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# Characterization of a Bifunctional PutA Homologue from *Bradyrhizobium japonicum* and Identification of an Active Site Residue that Modulates Proline Reduction of the Flavin Adenine Dinucleotide Cofactor<sup>†</sup>

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**ABSTRACT:** PutA is a bifunctional flavoenzyme in bacteria that catalyzes the four-electron oxidation of proline to glutamate. In certain prokaryotes such as *Escherichia coli*, PutA is also a transcriptional repressor of the proline utilization (*put*) genes and thus is trifunctional. In this work, we have begun to assess differences between bifunctional and trifunctional PutA enzymes by examining the PutA protein from *Bradyrhizobium japonicum* (BjPutA). Primary structure analysis of BjPutA shows it lacks the DNA-binding domain of *E. coli* PutA (EcPutA). Consistent with this prediction, purified BjPutA does not exhibit DNA-binding activity in native gel mobility shift assays with promoter regions of the *putA* gene from *B. japonicum*. The catalytic and redox properties of BjPutA were characterized and a reduction potential ( $E_m$ ) value of  $-0.132$  V (pH 7.5) was determined for the bound FAD/FADH<sub>2</sub> couple in BjPutA that is significantly more negative ( $\sim 55$  mV) than the  $E_m$  for EcPutA-bound FAD. The more negative  $E_m$  value thermodynamically limits proline reduction of the FAD cofactor in BjPutA. In the presence of phospholipids, reduction of BjPutA is stimulated, suggesting lipids influence the FAD redox environment. Accordingly, an  $E_m$  value of  $-0.114$  V (pH 7.5) was determined for BjPutA-bound FAD in the presence of polar lipids. The molecular basis for the lower reduction potential of FAD in BjPutA relative to EcPutA was explored by site-directed mutagenesis. Amino acid sequence alignment between BjPutA and EcPutA indicates only one difference in active site residues near the isoalloxazine ring of FAD: Val402 in EcPutA is substituted at the analogous position in BjPutA with Ala310. Replacement of A310 by Val in the BjPutA mutant A310V raised the reduction potential of bound FAD relative to wild-type BjPutA to an  $E_m$  value of  $-0.09$  V (pH 7.5). The  $>40$ -mV positive shift in the potential of the BjPutA mutant A310V suggests that the corresponding Val residue in EcPutA helps poise the FAD redox potential for thermodynamically favored proline reduction thereby allowing EcPutA to be efficiently regulated by proline availability. Limited proteolysis of BjPutA under reducing conditions shows FAD reduction does not influence BjPutA conformation indicating further that the redox dependent regulation observed with EcPutA may be limited to trifunctional PutA homologues.

Proline utilization is catalyzed by the flavoenzyme PutA (proline utilization A) in several microorganisms such as enteric bacteria *Escherichia coli* and *Salmonella typhimurium* and soil microbes *Pseudomonas putida* and *Bradyrhizobium japonicum* (1–5). The oxidation of proline is accomplished by successive FAD-<sup>1</sup> and NAD-dependent steps generating glutamate (2, 3, 6). In the first step, a proline dehydrogenase (PRODH) domain utilizes a noncovalently bound FAD to catalyze the two-electron oxidation of proline to form <sup>1</sup> $\Delta$ -pyrroline-5-carboxylate (P5C). In the second step, fol-

lowing hydrolysis of P5C to glutamate- $\gamma$ -semialdehyde (GSA), a pyrroline-5-carboxylate dehydrogenase (P5CDH) domain couples the oxidation of GSA with the reduction of NAD<sup>+</sup> to generate glutamate. A crystal structure of a truncated PutA protein from *E. coli* shows the PRODH domain is comprised of a  $\beta_3\alpha_8$  barrel involving residues 261–612 (7). The P5CDH domain has been mapped to residues 650–1130 using sequence alignments with other aldehyde dehydrogenase domains (8).

