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Discovery of the Membrane Binding Domain in Trifunctional Proline Utilization A

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Abstract

Escherichia coli proline utilization A (*EcPutA*) is the archetype of trifunctional PutA flavoproteins, which function both as regulators of the proline utilization operon and bifunctional enzymes that catalyze the four-electron oxidation of proline to glutamate. *EcPutA* shifts from a self-regulating transcriptional repressor to a bifunctional enzyme in a process known as functional switching. The flavin redox state dictates the function of *EcPutA*. Upon proline oxidation, the flavin becomes reduced, triggering a conformational change that causes *EcPutA* to dissociate from the *put* regulon and bind to the cellular membrane. Major structure/function domains of *EcPutA* have been characterized, including the DNA-binding domain, proline dehydrogenase (PRODH) and L-glutamate- γ -semialdehyde dehydrogenase catalytic domains, and an aldehyde dehydrogenase superfamily fold domain. Still lacking is an understanding of the membrane-binding domain, which is essential for *EcPutA* catalytic turnover and functional switching. Here, we provide evidence for a conserved C-terminal motif (CCM) in *EcPutA* having a critical role in membrane binding. Deletion of the CCM or replacement of hydrophobic residues with negatively charged residues within the CCM impairs *EcPutA* functional and physical membrane association. Furthermore, cell-based transcription assays and limited proteolysis indicate that the CCM is essential for functional switching. Using fluorescence resonance energy transfer involving dansyl-labeled liposomes, residues in the α -domain are also implicated in membrane binding. Taken together, these experiments suggest that the CCM and α -domain converge to form a membrane-

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Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

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binding interface near the PRODH domain. The discovery of the membrane-binding region will assist efforts to define flavin redox signaling pathways responsible for *EcPutA* functional switching.

Graphical Abstract

Proline and its metabolism have numerous significant roles in a myriad of cellular processes.^{1–4} Cellular energy is generated from the oxidative breakdown of proline to glutamate via the enzymes proline dehydrogenase (PRODH) and L-glutamate- γ -semialdehyde dehydrogenase (GSALDH), which in eukaryotes are localized in the mitochondrion.^{2,5,6} In humans, PRODH has been shown to influence cancer development and progression, with suppressor and pro-metastasis functions in early and late stage cancer, respectively.^{2,4,7–10} Inborn deficiencies in PRODH are associated with hyperprolineamia type 1,¹¹ risk of schizophrenia,^{11–13} and autism spectrum disorder.¹⁴ The role of proline metabolism in infectious diseases is of significant interest as proline has been shown to support growth and virulence of different pathogenic organisms including *Staphylococcus aureus*,^{15,16} *Helicobacter pylori*,^{17,18} *Cryptococcus neoformans*,¹⁹ and trypanosomes.^{20–22} The primary functions of proline during infection include utilization as an energy, carbon, and nitrogen source, as well as osmoregulation and stress resistance.

In Gram-negative bacteria, the oxidation of proline to glutamate is catalyzed by the enzyme proline utilization A (PutA), which has PRODH and GSALDH domains in one polypeptide.^{5,23} PutA enzymes are unique to Gram-negative bacteria, as eukaryotes and Gram-positive bacteria encode separate PRODH and GSALDH enzymes.⁵ The oxidation of proline to glutamate is initiated by PRODH, which generates δ^1 -pyrroline-5-carboxylate (P5C) (Figure 1A). PRODH is a FAD-dependent ($\beta\alpha$)⁸ barrel domain enzyme that couples the oxidation of proline to reduction of ubiquinone in the respiratory chain.^{5,24–26} GSALDH (aka P5C dehydrogenase and ALDH4A1) catalyzes the NAD⁺-dependent oxidation of L-glutamate- γ -semialdehyde (GSAL), which is generated in an intervening nonenzymatic step by hydrolysis of P5C.^{5,27} A substrate channel links the PRODH and GSALDH domains in PutAs, thereby preventing P5C/GSAL loss to the cellular milieu and improving efficiency of

glutamate production from pro-line.^{3,5,27–32} Recent evidence suggests that substrate channeling also occurs between separate PRODH and GSALDH enzymes from Gram-positive *Thermus thermophilus*, indicating that substrate channeling may be a fundamental feature of proline metabolism in other organisms that lack PutA.³³

PutA proteins are classified into three main types depending on the domain architecture (Figure 1B). PutAs in the type A class contain the two catalytic domains and are the best structurally characterized with high-resolution crystal structures of PutA from *Bradyrhizobium japonicum* (BjPutA),²⁷ *Geobacter sulfurreducens* (GsPutA),²⁸ and *Bdellovibrio bacteriovorus*.³⁴ Type B PutAs are distinguished by an additional C-terminal domain that was recently identified as an aldehyde dehydrogenase superfamily (ALDHSF) fold. Characterization of type B PutAs from *Sinorhizobium meliloti*²⁹ and *Corynebacterium freiburgense*³⁵ showed that the major function of the C-terminal ALDHSF domain is to stabilize aldehyde binding in the GSALDH active site and seal the substrate channel from bulk solvent.^{5,36} Type A PutAs, which lack the ALDHSF domain, have a different oligomeric structure than type B PutAs and form the substrate channel via domain-swapped dimerization.⁵ Finally, a group of PutA proteins classified as type C contain a N-terminal DNA-binding domain and are known as “trifunctional PutAs” (Figure 1B).⁵ The DNA-binding domain has a ribbon-helix-helix (RHH) fold and has been structurally characterized from *Escherichia coli*^{37,38} and *Pseudomonas putida*.³⁹

