed amounts of inhibitor. All assays were performed in 50 mM HEPES (pH 7.4) at 20 °C and followed by the decrease in absorbance at 278 nm from CoQ1 as above. To determine the type of inhibition and respective inhibition constants, data were fitted globally to the nonlinear forms of the competitive, noncompetitive, uncompetitive, and mixed inhibition equations along with their partial inhibition counterparts using the Enzyme Kinetics Module (1.1) from SYSTAT. Models that were selected to best represent the data are described below.

Initial velocity data with varying amounts of proline (5–200 mM), constant CoQ1 (200 μM), and different fixed concentrations (0.2, 2.6, and 10 mM) of the inhibitor, L-THFA, were fitted to the nonlinear form of the competitive inhibition equation, Eq. (2), where [S] is the varied initial proline concentration, Km is the Michaelis–Menten constant for proline, [I] is the l-THFA concentration, and KI is the competitive inhibition constant for l-THFA.

\[
\frac{v}{[E_T]} = \frac{k_{cat}[S]}{K_{m}(1 + \frac{[I]}{K_I}) + [S]}
\]

(2)

Initial velocity data with varying amounts of CoQ1 (5–300 μM), constant proline (100 mM), and different fixed concentrations of l-THFA (0.3, 0.6, 1.2, and 3.0 mM) were fitted to the uncompetitive inhibition model, Eq. (3), where [S] is the initial varied CoQ1.

**Fig. 1.** Structure of the PRODH domain of *E. coli* PutA (PDB 1tiw). (A) The (βα)8 barrel core structure of the PRODH domain is shown, highlighting the locations of the FAD cofactor (yellow) and THFA (cyan) bound at the si-face of FAD. (B) Illustration of the tight packing of the re-face of the FAD cofactor against β strands 4–6 (blue). Figures were generated with PyMOL [55]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Fig. 2.** Initial velocity pattern for PutA with proline as the variable substrate and CoQ1 as the fixed substrate. (A) Non-linear least squares fit to the ping-pong equation, Eq. (1), with 15 (circles), 25 (triangles), 50 (squares), and 200 μM (diamonds) CoQ1 and varied proline. The best fit parameters were kcat = 5.2 ± 0.3 s⁻¹, Km = 42 ± 4 mM proline, and Kinh = 112 ± 1μM CoQ1. (B) Double reciprocal plot of the fitted data in panel A with 15, 25, 50 and 200 μM CoQ1, from top to bottom.
concentration, $K_m$ is the Michaelis–Menten constant for CoQ$_1$, $[I]$ is the $\nu$-THFA concentration and $K_i$ is the uncompetitive inhibition constant [22].

$$ \frac{v}{[E]_i} = \frac{K_{cat}[S]}{K_m + [S](1 + \frac{[I]}{K_i})} \quad (3) $$

Initial velocity data with varying amounts of CoQ$_1$ (5–300 μM), constant proline (100 mM), and different fixed concentrations of Atopenin A5 (0, 20, 100, and 300 μM) were fitted to Eq. (2). Initial velocity data with varying amounts of proline (6.67–200 mM) constant CoQ$_2$ (110 μM) and fixed concentrations of Atopenin A5 (0, 25, and 300 μM) were fitted to Eq. (3), where $[S]$ is the varied proline concentration, $K_m$ is the Michaelis–Menten constant for proline, $[I]$ is the Atopenin A5 concentration, and $K_i$ is the uncompetitive inhibition constant. Dead-end inhibition data were also analyzed by Hanes–Woolf plots [33].

**Product inhibition**

Product inhibition studies were conducted by varying substrate concentrations at different fixed concentrations of a single product. All assays were conducted in 50 mM HEPES (pH 7.4) at 20 °C and followed by the decrease in absorbance at 278 nm from CoQ$_1$ as above using the Enzyme Kinetics Module (1.1) from SYSTAT. The data were fitted globally to all of the inhibition models mentioned above using the Enzyme Kinetics Module (1.1) from SYSTAT. The best fit models are described below. A product inhibition pattern was obtained by varying proline in fixed concentrations of PSC (0, 1.17, and 3.32 mM) and was best fit to the competitive inhibition model, Eq. (2). CoQ$_1$ was then varied in fixed PSC (0, 1.17, and 3 mM) and best fit to the uncompetitive inhibition model, Eq. (3). Product inhibition data were also analyzed by Hanes–Woolf plots [33].