Although the bifunctional enzymatic activity is shared in PutA from a variety of gram-negative bacteria, the control of *putA* expression is divergent. In *E. coli*, *S. typhimurium*, and *P. putida*, PutA is an autogenous transcriptional regulator

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<sup>1</sup> Abbreviations: FAD, flavin adenine dinucleotide; *put*, proline utilization; NAD<sup>+</sup>, nicotinamide adenine dinucleotide; PRODH, proline dehydrogenase; P5CDH, <sup>1</sup> $\Delta$ -pyrroline-5-carboxylate dehydrogenase; THFA, tetrahydro-2-furoic acid; P5C, <sup>1</sup> $\Delta$ -pyrroline-5-carboxylate; DCPIP, dichlorophenolindophenol; PC, phosphatidylcholine; GSA, glutamate- $\gamma$ -semialdehyde; SDS–PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; RHH, ribbon-helix-helix;  $E_m$ , reduction potential.

of the proline utilization (*put*) genes and thus is trifunctional (6, 9–11). It has been shown in *E. coli* and *S. typhimurium* that in the absence of proline, PutA binds to the *put* control DNA and represses expression of genes *putP* (encodes the high-affinity Na<sup>+</sup>-proline transporter) and *putA* which are transcribed in opposite directions from the *put* control region (6, 10). In the presence of proline, PutA associates with the membrane resulting in the activation of *put* gene expression (12–15). The DNA-binding domain of *E. coli* PutA (EcPutA) is in N-terminal residues 1–47 and apparently is a ribbon-helix-helix (RHH) fold suggesting PutA may be a member of the RHH superfamily (16). Sequence analysis to identify potential RHH domains in PutA from other bacteria has distinguished whether various PutA proteins are endowed with DNA-binding activity (16).

The activation of *put* gene expression in *E. coli* and *S. typhimurium* has been shown to be regulated by flavin reduction and subsequent PutA-membrane binding (12, 14, 15, 17, 18). Recently, it was demonstrated that oxidized PutA does not bind to *E. coli* polar lipid bilayers, whereas reduction of the FAD cofactor induces the formation of a strong PutA-membrane complex ( $K_d < 0.01$  nM) (18). Thus, FAD reduction generates a conformer of EcPutA that binds tightly to the membrane resulting in the dissociation of the PutA–DNA complex and *put* gene activation. We seek to determine whether FAD-dependent regulation of PutA conformation and membrane binding also occurs in PutA family members that are devoid of DNA binding activity. It is anticipated that functional and structural characterization of bifunctional PutA members will provide unique insights into the regulation of PutA from *E. coli*.

In this study, we chose to isolate and characterize PutA from the symbiotic nitrogen-fixing bacterium *B. japonicum* from which the *putA* gene was previously cloned by Straub et al. (5). After analyzing the *putA* gene organization, it appeared that PutA from *B. japonicum* (BjPutA) does not function as an autogenous transcriptional regulator but is regulated by PutR similar to *putA* gene regulation in *Agrobacterium tumefaciens* and *Rhodobacter capsulatus* (5, 19–21). PutR, a Lrp-type activator protein, activates *putA* gene expression in response to increased proline levels (19, 20). A potential PutR homologue is located upstream of the *putA* gene in *B. japonicum* (5). Sequence analysis also shows that BjPutA lacks the N-terminal RHH domain and thus is anticipated to be devoid of DNA-binding activity (16). In addition, initial work with BjPutA indicates that it will provide the first structural insights into the orientation of the PRODH and P5CDH domains as BjPutA crystals were recently obtained (Tanner, personal communication). Thus, a three-dimensional context will soon be available for interpreting studies with BjPutA.

Here, we confirm that BjPutA does not have DNA-binding activity and therefore is only a bifunctional enzyme. Kinetic and redox properties of BjPutA were determined and compared with EcPutA. The reduction potential ( $E_m$ ) for FAD bound to BjPutA is about 55 mV more negative than the  $E_m$  value for EcPutA. Partial proline reduction of FAD in BjPutA was also observed confirming the more negative  $E_m$  value. The molecular basis for the lower reduction potential of FAD in BjPutA relative to EcPutA was explored by characterizing the BjPutA mutant A310V. Analysis of the X-ray crystal structure of the PRODH domain from

EcPutA and amino acid sequence alignment between BjPutA and EcPutA indicates only one difference in active site residues near the isoalloxazine ring of FAD. Val402 in EcPutA is replaced by Ala at the analogous position (residue 310) in BjPutA. Surprisingly, a >40- mV positive shift in the  $E_m$  value of FAD bound to the BjPutA mutant A310V was observed suggesting that the corresponding Val in EcPutA has an important role in poising the redox potential of FAD and facilitating proline regulation of EcPutA.