Trifunctional PutAs repress transcription of the *putA* and *putP* (encoding a high affinity Na⁺-proline symporter)⁴⁰ genes of the *put* regulon in response to proline availability.^{37,41–43} As proline levels rise, PutA switches from a DNA-bound transcriptional repressor to a membrane-bound catabolic enzyme thereby relieving repression of the *put* regulon and increasing the breakdown of proline.⁴⁴ Previous work with PutA from *E. coli* (EcPutA) has shown proline reduction of the FAD cofactor triggers a conformational change that significantly enhances PutA–membrane associations effectively transforming PutA from a soluble DNA-binding protein to a membrane-bound enzyme.^{45–47} Reduction of the FAD cofactor alone is sufficient to induce conformational changes and membrane binding.⁴⁵ X-ray crystal structures of the EcPutA PRODH domain in different redox states and studies of PRODH active site mutants implicate a hydrogen bond network from the FAD N(5) position to the β 3- α 3 loop in the PRODH domain as critical for EcPutA functional switching.^{48–50} Mutation of hydrogen bonding residues in the network traps EcPutA as a transcriptional repressor with the mutants unable to localize to the membrane upon proline reduction of the FAD cofactor.^{48,50}

While significant progress has been made identifying residues critical for EcPutA functional switching, a major gap exists in understanding how EcPutA associates with membranes. Prior studies, however, have provided some important clues. Crystal structures of PutAs show three conserved ancillary domains (arm, α , and linker domains) near the PRODH catalytic domain.^{5,27–29,51} The proximity of the arm and α domains to the PRODH active site suggests they are positioned to respond to flavin reduction. In fact, proline reduction of EcPutA increases susceptibility to chymotrypsin cleavage at Arg234, implying that the surrounding region within the α -domain (residues 142–259) becomes more solvent exposed with flavin in the reduced state.^{46,52} It was also shown that loss of residues 58–233

eliminates *EcPutA* functional membrane associations while having no impact on PRODH activity.⁴⁶ Recently, a crystal structure of *GsPutA* showed a detergent molecule bound in the α -domain, supporting the idea of this domain being involved in membrane binding.²⁸ Although *GsPutA* is classified as a short bifunctional PutA (i.e., type A), it shares high structural homology with the *EcPRODH* domain, indicating a likely conserved function for the α -domain.²⁸

Another region implicated in *EcPutA*–membrane binding includes residues in the C-terminal end. A previous study showed that deletion of *EcPutA* residues 1295–1320 (*EcPutA*1–1294 mutant) generates a constitutive transcriptional repressor (*EcPutA*1–1294 mutant) that is devoid of functional membrane associations indicating an important role for C-terminal residues in *EcPutA*–membrane binding and functional switching.⁴⁴

To further understand how *EcPutA* switches function, we sought to identify the membrane-binding domain(s) of PutA. Here, we discover the essential function of a conserved C-terminal motif (CCM, residues 1300–1320, Figure 1C) in PutA membrane binding and identify residues that are critical for *EcPutA* functional switching. We also show evidence for the α -domain having an important role in PutA–membrane binding. Despite the large separation in primary sequence, we propose that residues of the CCM and α -domain converge spatially in the structure of PutAs to generate a membrane-binding interface.

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 and used for cloning and protein expression, respectively.

An *E. coli* BL21(DE3) strain that lacks PutA was used to test *in vivo* membrane interactions of PutA. A PutA deficient (*putA*[−]) *E. coli* strain of BL21(DE3) was generated by disrupting the *putA* gene with a kanamycin cassette (TargeTron Gene knockout system, Sigma). The primers used were 5′-AAAAAA-GCTTATAATTATCCTTACGCCTCCCGCAGGTGC-GCCCAGATAGGGTG-3′, 5′-CAGATTGTACAAATG-TGGTGATAACAGATAAGTCCCGCAGCCTAA-CTTACCTTTCTTTGT-3′ and 5′-TGAACGCAAGTTTCTAATTTTCGGTTAGGCGTC-GATAGAGGAAAGTGTCT-3′. The kanamycin cassette was inserted at 355 bp into the *putA* gene. Subsequent PCR analysis of the *putA* gene showed a 547 bp product, which contains 219 bp of the kanamycin cassette, demonstrating insertion of the kanamycin cassette into the *putA* gene. Western blot analysis of cell lysates using an antibody generated against the RHH domain as previously described⁴⁴ confirmed the lack of PutA expression.

Mutagenesis and Protein Purification

Site-directed mutants of *EcPutA* were made from the full-length construct of wild-type *EcPutA* (UniProt Accession ID:P09546) (*EcPutA*-pET14b) using Stratagene QuikChange or Invitrogen GeneTailor mutagenesis kits. The primers used for mutagenesis were purchased from Integrated DNA technologies and are listed in Table S1 (see Supporting Information).

C-terminal deletion mutants were generated by inserting a stop codon immediately after the codons for residues 1308, 1314, and 1317. All of the *EcPutA* mutants were confirmed by DNA sequencing. *EcPutA* wild-type and mutant proteins were overexpressed in *E. coli* strain BL21(DE3) pLysS and purified by Ni-NTA Superflow affinity (Qiagen) chromatography using a N-terminal 6xHis-tag as previously described.^{50,53} The N-terminal His-tag was retained for *EcPutA* wild-type and mutants in subsequent experiments. Purified proteins were stored in 50 mM Tris buffer containing 50 mM NaCl, 0.5 mM EDTA, 0.5 mM Tris (3-hydroxypropyl) phosphine (THP), and 10% glycerol (pH 7.5).

PRODH Kinetic Assays

The PRODH kinetic parameters of *EcPutA* wild-type and mutants were determined using dichlorophenolindophenol (DCPIP) as the terminal electron acceptor and phenazine methosulfate as the mediator (proline:DCPIP oxidoreductase assay) as previously described.³⁶ The steady-state parameters K_m and k_{cat} for proline were determined as previously described using *EcPutA* protein (0.01–0.3 μ M) and L-proline (0–700 mM) while keeping DCPIP fixed at 75 μ M. Assays were performed in 20 mM Tris-HCl buffer (pH 8.0) with 10% glycerol at 23 °C.

Limited Proteolysis of *EcPutA* Proteins

Limited digestion of *EcPutA* wild-type and mutants by chymotrypsin was performed as previously described.⁴⁹ Briefly, digests were performed with and without 20 mM proline in 50 mM potassium phosphate buffer (pH 7.5, 10% glycerol) for 1 h at 23 °C. After stopping the reactions with phenylmethanesulfonyl fluoride and hot SDS-PAGE sample buffer, samples were analyzed by SDS-PAGE (8% acrylamide). Resulting polypeptide bands were visualized with Coomassie Blue G-250 staining.