Product inhibition by CoQ$_2$H$_2$ was tested in assays varying CoQ$_2$ with fixed proline (30 mM) using a Cary 100 spectrophotometer inside an anaerobic glove box. CoQ$_2$H$_2$ was prepared from CoQ$_2$ by the method of Rieske under anaerobic conditions [33]. The concentration of CoQ$_2$H$_2$ was determined by the absorbance at 290 nm with $e_{290\text{nm}} = 4.14 \text{mM}^{-1} \text{cm}^{-1}$ [35].

**Solvent viscosity effects on steady-state parameters**

The effect of solvent viscosity on steady-state kinetic parameters was examined by varying the concentration of proline (0–400 mM) in assays with fixed CoQ$_1$ (300 μM) and increasing concentrations of $\nu$-sucrose (0.06, 0.65, 1.12, and 1.36 M) in 50 mM Tris (pH 7.5). The solvent viscosity relative to water at 20 °C ($\eta_{rel}$) was estimated to be 1, 2, 4, and 6, for assays with 0.06, 0.65, 1.12, and 1.36 M sucrose, respectively [36]. Data were fitted globally to Eq. (4), where $k_{cat}$ and $K_m$ have their usual meanings, $[S]$ represents the concentration of proline, $\eta_{rel}$ is the relative viscosity to water mentioned above, $m$ is a factor for the viscosity effect on $k_{cat}/K_m$, and $n$ is a factor for the viscosity effect on $k_{cat}$ [37]. Initial fitting gave $m = 7.6 \times 10^{-10}$. Thus, data were fitted with $m = 0$ and only $n$ was determined.

$$ \frac{v}{[E]_i} = \frac{K_{cat}[S]}{K_m + [S](1 + n[\eta_{rel} - 1])} \quad (4) $$

**PutA tryptophan fluorescence quenching**

The binding of the quinone analog, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) (Scheme 1B), to wild-type PutA was examined by intrinsic tryptophan fluorescence quenching of PutA. PutA (1 μM, 0.6 ml volume) in 50 mM potassium phosphate (pH 7.5) was excited at 295 nm while the fluorescence emission spectrum between 300 and 500 nm was collected using a Varian Eclipse spectrophotofluorometer. The PutA solution was titrated with increasing concentrations of DBMIB (0–20 μM) and the decrease in the fluorescence emission maximum of PutA at 338 nm was monitored. The observed fluorescence at 338 nm was corrected for the inner filter effect caused by the absorption of incident light by DBMIB at 295 nm using Eq. (5) [38]. In eq. (5), $F_{corr}$ and $F_{obs}$ are the corrected and observed fluorescence intensities, respectively, and $A_{rel}$ and $A_{o}$ are the respective absorbance values of DBMIB at the excitation (295 nm) and emission (338 nm) wavelengths.

$$ F_{corr} = F_{obs} \frac{A_{rel}}{A_{o}} $$

An equilibrium dissociation constant ($K_d$) for the PutA–DBMIB complex was determined by fitting the changes in fluorescence to Eq. (6), where $\theta$ is the fraction of DBMIB bound and $n$ is the number of binding sites.

$$ \theta = \frac{n[DDBMIB]_{free}}{K_d + [DBMIB]_{free}} \quad (6) $$

The concentration of free DBMIB ([DBMIB]$_{free}$) was calculated by Eq. (7).

$$ [DBMIB]_{free} = [DBMIB]_{total} - \theta[PutA]_{total} \quad (7) $$

$\theta$ was determined by $(F_0 - F)/(F_0 - F_{max})$, where $F_0$, $F$, and $F_{max}$ are the fluorescence intensities in the absence, presence, and extrapolated infinite amount of DBMIB, respectively. $F_{max}$ was determined by fitting the normalized fluorescence intensity to a general hyperbolic function.