## MATERIALS AND METHODS

**Chemicals.** All chemicals and buffers were purchased from Fisher Scientific and Sigma-Aldrich, Inc. DL-P5C was synthesized as previously described and quantitated using *o*-aminobenzaldehyde (22, 23). Restriction endonucleases and T4 DNA ligase were purchased from Fermentas and Promega, respectively. Molecular size standards used for calibrating size-exclusion columns were purchased from Sigma. BCA reagents used for protein quantitation were obtained from Pierce. All experiments used Nanopure water. EcPutA used in this study was purified as a 6× His tag fusion protein as described (18). The *putA* gene and upstream promoter sequences from *B. japonicum* in plasmid pPutArc were a generous gift from D. Kohl (Washington University, St. Louis, MO) (5). A putative regulatory DNA region (579 bp) upstream of the *putA* gene in *B. japonicum* was prepared by PCR amplification from the plasmid pPutArc using primers 5'-ACTTCCCTAGCCACCGGCAACG-3' and 5'-CGTAGGGGGCGGTGAAGGGAGGC-3' (5). Membrane vesicles were prepared from *E. coli* strain JT31 *putA*<sup>−</sup> as described (2). *E. coli* polar lipid extracts and phosphatidylcholine (PC) were purchased from Avanti Polar Lipids, Inc., and phospholipid suspensions were prepared as previously described (18). *E. coli* polar lipid suspensions were sonicated to form small unilamellar vesicles with diameters of around 50 nm. The resulting vesicles were centrifuged at 12 000g for 5 min, and the lipids from the supernatant were used for the experiments. Overall neutral PC lipid vesicles were prepared by passing the PC lipid suspension (15 mg/mL) through a 100 nm polycarbonate filter 30 times using a LipoFast microextruder. This extrusion technique has been shown to generate unilamellar vesicles from a variety of phospholipids (24). Lipid vesicles were stored at 4 °C.

**Subcloning, Purification, and Characterization of PutA from *B. japonicum*.** The *putA* gene from *B. japonicum* in plasmid pPutArc was subcloned into the vector pKA8H using primers 5'-GCCACAGGACACCATATGCCGAACATCCCGG-3' and 5'-GGAATTGGCGCGGCTCAGTCTCATGGCAGCATTA-3' that incorporated the restriction endonuclease sites *Nde*I and *Blp*I, respectively. The resulting construct, pKA8H-BjPutA, was verified by nucleic acid sequencing. The BjPutA mutant A310V was engineered by QuikChange site-directed mutagenesis using primers 5'-GACGGTTTTGGGCTCGTGATCCAGGCCTATCAG-3' and 5'-CTGATAGGCCTGGATCACGAGCCCCAAAACCGTC-3'.

Wild-type BjPutA and mutant A310V were overexpressed as N-terminal 8× His tag fusion proteins from pKA8H-BjPutA in *E. coli* strain BL21 DE3 pLysS and purified using a Ni<sup>2+</sup> NTA affinity column (Novagen) and anion-exchange chromatography (Hi-Trap Q-sepharose, Amersham Bio-

sciences) as previously described for EcPutA (25–27). The N-terminal 8× His tag was retained after purification. Purified BjPutA proteins were stored in 50 mM Tris (pH 7.5) containing 10% glycerol at  $-70^{\circ}\text{C}$ . The concentration of BjPutA proteins were determined using the BCA method (Pierce) with bovine serum albumin as the standard and spectrophotometrically using a molar extinction coefficient at 451 nm of  $13\,620\text{ M}^{-1}\text{ cm}^{-1}$  (25, 28). The molar extinction coefficient for wild-type BjPutA at 451 nm was determined as previously described (28). The molecular size of wild-type BjPutA was estimated by size-exclusion chromatography (Superdex-200 column) as described (16). Mass spectral analysis of BjPutA was performed in the Metabolomics Core Facility in the Redox Biology Center at the University of Nebraska-Lincoln. PRODH activity was measured using the proline:DCPIP oxidoreductase assay, and P5CDH activity was determined by reduction of  $\text{NAD}^{+}$  at 340 nm as described (28). The apparent  $K_i$  for inhibition of wild-type BjPutA by L-tetrahydro-2-furoic acid (L-THFA) was determined as described previously (26). Functional membrane association activity was measured as described for EcPutA by following formation of a yellow complex between P5C and *o*-aminobenzaldehyde at 443 nm (17). The chymotrypsin limited proteolysis of wild-type BjPutA and mutant A310V were performed in 50 mM Tris (pH 7.5) containing 5% glycerol as previously described (17). The 94-kDa product generated by limited chymotrypsin proteolysis of wild-type BjPutA was separated from full-length BjPutA by applying protease-treated BjPutA to a  $\text{Ni}^{2+}$  NTA affinity column. The 94-kDa product, which is missing the N-terminal 8× His tag, was collected in the flow-thru fractions and equilibrated in 50 mM Tris (pH 7.5) containing 10% glycerol. Nondenaturing gel electrophoretic mobility shift assays were used to test the binding of BjPutA to *putA* control DNA regions as described earlier (28).