Functional Membrane Association and Lipid Pull-Down Assays

Functional membrane association activity of wild-type *EcPutA* and the *EcPutA* variants was measured as previously described by monitoring the formation of a P5C/*o*-aminobenzaldehyde (*o*-AB) yellow adduct at 443 nm ($\epsilon = 2590 \text{ M}^{-1} \text{ cm}^{-1}$) in a 96-well plate.⁴⁸ Assays (200 μ L total volume) were performed at 23 °C in 50 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS) buffer (10 mM MgCl₂, 10% glycerol, pH 7.4) with 4 mM *o*-AB, 60 mM L-proline, and 0.02 mg/mL membrane vesicles prepared from *E. coli* strain JT31 *putA*[−]. Inverted *E. coli* membrane vesicles were prepared as reported by Abrahamson et al., frozen in liquid N₂, and stored at −70 °C until needed.⁵⁴

Lipid pull-down assays were performed as previously described.^{48,49} Assays were performed anaerobically under a nitrogen atmosphere in an anaerobic chamber (Belle Technology Glovebox) with vesicles prepared from *E. coli* polar lipids (Avanti Polar Lipids).^{48,49} 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 20 mM L-proline for 1 h and then centrifuged.⁴⁹ The soluble and lipid fractions were then analyzed by SDS-PAGE (10% gels) and stained with Coomassie Blue G-250. Partition coefficients for *EcPutA* wild-type and mutants were calculated as previously described by dividing the soluble/lipid (S/L) distribution ratio of the oxidized protein (S/L)_{ox} by the distribution ratio of the proline

reduced protein $(S/L)_{\text{red}}$.⁴⁴ The partition coefficient is thus the quantity $(S/L)_{\text{ox}}/(S/L)_{\text{red}}$ and indicates the extent to which proline induces PutA partitioning into the lipid fraction.⁴⁴ Complete reduction of the *EcPutA* wild-type and mutant proteins by 20 mM L-proline was confirmed by following reduction of the FAD cofactor at 450 nm by UV-visible spectroscopy.

Cell-Based Assays

Cell-based transcription repressor assays were performed as previously described in *E. coli* strain JT31 *putA-lacZ*-containing the *putC:lacZ* reporter gene (pUT03 construct) and constructs for *EcPutA* wild-type and mutants *EcPutA*1–1314, *EcPutA*1–1308, L1316D, and A1309D/L1316D (pUC18-*EcPutA*).⁴⁸ Cells were grown at 37 °C in minimal media containing ampicillin (50 µg/mL) and chloramphenicol (30 µg/mL) supplemented with 0 and 15 mM L-proline and grown to OD at 600 nm ~1.0. β -galactosidase assays were performed using cell extracts as described previously⁴⁸ with β -galactosidase activity reported in Miller units according to the method of Miller.⁵⁵

Membrane interactions were tested *in vivo* using triphenylte-trazolium chloride (TTC) proline indicator plates according to the method of Wood⁵⁶ and using the *E. coli* strain BL21(DE3) Kan^r *putA*⁻. *E. coli* cells were transformed with constructs *EcPutA*-pET14b, *EcPutA*(1–1308)-pET14b, *BjPutA*-pKA8H, and *BjPutA*(1–986)-pKA8H.³⁶ To test for functional membrane interactions, cells were grown in Luria–Bertani (LB) medium to an OD₆₀₀ of 0.5 and streaked on TTC-indicator agar medium containing L-proline (0.5%), peptone (0.2%), L-tryptophan (0.005%), thiamine (1 µg/mL), TTC (0.0025%) (w/v %), ampicillin (50 µg/mL), and kanamycin (25 µg/mL).

Förster Resonance Energy Transfer (FRET) Assays

Synthetic dansyl-PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-amine-*N*-(5-dimethylamino-1-naphthalenesulfonyl) lipids and *E. coli* polar lipid extract (67% phosphoethanolamine, PE; 23.2% phosphatidylglycerol, PG; and 9.8% cardiolipin) were purchased from Avanti Polar Lipids. Lipids were dried under N₂ and resuspended in 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4) containing 150 mM NaCl and 2 mM MgCl₂ (HEPES-NM buffer). The dansyl-PE lipid and *E. coli* polar lipid suspensions were then mixed to make a 5% dansyl-PE and 95% *E. coli* polar lipid suspension mixture at a final lipid concentration of 1 mg/mL. Lipid vesicles of the 5% dansyl-PE/95% *E. coli* lipid suspension were then prepared as previously described by passing the lipids through a 100 nm polycarbonate filter using a LiposoFast microextruder.⁵⁷ For the fluorescence measurements, *EcPutA* wild-type and mutants (1 µM) were incubated for 5 min with 50 µg of 5% dansyl-PE/95% *E. coli* liposomes in HEPES-NM buffer at 23 °C. Fluorescence emission was measured from 305 to 580 nm using an excitation wavelength of 295 nm (f_{ex} 295 nm). L-Proline (5 mM final concentration) was then added to the liposome suspension and incubated for 1 min. After incubation with proline, the emission spectrum was recorded again (f_{ex} 295 nm). Normalized percent FRET change was calculated by normalizing the change in emission at 520 nm between 0 and 5 mM proline relative to the fluorescence emission change observed with *EcPutA* wild-type. Statistical

significance was determined using a two-tailed *t* test using Sigma Plot 12.0 software for which a *p*-value <0.05 indicated a significant difference in FRET change.

RESULTS

The Conserved C-terminal Motif is Essential for Membrane Binding

Because the *EcPutA*1–1294 mutant was shown previously to lack membrane binding, residues 1295–1320 were analyzed further for potential membrane interactions.⁴⁴ In a recent study a conserved C-terminal motif (CCM) was identified and shown to have a role in substrate channeling in PutA from *Rhodobacter capsulatus* (*RcPutA*) and *Bradyrhizobium japonicum* (*BjPutA*).³⁶ We performed a multiple sequence alignment of PutAs (79 total) to investigate the conservation of hydrophobic residues in the CCM of *EcPutA*, which includes residues 1300–1320. A stretch of conserved hydrophobic residues were found in the CCM of *EcPutA* beginning at residue 1308 with the most highly conserved residues corresponding to Ala1309 and Leu1316 (Figure 1C). The multiple sequence alignment of the CCM is provided in Figure S1, Supporting Information.

