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

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ABSTRACT

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 Δ^1-pyrroline-5-carboxylate (P5C) 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_2. The kinetic mechanism of the PRODH reaction was investigated use a variety of steady-state approaches. Initial velocity patterns measured using proline and CoQ_1, 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.

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),[1] a membrane-associated flavoenzyme, which in eukaryotes 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 bacteriods, 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 Δ^1-pyrroline-5-carboxylate (P5C) 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 $(\beta\alpha)_8$ 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 *si*-face of flavin (Fig. 1A). The $(\beta\alpha)_8$ barrel core is also found in monfunctional PRODHs from Gram-positive bacteria and is predicted to be the catalytic core in human PRODH [20].

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[1] *Abbreviations used*: FAD, flavin adenine dinucleotide; *put*, proline utilization; NAD^+, nicotinamide adenine dinucleotide; PRODH, proline dehydrogenase; P5CDH, Δ^1-pyrroline-5-carboxylate dehydrogenase; P5C, Δ^1-pyrroline-5-carboxylate; THFA, tetrahydro-2-furoic acid; DCPIP, dichlorophenolindophenol; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; GSA, γ-glutamate semialdehyde; PCD, protocatechuate dioxygenase; PCA, protocatechuic acid; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; BCA, bicinchoninic acid; CoQ, ubiquinone; *o*-AB, *o*-aminobenzaldehyde; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone.

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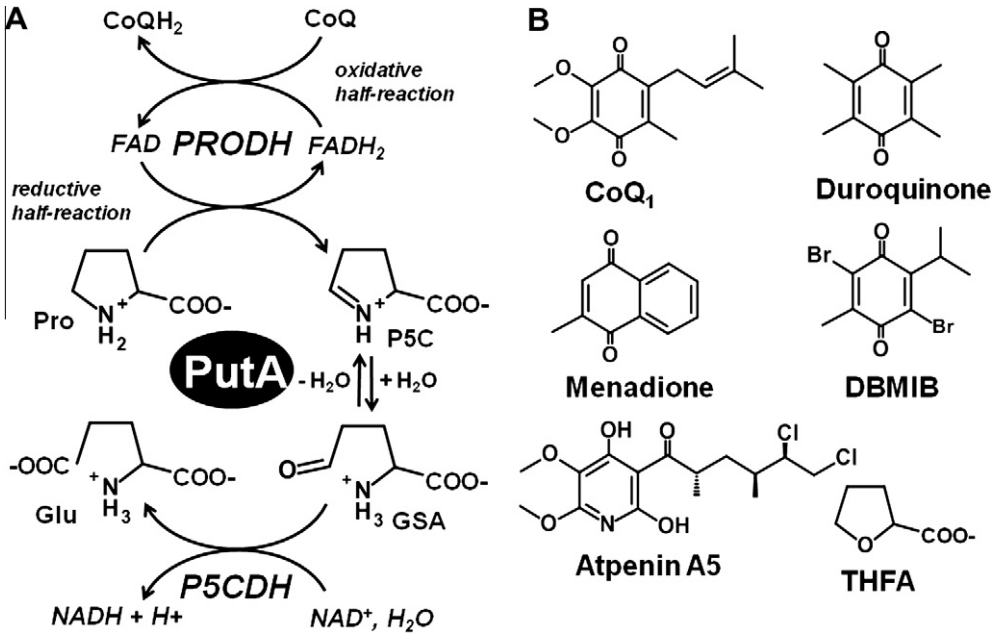

Scheme 1. Overall reaction catalyzed by PutA. (A) (Top) The PRODH domain of PutA catalyzes the proline:ubiquinone oxidoreductase reaction which involves the oxidation of proline and subsequent reduction of CoQ. (Bottom) The P5CDH domain of PutA catalyzes the oxidation of glutamate semialdehyde to glutamate with the concomitant reduction of NAD^+. (B) Chemical structures of CoQ_1, ubiquinone analogs, and THFA.

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, PRODH and P5CDH domains 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 proline mediated reduction of the flavin cofactor and subsequent 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]. DL-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]. P5C concentrations were determined by measuring the formation of the covalent complex with *o*-aminobenzaldehyde (*o*-AB) at 443 nm (ε_{443nm} = 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 Nanopure 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, CoQ_1, CoQ_2, and CoQ_4 were measured by the decrease in quinone absorbance in the UV range using the following molar extinction coefficients for the quinones: duroquinone (ε_{271nm} = 18.5 $mM^{-1}cm^{-1}$), menadione (ε_{262nm} = 14 $mM^{-1}cm^{-1}$), CoQ_1 (ε_{278nm} = 14.5 $mM^{-1}cm^{-1}$), CoQ_2 (ε_{282nm} = 8 $mM^{-1}cm^{-1}$), and CoQ_4 in 468 μM Triton X-100 (ε_{282nm} = 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 CoQ_4. 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