Proline titrations of wild-type BjPutA in the absence and presence of lipid vesicles were performed at  $20^{\circ}\text{C}$  in 50 mM potassium phosphate buffer (pH 7.5) containing 5% glycerol under anaerobic conditions. The BjPutA–proline mixtures were equilibrated 30 min prior to recording each spectrum. BjPutA mutant A310V was titrated with proline in the absence of lipid vesicles under identical conditions used for wild-type PutA, and the data were analyzed as previously described assuming the formation of a reduced PutA–P5C complex (28).

**Spectroelectrochemistry.** Potentiometric measurements of EcPutA, wild-type BjPutA, and BjPutA mutant A310V were performed using a three-electrode single compartment spectroelectrochemical cell containing a gold foil working electrode, a silver/silver chloride reference electrode, and a silver chloride counter electrode (28, 29). The measurements were performed under a nitrogen atmosphere in a Belle Technology glovebox. PutA and lipid vesicle solutions were kept overnight in the glovebox prior to potentiometric measurements. All potential values are reported relative to the normal hydrogen electrode and were determined in the reductive and oxidative directions. Potentiometric measurements were recorded at  $20^{\circ}\text{C}$  in 50 mM potassium phosphate (pH 7.45–7.5) and 5% glycerol. The pH of the protein solutions was measured at the end of each potentiometric titration. The phospholipid concentrations in the potentiometric titrations were 0.2 mg/mL or about 0.24–0.26 mM resulting in a 10:1

molar ratio of lipid to BjPutA. From preliminary surface plasmon resonance studies, an upper limit for the dissociation constant of the BjPutA–lipid complex was estimated to be  $<10\text{ nM}$ ; thus, BjPutA is anticipated to be fully saturated with lipids during the potentiometric experiments (data not shown). Methyl viologen (0.1 mM) and potassium ferrocyanide (0.1 mM) were used as mediator dyes in all potentiometric experiments. Pyocyanine ( $E_m = -0.04\text{ V}$ , pH 7.5) (5  $\mu\text{M}$ ) and indigo disulfonate ( $E_m = -0.109\text{ V}$ , pH 7.5) (2–3  $\mu\text{M}$ ) were used as indicator dyes in experiments with EcPutA and BjPutA mutant A310V, while indigo disulfonate, 1,4-hydroxynaphthoquinone ( $E_m \sim -0.139\text{ V}$ , pH 7.0) and anthraquinone-2,6-disulfonate ( $E_m \sim -0.184\text{ V}$ , pH 7.0) were used as indicator dyes in potentiometric experiments with wild-type BjPutA (28). Equilibration of the system in the potentiometric experiments was considered to be obtained when the measured potential change was less than 1 mV in 5 min; this was typically 1–2 h. The UV–visible spectra in each experiment were recorded from 300 to 800 nm on a Cary 100 spectrophotometer. The absorbance at 451 nm was used to monitor the amount of oxidized and reduced FAD. Clean spectra of BjPutA in the potentiometric experiments were obtained by subtracting the spectra of the mediator and indicator dyes in the absence of protein which were measured under identical conditions in the absence and presence of lipid vesicles. To ensure the system was reversible and at equilibrium, the solution was reoxidized during the potentiometric experiments by switching the applied potential from  $-0.8$  to  $+0.8\text{ V}$ . The  $E_m$  and  $n$  values were calculated by fitting to a nonlinear form of the Nernst equation as described with typical error in the reported reduction potential values of  $\pm 2\text{--}5\text{ mV}$  (26).