The potential role of the CCM in membrane binding was examined by generating the CCM deletion mutant, *EcPutA*1–1308. The ability of *EcPutA*1–1308 to interact with membranes was first tested by cell-based assays using TTC-proline indicator medium.⁵⁶ *E. coli* cells deficient in PutA (*putA*[−]) containing either *EcPutA* wild-type or *EcPutA*1–1308 constructs were tested for the ability to couple proline utilization with the respiratory chain by following reduction of TTC, a redox dye that acts as an electron acceptor for dehydrogenases and membrane respiratory complexes.^{58,59} Figure 2 shows that cells with wild-type *EcPutA* turn red indicating TTC reduction, whereas cells with *EcPutA*1–1308 are white, suggesting that *EcPutA*1–1308 is deficient in membrane binding. To further corroborate the role of CCM in membrane binding, *E. coli putA*[−] cells were transformed with *BjPutA* wild-type and the CCM deletion mutant *BjPutA*1–986. Previously, *BjPutA*1–986 was shown to exhibit PRODH and GSALDH activity similar to that of wild-type *BjPutA*.³⁶ However, the membrane binding activity was not examined. Figure 2 shows that cells with wild-type *BjPutA* generate a red color whereas cells containing *BjPutA*1–986 remain white, further implicating the CCM as critical for PutA-membrane interactions.

Roles of CCM Residues in Functional Membrane Associations

To further examine the membrane-binding role of the CCM in *EcPutA*, two other truncation mutants besides *EcPutA*1–1308 were generated. As detailed in Figure 3, these truncations included mutants *EcPutA*1–1314 and *EcPutA*1–1317. In addition, point mutations were generated by replacing hydrophobic residues (Ala1309 and Leu1316) in the CCM region with Asp as summarized in Figure 3. The ability of these mutants to functionally associate with *E. coli* membrane vesicles was first determined by PRODH activity assays in which membrane vesicles are the terminal electron acceptor. The ability of *EcPutA* to couple proline oxidation with reduction of ubiquinone in the membrane during catalytic turnover is indicated by formation of a P5C/*o*-AB yellow complex, which is followed at 443 nm. Truncation of C-terminal residues resulted in a trend of decreased P5C/*o*-AB formation with *EcPutA*1–1308 showing no measurable activity (Figure 3). In assays using DCPIP as the

terminal electron acceptor, the truncation mutants exhibited PRODH activity similar to that of wild-type *EcPutA* (Table 1). These results indicate that the decrease in P5C production with the truncation mutants is due to an attenuated ability to functionally utilize membrane vesicles as an electron acceptor, rather than a general loss of PRODH activity. Substitution of Ala1309 or Leu1316 with Asp also resulted in lower functional membrane activity (Figure 3B). In the *EcPutAA1309D/L1316D* double mutant, functional membrane association activity was abolished, similar to that observed for the truncation mutant *EcPutA1–1308*. The mutants *EcPutAL1316D* and *EcPutAA1309D/L1316D* exhibit PRODH:DCPIP oxidoreductase activity similar to that of wild-type *EcPutA*, indicating that the loss of activity with membranes is due to dysfunctional membrane binding (Table 1).

CCM Alteration Attenuates Physical Membrane Association

Next, we sought to determine if the loss of functional association activity results from loss of physical associations between the mutants and membrane vesicles. To this extent, we examined the ability of the *EcPutA* mutants to physically interact with liposomes using lipid pull-down assays in the absence and presence of proline. Without proline, wild-type *EcPutA* resides in the soluble fraction but upon adding proline, *EcPutA* sediments with the liposomes (made from *E. coli* polar lipids) after centrifugation (Figure 4A). The distribution of *EcPutA* between the lipid and soluble fractions was quantified by densitometry and is plotted in Figure 4B. A partition coefficient was then determined as a measure of the *EcPutA* shift from soluble to lipid fraction upon proline reduction of the flavin.⁴⁸

Figure 4 shows the results of lipid-pull down assays with the different *EcPutA* mutants and the corresponding distribution in the soluble and lipid fractions. The amount of PutA protein in the lipid fraction after adding proline was generally lower for the *EcPutA* mutants relative to that of wild-type *EcPutA*. Mutants *EcPutA1–1308* and *EcPutAA1309D/L1316D* exhibited the lowest abundance in the lipid fraction indicating deficient lipid interactions. These results are consistent with the functional membrane association assays in which no measurable activity was observed with mutants *EcPutA1–1308* and *A1309D/L1316D*. The partition coefficient for *EcPutA* wild-type is 8.8, while the *A1309D/L1316D* mutant has the lowest value of 1.2 (Figure 4B). A lower partition coefficient value relative to wild-type *EcPutA* is consistent with weak proline induction of membrane binding in the mutant *A1309D/L1316D*. These results further demonstrate that loss of CCM residues in *EcPutA*, or replacement of hydrophobic residues with negatively charged aspartate residues in the CCM, impairs *EcPutA* membrane binding.

To further explore *EcPutA* membrane interactions, we utilized a FRET approach with *EcPutA* intrinsic tryptophan fluorescence as the donor (emission $\lambda_{\max}$ 348 nm) and fluorescent dansyl liposomes as the acceptor (excitation $\lambda_{\max}$ 336 nm, emission $\lambda_{\max}$ 520 nm). *EcPutA* wild-type was first incubated with dansyl-labeled liposomes, and the fluorescence spectrum was recorded. Next, the wild-type *EcPutA*–liposome mixture was titrated with increasing amounts of proline (0–10 mM) (Figure 5). The intrinsic Trp fluorescence decreased as a function of proline, whereas the dansyl-labeled liposomes increased in emission at 520 nm. These results are consistent with proline reduction of the FAD cofactor triggering *EcPutA*–membrane binding as indicated by increased FRET

between Trp residues (donor) and the dansyl-labeled liposomes (acceptor). Thus, *EcPutA*–lipid binding results in excitation of the liposomes by Trp fluorescence emission leading to increased dansyl fluorescence of nearly 13% with wild-type *EcPutA* (λ_{max} 520 nm).