**Incubation of PutA with CoQ$_2$ and CoQ$_2$H$_2$**

PutA (60 μM) in 50 mM potassium phosphate buffer (pH 7.4, 50 mM NaCl, and 10% glycerol) was incubated with increasing amounts of CoQ$_2$ up to a final concentration of 50 μM CoQ$_2$. The spectrum of the final PutA (60 μM)-CoQ$_2$ (50 μM) mixture was recorded on a Cary 100 spectrophotometer (300–700 nm). To subtract the spectrum of CoQ$_2$, a 50 μM CoQ$_2$ solution without PutA was used to baseline the instrument. To test PutA activity with CoQ$_2$H$_2$, oxidized PutA (20 μM after mixing) was degassed and mixed with 260 μM of CoQ$_2$H$_2$ under anaerobic conditions in the glove box. After mixing, changes in absorbance of the FAD cofactor were monitored (300–700 nm) for 45 min using a Cary 100 spectrophotometer in the glove box and spectral kinetics software.

<table>
<thead>
<tr>
<th>Analog</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duroquinone</td>
<td>147 ± 27</td>
<td>1.2 ± 0.1</td>
<td>8400 ± 1700</td>
</tr>
<tr>
<td>Menadione</td>
<td>79 ± 5</td>
<td>3.5 ± 0.1</td>
<td>44,000 ± 3100</td>
</tr>
<tr>
<td>CoQ$_2$</td>
<td>110 ± 15</td>
<td>3.4 ± 0.2</td>
<td>31,000 ± 4600</td>
</tr>
<tr>
<td>CoQ$_2$H$_2$</td>
<td>16 ± 4</td>
<td>1.4 ± 0.1</td>
<td>87,000 ± 23,000</td>
</tr>
<tr>
<td>CoQ$_4$</td>
<td>34 ± 4</td>
<td>0.22 ± 0.04</td>
<td>6700 ± 1400</td>
</tr>
</tbody>
</table>

* Assays were performed using 200 mM proline.
Results

Alternative substrates and initial velocity pattern

The kinetic parameters $K_m$ and $k_{cat}$ for PutA with different ubiquinone analogs were determined using a saturating concentration of proline. Table 1 shows $k_{cat}$ values in the range 0.2–3.5 s$^{-1}$ with the lowest activity observed with CoQ4. The highest catalytic efficiency ($k_{cat}/K_m$) was observed with menadione and CoQ2. These results indicate that although the isoprene side chain is not essential for PutA reactivity with quinones, it does influence the catalytic efficiency of PutA.

Table 2 shows that the $k_{cat}/K_m$ for proline with different electron acceptors under saturating conditions varies from 36 M$^{-1}$s$^{-1}$ with menadione to 287 M$^{-1}$s$^{-1}$ using membrane vesicles.

The steady-state reaction mechanism of PutA was investigated by performing assays in which CoQ1 (15–200 µM) and proline (5–200 mM) concentrations were varied. Fig. 2 shows that the data fit well to the ping-pong mechanism, Eq. (1). The kinetic parameters were estimated by fitting the data to a ping-pong mechanism with $k_{cat}$ of 5.2 s$^{-1}$ and $K_m$ values of 42 mM and 112 µM for proline and CoQ1, respectively. Slight substrate inhibition by proline was observed in assays using low CoQ1 (15 µM) and high proline concentration (200 mM).

Dead-end and product inhibition experiments

Dead-end inhibition studies were performed to rule out an obligatory ternary mechanism. The inhibition of PutA was first evaluated with L-THFA, an isostructural analog of proline (see Scheme 1B). The type of inhibition and $K_I$ values for L-THFA versus proline and CoQ1 are listed in Table 3. L-THFA is a competitive inhibitor of PutA with respect to proline (Fig. S1A). When varying CoQ1, L-THFA shows inhibition of PutA that is best fit by an equation for uncompetitive inhibition (Fig. 3A).

Fig. 4. Product inhibition pattern with P5C. (A) A non-linear least squares fit to the competitive inhibition equation, Eq. (2), with 0 (circles), 1.17 (open circles), 3.3 mM (inverted triangles) P5C and varied proline. Best fit parameters were $k_{cat} = 1.40 ± 0.04$ s$^{-1}$, $K_m = 36.7 ± 3.3$ µM CoQ1, and $K_I = 1.0 ± 0.07$ mM P5C. Note that these assays contained approximately 500 mM NaCl which diminished $k_{cat}$.