## RESULTS

**BjPutA Amino Acid Sequence and Properties of Wild-Type BjPutA.** The original nucleic acid sequence data (GenBank accession number X90401) of the *putA* gene from *B. japonicum* predicts a polypeptide of 1016 amino acids which, including the 8× His tag (1036 total amino acids), would have a molecular mass of 113 350 Da (5). The molecular mass of purified BjPutA, however, is 109 858 Da. A more recent nucleic acid sequence of the *putA* gene (GenBank accession number NP\_773901) from *B. japonicum* strain USDA110 predicts a 999 amino acid polypeptide which, along with the 8× His tag (1019 total amino acids), has an anticipated molecular mass of 109 856 Da (30). Nucleic acid sequencing of the *putA* clone failed to resolve the discrepancy since it was difficult to obtain reliable sequencing data in the questionable regions. The conflicting data between the two sequences mainly included the coding regions of residues 519–600, 712–762, and 1006–1019 of the *putA* sequence from *B. japonicum* strain USDA 110. Eventually, preliminary X-ray crystallography data from BjPutA confirmed that the amino acid sequence of purified BjPutA matches that predicted from the *B. japonicum* USDA110 nucleic acid sequence data (Tanner, personal communication). In addition, the theoretical *pI* value of BjPutA from the original sequence is  $>10$  suggesting a highly basic protein. However, BjPutA was purified by anion-exchange chromatography (pH = 8.1) consistent with a theoretical *pI* value of around 6.5 from the newly deposited *putA* sequence from *B. japonicum* USDA110. Figure 1 shows