FRET experiments were then performed with the *EcPutA* mutants *EcPutA*1–1308, *EcPutA*1–1314, *EcPutA*1–1317, L1316D, and A1309D/L1316D. Figure 5B shows the proline dependent increase in FRET relative to that observed with wild-type *EcPutA*. *EcPutA*1–1317 showed FRET similar to that of wild-type, whereas with other mutants FRET was significantly reduced. The FRET change of mutants *EcPutA*1–1308 and A1309D/L1316D was <26% of wild-type indicating a severe loss in liposome binding. The lower changes in FRET with the *EcPutA* mutants are not a result of lower PRODH activity, as the PRODH activity of the mutants are similar to that of wild-type *EcPutA* (Table 1). It is also important to note that the FAD cofactors of all the mutants were fully reduced by the proline concentration used for the FRET experiments (data not shown). Thus, the FRET behavior of the *EcPutA*1–1308 and *EcPutA* A1309D/L1316D mutants are consistent with the other results above, altogether indicating that C-terminal residues 1309–1320 have a critical role in *EcPutA*–membrane binding.

Role of CCM Residues in Functional Switching

To examine the effects of CCM truncations on the *in vivo* transcriptional repressor activity of *EcPutA*, we performed cell-based reporter assays. In agreement with our previous studies,^{44,48} repression of the *putC::lacZ* reporter gene is attenuated upon proline addition as wild-type *EcPutA* binds to the membrane rather than to *put* control DNA (Figure 6). In contrast, repression of the reporter gene is not relieved by proline in cells expressing *EcPutA*1–1314, *EcPutA*1–1308, or L1316D, providing further evidence that these mutants are unable to associate with the membrane. Intriguingly, *EcPutA* A1309/L1316D acts as a “super-repressor”, where there is no discernible expression of the reporter in the absence or presence of proline. Thus, dysfunctional membrane binding impairs the ability of the *EcPutA* mutants to function as a proline-responsive transcriptional repressor.

We then explored whether CCM mutants were defective in proline-dependent conformational changes. Limited proteolysis of wild-type *EcPutA* in the absence and presence of proline generates distinct proteolysis patterns for the oxidized and proline-reduced forms of *EcPutA* (Figure 7). In agreement with our previous studies, proline induces a change from a 135-kDa band (oxidized) to a 119-kDa band (proline-reduced) (Figure 7).^{46,49} Limited proteolysis was then performed with the *EcPutA* mutants A1309D, A1309D/L1316D, and *EcPutA*1–1308 (Figure 7). *EcPutA* A1309D mutant exhibited a distinct 119-kDa band with proline similar to that of wild-type. *EcPutA*1–1308 and A1309D/L1316D, however, exhibited no change with proline in the chymotrypsin proteolysis pattern and instead had only a major 111-kDa band in the absence and presence of proline. These results indicate that *EcPutA* mutants *EcPutA*1–1308 and A1309D/L1316D are unable to undergo a proline-dependent conformational change similar to that of wild-type. Thus, alteration of the CCM by point mutation or truncation inhibits conformational changes that are associated with membrane-binding and redox-sensitive functional switching of *EcPutA*.

Membrane Binding Regions of *EcPutA*

We next explored other regions in PutA for possible membrane interactions by utilizing a FRET mapping approach. Residues in different domains were mutated to Trp, while existing Trp residues were mutated to Phe. If a mutated residue is within ~24 Å (estimated Förster distance for the Trp/dansyl liposome pairing)^{60,61} of the membrane, the FRET signal is expected to change, thus indicating membrane interactions within that region. The addition of Trp in the membrane-binding domain should increase FRET, whereas removal of Trp from the membrane-binding domain should decrease FRET. Substitutions at residues far from the PutA–membrane interaction surface would have no impact on FRET.

Residues in the α -domain and the CCM were the primary focus of mutagenesis for the FRET study. Within the α -domain, residues Phe259, Phe205, and Met255 were mutated to Trp, while Trp194 and Trp211 were mutated to Phe. In the CCM, we mutated the terminal Gly1320 residue to Trp. In addition, we also performed mutagenesis of residues in the auxiliary arm domain, as this domain is found within the Ser58–Asn233 region implicated in previous studies to be vital for membrane binding.⁴⁶ The auxiliary arm contains no Trp residues; therefore we introduced Trp at residues Tyr129 and Leu131. We also mutated residue Trp1185 to Phe in the ALDHSF domain.

Figure 8 shows that *EcPutA* mutants W194F and W211F in the α -domain exhibited a decrease in FRET change with proline relative to wild-type *EcPutA*. A double mutant of these residues (W194F/W211F) resulted in a more pronounced decrease in FRET change. These results suggest that the α -domain interacts with the membrane and that Trp194 and Trp211 are significant contributors to the FRET signal. To test the impact of inserting Trp residues in the α -domain, *EcPutA* mutants F259W, F205W, and M255W were also examined. All of these mutants generated a higher FRET signal change with proline, and double mutants F259W/F205W and F259W/M255W led to further increases in FRET. These results strongly indicate that the α -domain interacts with membranes.

Adding a Trp residue to the CCM in *EcPutA* mutant G1320W increased the proline-induced FRET change relative to wild-type *EcPutA*. This result provides additional evidence for the CCM being involved in *EcPutA*–membrane binding. The PRODH domain was also tested by replacing Trp396 with Phe. The *EcPutA* mutant W396F exhibited a significant decrease in FRET with proline, indicating that the PRODH domain associates with the membrane, which is required for catalytic turnover. Mutations that did not significantly impact FRET were Y129W and L131W of the arm domain and W1185F of the ALDHSF domain. These results indicate that the regions surrounding these residues do not associate with the membrane.