Table 2

<table>
<thead>
<tr>
<th>Analog</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duroquinone</td>
<td>27 ± 2</td>
<td>1.00 ± 0.02</td>
<td>37 ± 2.8</td>
</tr>
<tr>
<td>Menadione</td>
<td>67 ± 6</td>
<td>2.4 ± 0.1</td>
<td>36 ± 3.6</td>
</tr>
<tr>
<td>CoQ$_1$</td>
<td>47.5 ± 2.5</td>
<td>3.30 ± 0.05</td>
<td>70 ± 3.8</td>
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<tr>
<td>CoQ$_2$</td>
<td>9.6 ± 1.3</td>
<td>1.10 ± 0.03</td>
<td>115 ± 16</td>
</tr>
<tr>
<td>CoQ$_4$</td>
<td>2.3 ± 0.5</td>
<td>0.20 ± 0.01</td>
<td>87 ± 19</td>
</tr>
<tr>
<td>Membrane vesicles</td>
<td>1.5 ± 0.3</td>
<td>0.43 ± 0.02</td>
<td>287 ± 59</td>
</tr>
</tbody>
</table>

* Parameters were estimated from assays which follow o-AB-P5C complex formation.

Fig. 3. Dead-end inhibition initial velocity patterns for PutA. (A) Uncompetitive inhibition of PutA by THFA versus CoQ$_1$. Non-linear least squares fit to the uncompetitive inhibition equation, Eq. (3), displayed as a Hanes–Woolf plot with 0 (circles), 0.3 (open circles), 0.6 (inverted triangles), and 1.2 mM (open inverted triangles) L-THFA and varied CoQ$_1$. The best fit parameters were $K_m = 130 ± 8$ µM CoQ$_1$, $k_{cat} = 4.4 ± 0.1$ s$^{-1}$, $K_I = 0.39 ± 0.02$ mM. (B) Uncompetitive inhibition of PutA by Atpenin A5 versus proline. A non-linear least squares fit to Eq. (3) displayed as a Hanes–Woolf plot with 0 (open circles), 25 (closed circles), and 300 µM (inverted triangles) Atpenin A5 and varied proline. Best fit parameters are $k_{cat} = 3.7 ± 0.1$ s$^{-1}$, $K_m = 60 ± 3$ mM proline, and $K_I = 124 ± 8$ µM Atpenin A5.

Table 3

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Variable substrate</th>
<th>Type of inhibition</th>
<th>$K_I$ (mM)</th>
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<tbody>
<tr>
<td>THFA</td>
<td>Proline</td>
<td>Competitive</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>THFA</td>
<td>CoQ1</td>
<td>Uncompetitive</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>Atpenin A5</td>
<td>Proline</td>
<td>Uncompetitive</td>
<td>124 ± 2</td>
</tr>
<tr>
<td>Atpenin A5</td>
<td>CoQ1</td>
<td>Competitive</td>
<td>97 ± 8</td>
</tr>
<tr>
<td>P5C</td>
<td>Proline</td>
<td>Competitive</td>
<td>0.64 ± 0.04</td>
</tr>
<tr>
<td>P5C</td>
<td>CoQ1</td>
<td>Uncompetitive</td>
<td>1.0 ± 0.07</td>
</tr>
</tbody>
</table>

Fig. 3A Hanes–Woolf replot of the data shows a parallel line pattern indicating P5C is competitive with proline.

Fig. 4. Product inhibition pattern with P5C. (A) A non-linear least squares fit to the competitive inhibition equation, Eq. (2), with 0 (circles), 1.17 (open circles), 3.3 mM (inverted triangles) P5C and varied proline. Best fit parameters were $k_{cat} = 1.40 ± 0.04$ s$^{-1}$, $K_m = 36.7 ± 3.3$ µM CoQ1, and $K_I = 1.0 ± 0.07$ mM P5C.
Inhibition of PutA with the ubiquinone analog, Atpenin A5 [39], was examined by varying proline and CoQ1 (see Scheme 1B). Atpenin A5 is competitive versus CoQ with $K_i = 97 \mu M$ (Table 3 and Fig. S1B). With respect to proline, Atpenin A5 exhibited uncompetitive inhibition of PutA (Table 3 and Fig. 3B). The results from the steady-state dead-end inhibition profiles using L-THFA and Atpenin A5 are consistent with a ping-pong mechanism [40].