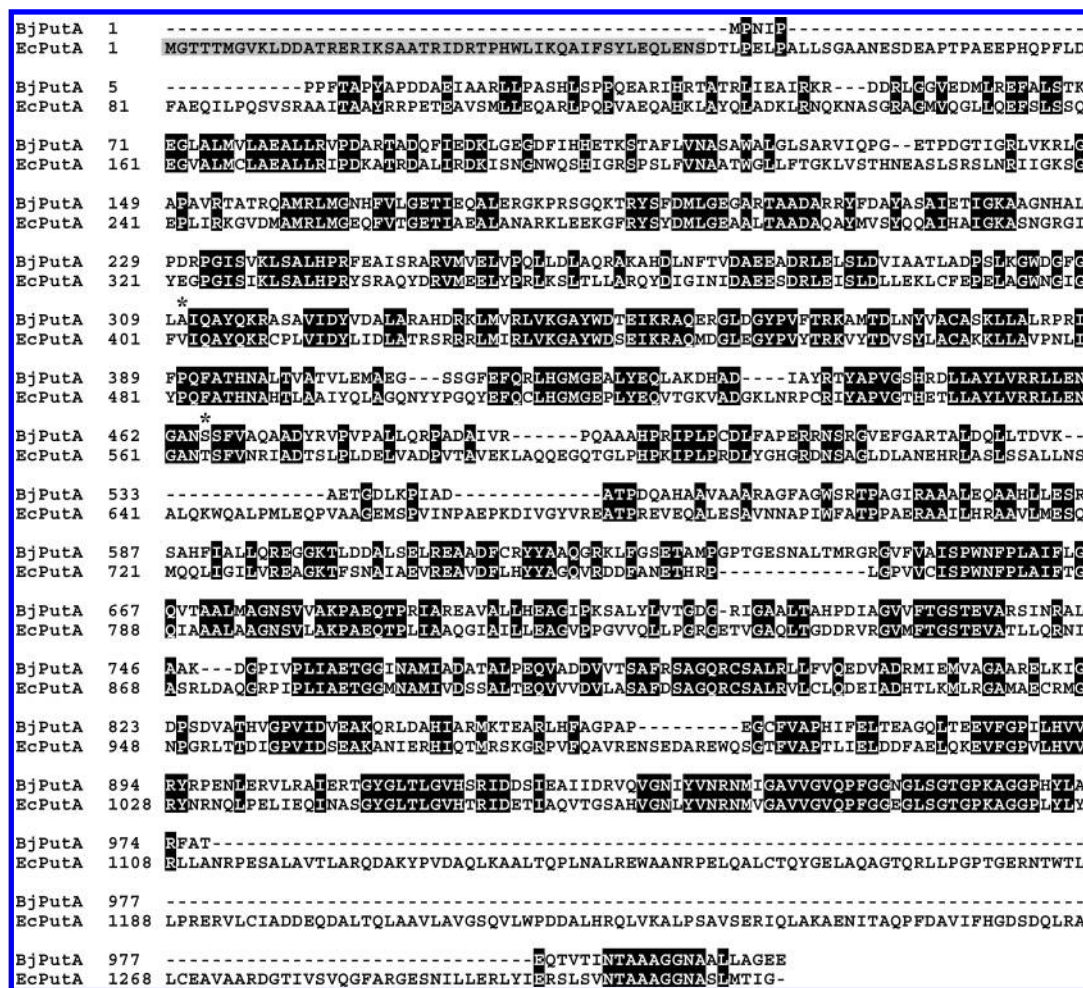


FIGURE 1: Amino acid sequence alignment of PutA from *Bradyrhizobium japonicum* (BjPutA) and *Escherichia coli* (EcPutA). Black boxes indicate identical residues shared between BjPutA (GenBank accession number NP\_773901) and EcPutA (GenBank accession number P09546), and residues shaded in gray indicate the DNA-binding domain of EcPutA (residues 1–47). The asterisks above the letters indicate the two FAD-binding residues that are not identical in BjPutA and EcPutA. The alignment was made using BioEdit and ClustalW.

an alignment of the predicted BjPutA amino acid sequence from *B. japonicum* USDA110 with EcPutA. The two proteins share 37% sequence identity with high conservation in the PRODH and P5CDH domains. The sequence identity in the PRODH domains between BjPutA (residues 169–507) and EcPutA (residues 261–612) is 52%, while the sequence identity in the P5CDH domains is 46% (BjPutA residues 533–977 and EcPutA residues 650–1130). Two significant differences between the two proteins are that BjPutA lacks the RHH domain (EcPutA residues 1–47) and EcPutA residues 1112–1299. The RHH motif in EcPutA is responsible for DNA-binding activity and is also critical for stabilizing the dimeric structure of EcPutA (16). Deletion of the RHH domain disrupts the EcPutA dimer, consistent with known properties of the RHH motif which forms a hydrophobic dimerization core (16). Despite lacking the RHH motif, BjPutA purifies as a dimer suggesting a different mode of dimer formation. A molecular mass of ~220 kDa was estimated for BjPutA by size-exclusion chromatography consistent with a dimeric structure (data not shown). To confirm that BjPutA is devoid of DNA binding activity, we tested whether BjPutA could bind to potential regulatory regions (579 bp) upstream of the *putA* gene in *B. japonicum*. Gel mobility shift assays showed no binding of BjPutA to the promoter DNA (5 nM) even at 2  $\mu$ M of BjPutA protein.

Table 1: Kinetic Constants for PRODH Activity and Reduction Potentials in BjPutA and EcPutA<sup>a</sup>

enzyme	$K_m$ (mM)	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (s <sup>-1</sup> M <sup>-1</sup> )	$E_m$ (mV)
BjPutA				
wild-type	150 ± 11	5.6 ± 0.7	37	-132 ± 3
+ polar lipids	45 ± 5	18 ± 2	400	-114 ± 5
+ PC lipids	88 ± 15	11 ± 0.6	125	-127 ± 4
A310V	156 ± 23	8.4 ± 0.2	54	-90 ± 5
EcPutA				
wild-type <sup>b</sup>	100	12	120	-77
+ polar lipids	92 ± 13	10.5 ± 0.9	114	-81 ± 4

<sup>a</sup> Potentiometric measurements were determined in potassium phosphate buffer (pH 7.5) at 20 °C. <sup>b</sup> Values from ref 26.

BjPutA was also not observed to bind to the *put* control DNA region from *E. coli*. Thus, as anticipated, we did not observe any evidence for DNA binding activity in BjPutA consistent with primary structure analysis that shows BjPutA lacks the RHH domain of EcPutA.

The PRODH specific activity for wild-type BjPutA determined by proline:DCPIP oxidoreductase assays is around 2.5–3.0 U/mg with a turnover number ( $k_{cat}$ ) that is a 2-fold lower than EcPutA and is summarized in Table 1 (26). The PRODH activity reached a plateau at pH ~ 8.0. In addition, an apparent  $K_i$  of 0.1 mM for the competitive inhibition of BjPutA by L-THFA, an isostructural analogue