To confirm that FRET changes observed with the mutants in the α -domain and CCM are not caused by changes in PRODH activity, the steady-state kinetic parameters for PRODH activity were determined. Table 1 shows that all of the mutants in this study exhibited PRODH activity similar to that of wild-type *EcPutA*. We also confirmed that mutants exhibiting decreased FRET are capable of functionally interacting with the membrane. Figure 9 shows that *EcPutA* mutants W396F, W194F, W211F, and W194F/W211F have functional membrane binding activity similar to that of the wild-type *EcPutA*, indicating that

the decrease in FRET observed with these mutants is not caused by impaired membrane interactions.

DISCUSSION

The mechanism by which trifunctional PutAs functionally switch from a transcription factor to an active membrane-bound enzyme is currently unknown. In order to determine the mechanism, the membrane-binding domain of PutA needs to be established. Here, we located the regions of *Ec*PutA involved in membrane binding. We show that residues of the CCM are of crucial importance for *Ec*PutA functional membrane interactions. Truncation of *Ec*PutA to residue 1314 attenuates its ability to functionally associate with membrane vesicles, and further deletion to residue 1308 (*Ec*PutA1–1308) completely abolishes membrane-binding activity. Also, deleting the CCM in *Bj*PutA (*Bj*PutA1–986) eliminates TTC reduction in cell-based assays, suggesting that membrane binding is a conserved role of the CCM in all three PutA types. Crystal structures of type A and type B PutAs show that the CCM is part of a lid that helps seals the substrate channel from the bulk medium.^{27–29,34,35} Thus, it appears that the CCM has a dual function in PutAs.

The CCM was further examined in *Ec*PutA by replacing highly conserved Ala1309 and Leu1316 with a negatively charged Asp residue to potentially disrupt hydrophobic PutA–membrane interactions. The *Ec*PutA mutants A1309D and L1316D had attenuated functional membrane association activity, while the double mutant A1309D/L1316D showed no activity similar to that of *Ec*PutA1–1308. Lipid pull-down assays and FRET experiments also showed that substituting Asp at hydrophobic residues in the CCM severely impairs *Ec*PutA–membrane binding. These results are consistent with PutA–membrane binding involving hydrophobic interactions as Surber and Maloy originally proposed.⁶² Other studies have also concluded *Ec*PutA and *Bj*PutA membrane binding involves hydrophobic interactions. Isothermal titration calorimetry revealed *Bj*PutA–membrane binding is entropy driven consistent with strong hydrophobic forces being involved.⁵⁷ However, *Bj*PutA also exhibits an electrostatic dependence on lipid binding, whereas *Ec*PutA does not discriminate between differently charged membranes.^{45,57} From the results here, it seems that highly conserved residues Ala1309 and Leu1316 of *Ec*PutA have a key role in driving hydrophobic PutA–membrane binding.

In addition to disrupting membrane binding, *Ec*PutA mutants *Ec*PutA1–1308 and A1309D/L1316D were shown not to undergo a proline-dependent conformational change as observed for wild-type *Ec*PutA. In *Ec*PutA, proline induces a conformational change that is concomitant with PutA–membrane binding. The lack of a proline-induced conformational change in *Ec*PutA mutants *Ec*PutA1–1308 and A1309D/L1316D is consistent with the loss of membrane binding. *In vivo* cell-based assays showed that *Ec*PutA1–1308 and A1309D/L1316D do not functionally switch. Interestingly, the A1309D/L1316D mutant appears to behave as a super-repressor of the *put* regulon.

Besides the CCM, we also further demonstrated that the α -domain and the PRODH domain are involved in *Ec*PutA membrane binding using a FRET mutagenesis mapping approach. Dansyl liposomes are a useful tool for monitoring protein–membrane interactions.^{60,63–66}

Interactions between lipid vesicles and synaptotagmin upon calcium addition were measured using intrinsic tryptophan fluorescence from synaptotagmin and dansyl liposomes.⁶³ Synaptotagmin and dansyl-PE liposomes incubated in the presence of calcium showed a higher FRET response than synaptotagmin and dansyl liposomes alone. Dansyl lipids were also used as the acceptor in FRET assays examining the membrane binding capabilities of Factor VIII.⁶⁵ Here, we utilized the tryptophan donor/dansyl acceptor system to create a series of mapping mutants. By introducing or removing Trp residues, we altered the Trp donor fluorescence, thereby manipulating dansyl fluorescence emission if the mutation was at a residue near the membrane-binding site. Replacing Trp residues in the α -domain and PRODH domain with Phe decreased FRET indicating that these Trp residues are within the membrane-binding region. Substituting in Trp residues in the α -domain caused a FRET increase, further implicating the α -domain in membrane binding and corroborating with previous studies indicating a membrane-binding region within residues 58–223.⁴⁶ Adding a Trp residue in the CCM at Gly1320 (G1320W) also increased FRET further verifying a membrane-binding role for the CCM. In contrast, adding Trp residues within the arm domain or mutating Trp1185 to phenylalanine in the ALDHF domain did not change FRET, indicating these regions are not involved in membrane binding. In summary, our results provide strong evidence for the α -domain, PRODH domain, and the CCM as being critical for *EcPutA* membrane interactions.

The residues examined in this study are highlighted in a homology model of the catalytic module of *EcPutA* in Figure 10. The homology model was built using SWISS-MODEL⁶⁷ with the coordinates of the crystal structure of PutA (PDB entry 5KF6) from *Sinorhizobium meliloti* (*SmPutA*) as the template.²⁹ Although *SmPutA* is a type B PutA, it has 60% sequence identity to *EcPutA* and contains all of the domains being probed in the FRET experiments.²⁹ The model contains residues 85–1316, which includes all but the DNA-binding domain.