Product inhibition studies were next performed to help distinguish between one- and two-site ping pong mechanisms. Dead-end inhibition patterns for one-site and two-site ping pong mechanisms are identical so they cannot distinguish between these two mechanisms [40]. Product inhibition, however, exhibits opposite patterns [40]. The inhibition of PutA with the products, P5C and CoQ$_1$, was studied under conditions in which proline or CoQ$_1$ was varied. The inhibition patterns of P5C versus proline and PSC versus CoQ$_1$ were best fit to competitive and uncompetitive inhibition models, respectively (Table 3 and Fig. 4). The product inhibition pattern with PSC is suggestive of a two-site ping-pong mechanism.

The effect of CoQ$_1$H$_2$ on PRODH activity was next examined but inhibition was not observed with up to 800 $\mu M$ CoQ$_1$H$_2$. CoQ$_1$H$_2$ (260 $\mu M$) was also incubated with PutA (20 $\mu M$) under anaerobic conditions with the FAD spectrum of oxidized PutA monitored for possible reduction by CoQ$_1$H$_2$. After 45 min of incubation, reduction of the FAD cofactor by CoQ$_1$H$_2$ was not apparent (Fig. S2).

**Viscosity dependence of the steady-state parameters**

To explore whether diffusive steps are rate limiting during turnover, the effect of viscosity on $k_{cat}/K_m$ and $k_{cat}$ was analyzed by performing steady-state assays in the presence of increasing amounts of the viscogen, $\alpha$-sucrose [37,41]. $\alpha$-Sucrose was added to the assay buffer to generate viscosities relative to water ($\eta_{rel}$, 20 $^\circ$C) of 1, 2, 4, and 6. Fig. 5A shows the effect of viscosity on $k_{cat}$ by fitting the steady-state kinetic parameters to Eq. (4). Fitting the data globally to Eq. (4) gave $n = 0.15 \pm 0.02$, indicating that increasing viscosity in the medium decreases $k_{cat}$ by 15% of its initial value when $\eta_{rel}$ increases by one unit. The change in $k_{cat}$ as a function of $\eta_{rel}$ was also evaluated by plotting the ratio of $(k_{cat}) \div (k_{cat})$ against $\eta_{rel}$ (20 $^\circ$C), where $(k_{cat})$ and $(k_{cat})$ are the catalytic constants determined in the absence and presence of viscogen, respectively (Fig. S5B). If $k_{cat}$ is 100% diffusion limited, the slope of this plot will be unity; otherwise, the slope will be $<$1. The slope of the plot of $(k_{cat}) \div (k_{cat})$ versus $\eta_{rel}$ is 0.2 indicating a small decrease in $k_{cat}$ with increased viscosity of the solution. From these results, it appears a product diffusion step limits $k_{cat}$ by about 20%.

**Spectroscopic analysis of quinone binding**

The binding of quinone to PutA was studied by fluorescence quenching using the brominated quinone analog, DBMIB, which PutA can utilize as an alternate substrate ($k_{cat} = 4 s^{-1}$). Previous studies have shown that DBMIB is superior to CoQ in quenching protein tryptophan fluorescence, thus, making it a useful analog for monitoring quinone-protein binding [42]. DBMIB caused quenching of PutA tryptophan fluorescence at 338 nm in a saturating manner, from which a $K_d$ of 5.7 $\pm$ 0.5 $\mu M$ ($n = 0.88 \pm 0.03$) for the DBMIB–PutA complex was estimated (Fig. 6).

To gain insight into the spatial relationship between quinone and proline binding sites, the DBMIB test was repeated in the presence of THFA. As described above, THFA is a competitive inhibitor with respect to proline and as shown in Fig. 1A, THFA is known to occupy the proline binding site [19]. Thus, if proline and quinone have overlapping binding sites, one expects the $K_d$ for DBMIB to increase in the presence of THFA. The $K_d$ value for DBMIB binding to PutA in the presence of 200 mM THFA is 4.9 $\pm$ 0.4 $\mu M$ ($n = 0.83 \pm 0.03$), which is nearly identical to the value obtained in the absence of THFA. This result is consistent with proline and ubiquinone having separate binding sites.

Given that substrate binding to flavoproteins often perturbs the flavin spectrum, oxidized PutA was titrated with CoQ$_2$ [43]. The flavin spectrum was unperturbed, suggesting that CoQ$_2$ does not significantly influence the flavin active site environment (Fig. S3). Addition of DBMIB to PutA also did not perturb the flavin spectrum. Because DBMIB is an alternate substrate and is shown to bind PutA by tryptophan fluorescence (Fig. 6), the lack of changes in the flavin spectrum with CoQ$_2$ or DBMIB is not due to the absence of binding, but rather suggests that the ubiquinone binding site is located at a position that is too distant from the isoalloxazine ring to significantly alter the flavin spectrum.