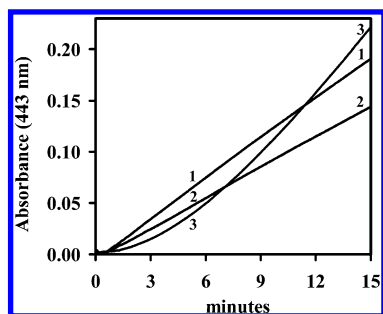


FIGURE 2: Functional membrane association assays. Full-length BjPutA (10  $\mu\text{g/mL}$ ) (1), protease-treated BjPutA (10  $\mu\text{g/mL}$ ) (2), and EcPutA (10  $\mu\text{g/mL}$ ) (3) were incubated with 60 mM proline, 4 mM *o*-aminobenzaldehyde, and inverted membrane vesicles from *E. coli* strain JT31 *putA*<sup>−</sup> (0.1 mg/mL membrane protein) in 20 mM Mops buffer (pH 7.5) at 23 °C. The reactions were monitored at 443 nm to determine the PRODH activity for each protein. The calculated specific activities for full-length BjPutA (1), protease treated BjPutA (2), and EcPutA (3) are 0.05, 0.04, and 0.08 unit/mg membrane protein, respectively.

of proline, was determined which is similar to EcPutA (26). The P5CDH specific activity is around 1.2 U/mg (pH 7.0) with an apparent  $K_m$  value of  $3.7 \pm 0.6$  mM DL-P5C. Considering the substrate for the P5CDH step is most likely the open chain form, GSA, the actual  $K_m$  value may be lower. At pH 7.0, the P5C/GSA equilibrium ratio is about 5:1; thus, the  $K_m$  value for P5CDH activity in BjPutA is most likely 1 mM (31). The P5CDH activity observed with BjPutA is almost 4-fold higher relative to EcPutA (0.3 U/mg) (28). Differences in aldehyde dehydrogenase activity between BjPutA and EcPutA were also observed with an alternative substrate, succinic acid semialdehyde. The aldehyde dehydrogenase specific activity with succinic acid semialdehyde was 0.04 and 0.006 U/mg for BjPutA and EcPutA, respectively. A  $K_m$  of  $12 \pm 1$  mM was estimated for succinic acid semialdehyde dehydrogenase activity in BjPutA. Thus, with both substrates, BjPutA exhibits higher ADH specific activity.

The spectroscopic properties of the bound FAD in wild-type BjPutA are similar to EcPutA with wavelength maxima at 451 and 378 for the two main FAD bands (see Figure 4, curve 1) (28). The molar extinction coefficient of the bound FAD was estimated to be  $13.62 \pm 0.42 \text{ M}^{-1} \text{ cm}^{-1}$  at 451 nm. The FAD to polypeptide ratio was estimated to be about 0.55–0.6 mol of FAD per polypeptide. This ratio is lower than EcPutA which typically has 0.8–0.85 mol of FAD per polypeptide (28). Attempts to increase the FAD content by reconstitution were unsuccessful. Interestingly, the molar extinction coefficient at 278 nm for the denatured BjPutA polypeptide is only around  $59\,054 \text{ M}^{-1} \text{ cm}^{-1}$  due to a lower Trp content of 0.5% relative to EcPutA which has a Trp content of 0.9% and a molar extinction coefficient of  $122\,562 \text{ M}^{-1} \text{ cm}^{-1}$  at 278 nm (28).

**Membrane Functional Association Assays and Conformational Changes in Wild-Type BjPutA.** Wild-type BjPutA exhibited functional membrane association activity using *E. coli* membrane vesicles with a specific activity of 0.05 U/mg of membrane protein or 0.5 U/mg of BjPutA as shown in Figure 2. The  $K_m$  for proline determined by membrane functional association assays was about  $1.1 \pm 0.2$  mM proline which is similar to the  $K_m$  value (4.3 mM proline) for membrane-associated PRODH activity in EcPutA

(32). The effects of lipids on the steady-state kinetic parameters of BjPutA were also examined by proline:DCPIP oxidoreductase assays performed in the presence of *E. coli* polar lipids in which  $K_m$  and  $k_{\text{cat}}$  values of 45 mM proline and  $18 \text{ s}^{-1}$  were estimated, respectively (Table 1). Thus, in the presence of lipid vesicles, BjPutA is transformed into a more efficient enzyme with  $k_{\text{cat}}/K_m$  increasing around 10-fold from 37 to  $400 \text{ s}^{-1} \text{ M}^{-1}$  as shown in Table 1. Enhanced PRODH kinetic properties were not observed with EcPutA in the presence of polar lipids.