Although separated in primary sequence, the model shows that the α -domain and the CCM—two regions implicated here in membrane binding—converge spatially near the FAD. The residues identified by FRET as near the membrane interaction surface are in the CCM, α -domain, and the bottom of the PRODH domain (Trp396) (red spheres in Figure 10). The intersection of 24-Å radius spheres centered on these residues defines a target that is consistent with all the FRET data and presumably within the membrane-association region (white sphere in Figure 10). Importantly, the trilateration target is outside of the 24-Å radii spheres centered on the FRET-negative residues (blue spheres in Figure 10). This calculation predicts a junction formed by the CCM, α -domain, PRODH domain, and GSALDH domain as potentially involved in membrane binding (Figure 10B,C). As mentioned above, the α -domain is also the binding site for a detergent molecule observed in a *GsPutA* structure (PDB entry 4NMC). Figure 10A shows the location of the detergent-binding site from *GsPutA* in the *EcPutA* homology model. Interestingly, this region includes helix α 8 of the PRODH active site, which has been shown to undergo conformational changes in response to proline binding and flavin reduction.^{28,49} Because of its flexibility, helix α 8 has been suggested to help transmit the flavin redox state to the membrane-binding domain. The model in Figure 10 is consistent with this notion. Also, the CCM being adjacent to the α -

domain and helix $\alpha 8$ provides clues into why deletion of the CCM may impair proline-induced conformational changes and membrane binding of *EcPutA*.

Now that a membrane-binding interface of *EcPutA* has been identified, it may be possible to map a hydrogen bond network between the FAD cofactor and the membrane-binding interface. We propose that when the FAD is reduced by proline, conformational changes in *EcPutA* enable the α -domain and CCM to interact with the membrane. Determining the mechanism of this process will generate novel insights into flavin-based redox functional switching and may be useful for inhibiting proline metabolism in pathogenic organisms that utilize trifunctional PutAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ALDHSF	aldehyde dehydrogenase superfamily
CCM	C-terminal motif
dansyl-PE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine- <i>N</i> -(5-dimethylamino-1-naphthalenesulfonyl)
DCPIP	dichlorophenolindophenol
BjPutA	proline utilization A from <i>Bradyrhizobium japonicum</i>
EcPutA	proline utilization A from <i>Escherichia coli</i>
FAD	flavin adenine dinucleotide
FRET	Förster resonance energy transfer
GsPutA	proline utilization A from <i>Geobacter sulfurreducens</i>
GSAL	glutamate- γ -semialdehyde
GSALDH	GSAL dehydrogenase
HEPES	<i>N</i> -(2-hydroxyethyl)-piperazine- <i>N'</i> -2-ethanesulfonic acid
MOPS	3-(<i>N</i> -morpholino)-propanesulfonic acid
NAD⁺	nicotinamide adenine dinucleotide
<i>o</i>-AB	<i>o</i> -aminobenzaldehyde

PE	phosphoethanolamine
PG	phosphatidylglycerol
PRODH	proline dehydrogenase
P5C	¹ -pyrroline-5-carboxylate
PutA	proline utilization A
RcPutA	proline utilization A from <i>Rhodobacter capsulatus</i>
RHH	ribbon–helix–helix
SDS-PAGE	sodium dodecyl sulfate poly-acrylamide gel electrophoresis
SmPutA	proline utilization A from <i>Sinorhizobium meliloti</i>
TTC	triphenyltetrazolium chloride

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Figure 1.

(A) Reactions catalyzed by PRODH and GSALDH resulting in the oxidation of proline to glutamate. (B) Domain architectures of PutAs. *EcPutA* (1320 amino acids) is classified as type C. (C) Results from a multiple sequence alignment of PutAs showing the conservation of residues in the CCM. The alignment was computed using the T-Coffee server and visualized using WebLogo software. Residue numbering is according to the *EcPutA* sequence. The GenBank accession numbers of the aligned PutA sequences and T-Coffee alignment of these residues are provided in Table S2, Supporting Information.

Figure 2.

Cell-based membrane interactions. *E. coli* strain BL21(DE3) *putA*⁻ was transformed with wild-type *E. coli* (*EcPutA*) and *B. japonicum* (*BjPutA*) PutA constructs and conserved C-terminal motif (CCM) deletion mutant *EcPutA*1–1308 and *BjPutA*1–986 constructs. Cells were plated on triphenyltetrazolium chloride (TTC)-indicator agar medium containing L-proline and grown for 20 h at 37 °C. Red colonies indicate the ability to utilize proline as a respiratory substrate and functional PutA–membrane interactions.

Figure 3.

Functional membrane binding analysis of *EcPutA* C-terminal mutants. (A) Sequences of the CCM deletions in *EcPutA* truncation mutants. Locations of residues mutated to aspartate (Ala1309 and Leu1317) are indicated in red. (B) Functional membrane association activity of *EcPutA* wild-type and mutants was measured by incubating 4 μ g of *EcPutA* protein with 0.1 mg/mL *E. coli* (strain JT31 *putA*⁻) inverted membrane vesicles, 60 mM proline, and 4 mM *o*-AB in 20 mM MOPS (pH 7.5). All reactions were performed at 23 °C, and absorbance was monitored at 443 nm, which follows *o*-AB-P5C adduct formation.

Figure 4.

Lipid binding assays of *EcPutA* CCM mutants. (A) Lipid pull-down assays were performed with *EcPutA* wild-type and mutants (0.3 mg/mL) in 10 mM HEPES buffer (pH 7.5, 150 mM NaCl) with *E. coli* polar lipids (0.8 mg/mL) for 1 h in the absence (Ox) and presence (Red) of proline (20 mM). The soluble and lipid fractions were separated by Air-fuge ultracentrifugation and analyzed by SDS-PAGE as previously described.⁴⁸ (B) Protein bands from SDS-PAGE were quantified using ImageJ software. The percent distribution of *EcPutA* in the soluble and lipid fractions is shown for the oxidized and proline reduced samples as different colored bars (black, oxidized soluble; red, oxidized lipid; green, proline reduced soluble; yellow, proline reduced lipid). A partition coefficient calculated as $(S/L)_{\text{ox}}/(S/L)_{\text{red}}$ as previously described⁴⁸ is a measure of the extent that *EcPutA* partitions into the lipids in the presence of proline and is indicated by open circles.

Figure 5.