**Discussion**

The $k_{cat}/K_m$ values for the ubiquinone analogs varied by 10-fold with the lowest and highest values determined for CoQ$_2$ and CoQ$_3$, respectively. The $k_{cat}$ values were similar with all the ubiquinone analogs tested, with CoQ$_6$ being the exception. The physiological ubiquinone substrate in the E. coli cytoplasmic membrane has 8 isoprene units (CoQ$_9$). Thus, it is somewhat surprising that CoQ$_4$ appears to be the least efficient substrate. However, the presence of a membrane or a detergent micelle could considerably affect the outcome of such studies [44]. Interestingly, PutA is able to utilize duroquinone indicating that the isoprene side chain is not essential for PutA reactivity with quinones. Other studies have drawn similar conclusions with the efficacy of the isoprene side chain length [42,45]. Slight substrate inhibition was observed

![Fig. 5. Effect of increasing solvent viscosity on steady-state kinetics. (A) Non-linear least squares fit to Eq. (4) with $0.0, 0.65, 1.12, 1.36 M$ D-sucrose which corresponds to a viscosity relative to water at 20 $^\circ$C ($\eta_{rel}$) of 1, 2, 4, and 6 in varied proline concentrations [35]. (B) The ratio $(k_{cat}) \div (k_{cat})$ was plotted against $\eta_{rel}$, where $(k_{cat})$ is the catalytic constant in the absence of viscogen, $(k_{cat})$ is the catalytic constant in the presence of viscogen, and $\eta_{rel}$ is the relative viscosity to water at 20 $^\circ$C. The data were fitted to a line to yield a slope value of $0.2 \pm (5 \times 10^{-5})$ for which slope $= \Delta(k_{cat}) \div (k_{cat})/A(\eta_{rel})$.](image-url)
necessary for proper conclusions to be drawn as ternary mechanisms and product inhibition studies were not conducted, which are necessary for proper conclusions to be drawn as ternary mechanisms and product inhibition studies were not conducted, which is not competent in catalysis. Other enzymes that adhere to a two-site ping pong mechanism have displayed more competitive substrate inhibition including the original mechanism by Northrop [46].

Our kinetic data with E. coli PutA show evidence for a two-site ping-pong mechanism for the proline:ubiquinone oxidoreductase reaction. A previous study with PutA from Salmonella typhimurium indicated a ping pong mechanism as well [17], however, dead-end and product inhibition studies were not conducted, which are necessary for proper conclusions to be drawn as ternary mechanisms can still produce parallel lines on a Lineweaver–Burk plot [32]. Furthermore, product inhibition studies are required to further distinguish between one-site and two-site ping-pong mechanisms [40]. Our results with the dead-end inhibitors show THFA is competitive with respect to proline as expected from the X-ray structure of the PRODH domain (Fig. 1A), and uncompetitive with respect to CoQ1, indicating a ping pong mechanism as well [17].

Dead-end and product inhibition patterns are rate limiting [41]. It was found that the viscosity effect on $k_{cat}/K_m$ was negligible, while $k_{cat}$ decreased 15–20% per unit viscosity. The lack of a strong viscosity effect on the kinetic parameters suggests that the overall reaction is not limited by diffusive steps, but most likely by a chemical step. The viscosity data also supports a rapid equilibrium assumption for the steady-state mechanism of PutA.

In summary, our steady-state kinetic data show a rapid equilibrium two-site ping pong mechanism for proline:ubiquinone oxidoreductase activity in PutA. Because sequence motifs in the PRODH catalytic core that are important for FAD and proline binding are shared among bacterial PutAs and eukaryotic PRODH enzymes, we predict that eukaryotic enzymes such as human PRODH will follow a similar kinetic mechanism found for E. coli PutA [1].

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Appendix A. Supplementary data


References


Fig. 6. Tryptophan fluorescence quenching of PutA with DBMIB. One micro molar solution of PutA was titrated with DBMIB while monitoring tryptophan fluorescence (ex. 295 nm, em 338 nm). Data were fitted to Eq. (6). Inner filter effects caused by DBMIB were corrected as discussed in materials and methods.