FRET analysis of *EcPutA*–lipid binding. (A) Wild-type *EcPutA* (1 μ M) was incubated with 0.025 mg/mL *E. coli* polar liposomes (containing 5% dansyl-PE) in 10 mM HEPES buffer (150 mM NaCl, 2 mM MgCl₂, pH 7.5) and titrated with 0–10 mM proline. The samples were excited at 290 nm ($\lambda_{\text{ex}} = 290$ nm), and the emission spectra of the Trp residues and the dansyl liposomes were monitored at 340 and 520 nm, respectively. With 10 mM proline, the decrease in Trp fluorescence was 14.4%, and the corresponding increase in dansyl emission was 12.7%. (B) *EcPutA* wild-type and mutants (1 μ M) were incubated with 0.025 mg/mL *E. coli* polar vesicles (containing 5% dansyl-PE) in 10 mM HEPES buffer (150 mM NaCl, 2 mM MgCl₂, pH 7.5) with and without 5 mM proline. FRET was monitored at $\lambda_{\text{em}} = 520$ nm using $\lambda_{\text{ex}} = 290$ nm. Shown is the relative change in fluorescence emission of the dansyl liposomes as a function of proline with the mutants normalized to wild-type *EcPutA*. Values are the mean of three independent experiments $\pm$ SD (*, $p < 0.05$).

Figure 6.

Cell-based functional switching assays. Cell-based transcriptional repressor assays in *E. coli* strain JT31 *putA*⁻ *lacZ*⁻ cells containing the *putC::lacZ* reporter gene plasmid and expression constructs for EcPutA wild-type and mutants EcPutA1–1314, EcPutA1–1308, L1316D, and A1309D/L1316D. Cells were grown at 37 °C in minimal media supplemented with 0 and 15 mM proline. β -Galactosidase activity is reported as the average of four independent experiments $\pm$ standard errors. BD, below detection.

Figure 7.

Limited proteolysis of wild-type *EcPutA* and mutants A1309D, A1309D/L1316D, and *EcPutA*1–1308. Purified proteins (1 mg/mL) were digested with chymotrypsin (10 μ g/mL) for 1 h in 50 mM potassium phosphate (pH 7.5) alone (OX) and in reactions containing 20 mM proline (PRO). Digested protein fragments (10 μ g) were separated by denaturing gel electrophoresis (7.5% acrylamide), and gels were stained with Coomassie Blue. The left-hand lane of each gel is undigested *EcPutA* protein (144 kDa band).

Figure 8.

FRET mapping of *EcPutA* membrane interactions. Wild-type *EcPutA* and mutants (1 μ M) were incubated with inverted *E. coli* polar vesicles (with 5% dansyl-PE incorporated) (0.025 mg/mL) in 10 mM HEPES buffer (150 mM NaCl, 2 mM MgCl₂, pH 7.5) and treated with and without 10 mM proline. Fluorescence emission was monitored at $\lambda_{em} = 520$ nm using a $\lambda_{ex} = 290$ nm. (Top panel) The coloring of the *EcPutA* domain map matches the colors of the FRET data (bottom panel). FRET data are plotted as the relative change in fluorescence emission as a function of proline with the mutants normalized to wild-type *EcPutA*. Data are the mean $\pm$ SD from four independent experiments (*, $p < 0.05$).

Figure 9.

Functional membrane association assays. *EcPutA* wild-type and mutant proteins (4 μ g) were incubated with *E. coli* (strain JT31 *putA*⁻) inverted membrane vesicles (0.1 mg/mL membrane protein) in 20 mM MOPS (pH 7.5) containing 60 mM proline and 4 mM *o*-AB. All reactions were performed at 23 °C, and absorbance was monitored at 443 nm, which follows *o*-AB-P5C adduct formation. Plotted is the concentration of *o*-AB-P5C complex formed per mg of *EcPutA* protein.

Figure 10.

Structure-based summary of FRET mapping data. (A) Homology model of the catalytic module of *EcPutA*. The model includes residues 85–1316. Red spheres indicate residues that are within the Förster distance of the *EcPutA*–membrane interaction surface (positive FRET hits). Blue spheres indicate residues outside of the Förster distance of the *EcPutA*–membrane interaction surface (negative FRET hits). The white sphere indicates the target area obtained by trilateration of the positive FRET hits. (B) Zoom of the boxed area indicated in panel A, which contains the target area from trilateration. (C) Surface representation of the zoom region highlighting the convergence of three domains.

Table 1PRODH Kinetic Parameters^a for *EcPutA* Wild-Type and Mutants

<i>EcPutA</i>	K_m (mM)	k_{cat} (s ⁻¹)		k_{cat}/K_m (M ⁻¹ s ⁻¹)
wild-type	166.7 ± 4.8	10.5 ± 0.1		63.2 ± 1.9
<i>EcPutA</i> 1-1308	119.6 ± 5.6	12.1 ± 0.2		104 ± 9
<i>EcPutA</i> 1-1314	171.4 ± 7.9	14.8 ± 0.3		86.5 ± 4.4
<i>EcPutA</i> 1-1317	238.1 ± 22.4	14.0 ± 0.6		58.8 ± 6.1
L1316D	170.6 ± 13.3	18.7 ± 0.6		109.3 ± 9.2
A1309D/L1316D	141.8 ± 5.6	10.9 ± 0.3		76.8 ± 3.3
W194F	125.6 ± 36.2	6.4 ± 0.5		50.8 ± 18.8
W211F	188.0 ± 40.8	6.9 ± 0.5		36.6 ± 10.5
W194F/W211F	60.1 ± 12.3	4.9 ± 0.3		80.7 ± 21.3
W396F	237.6 ± 65.8	10.9 ± 1.6		46.1 ± 19.5
W1185F	92.2 ± 19.4	13.7 ± 1.0		143.9 ± 40.6
F259W	103.9 ± 20.6	9.6 ± 0.7		92.6 ± 25.2
M255W/F259W	312.9 ± 105	9.9 ± 1.6		31.6 ± 15.7
F205W/F259W	220.1 ± 74.2	3.3 ± 0.6		14.9 ± 7.6
G1320W	100.4 ± 18.2	7.8 ± 0.5		77.7 ± 19.4
L131W	287 ± 10.3	4.9 ± 1.1		17.1 ± 4.5
Y129W	157 ± 15.5	17.0 ± 0.1		108.2 ± 11.4

^aKinetic parameters were determined in 20 mM Tris-HCl buffer (pH 8.0, 10% glycerol) containing 75 μ M DCPIP and varying proline concentrations at 23 °C.