A characteristic of the membrane functional association assays in previous work with EcPutA has been a lag phase ( $\sim 5$  min) prior to the linear production of P5C (see Figure 2) (32). This nonlinear phase of the reaction most likely reflects the formation of a stable enzyme–membrane complex (12, 32). In fact, the rate of approach to steady-state activity was used by Wood to infer a redox mechanism for the membrane association of EcPutA (12). Wood found that incubating EcPutA under reducing conditions enhanced membrane association kinetics (12). These observations implied that formation of an enzyme–membrane complex involved an obligatory conformational change in EcPutA. Interestingly, we did not observe a similar lag phase in the BjPutA membrane association assays (see Figure 2) suggesting BjPutA is already membrane-associated or that its membrane association kinetics are much faster relative to EcPutA.

The mechanism by which EcPutA switches from a soluble DNA-binding protein to a membrane-bound enzyme has been shown by limited proteolysis to involve a FAD redox-dependent conformational change (15, 17). Three conformers of EcPutA have been identified: (1) oxidized PutA, (2) ligand bound PutA, and (3) reduced PutA (17). Reduction of the FAD cofactor in EcPutA by proline or sodium dithionite generates conformer 3 which subsequently binds to the membrane resulting in the activation of *put* gene expression (18). To examine whether BjPutA undergoes a similar redox-dependent conformational change, we subjected BjPutA to limited proteolysis in the presence of proline, the nonreducing substrate analogue L-THFA, and sodium dithionite and analyzed the products by 7.5% SDS–PAGE. Figure 3 shows the major polypeptide products from limited proteolysis of BjPutA with chymotrypsin under different conditions. Oxidized BjPutA shows a major band around 104 kDa and a minor band at  $\sim 94$  kDa. Upon reduction of BjPutA with sodium dithionite, no change in the proteolysis pattern is observed relative to oxidized BjPutA. Thus, unlike EcPutA in which reduction of FAD is the principal modulator of EcPutA conformation, reduction of the FAD cofactor in BjPutA has no effect on the limited chymotrypsin proteolysis of BjPutA. Ligand binding to BjPutA, however, was shown to favor the formation of the 94-kDa fragment. In the presence of proline and the nonreducing ligand L-THFA, the 104-kDa band disappears and the 94-kDa band predominates. Mass spectral analysis of the 94-kDa band estimated that approximately 42 N-terminal residues (including the  $8 \times \text{His}$  tag) and 26 C-terminal residues are removed with cleavage sites at L23 and F975 in BjPutA. The calculated decrease in mass is around 7.3 kDa resulting in a polypeptide of 102.6 kDa which is slightly higher than the mass estimated by SDS–PAGE ( $\sim 94$ -kDa).



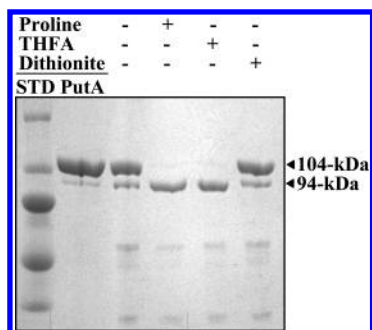


FIGURE 3: Effects of ligand binding and FAD reduction on the protease susceptibility of BjPutA. Purified BjPutA (1 mg/mL) was digested with chymotrypsin (10  $\mu$ g/mL) alone and in the presence of proline (5 mM), L-THFA (5 mM), and sodium dithionite (10 mM) in 50 mM potassium phosphate buffer (pH 7.5) for 1 h at room temperature. The protease digestion in the presence of dithionite was performed under anaerobic conditions. The reaction products were analyzed by denaturing gel electrophoresis (7.5% acrylamide) and stained with Coomassie Blue. The two left-hand lanes show the molecular mass standards of 150, 100, 75, 50, and 37 kDa and purified BjPutA prior to treatment with chymotrypsin.

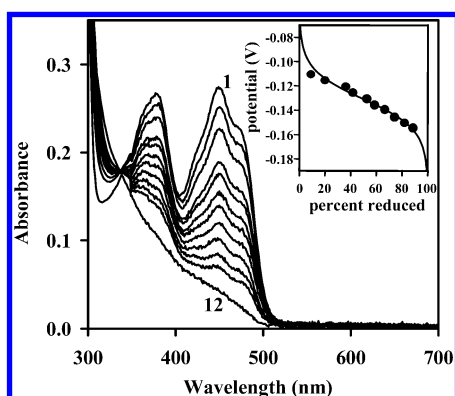


FIGURE 4: Spectroelectrochemical properties of BjPutA-bound FAD. Potentiometric titration of BjPutA (20  $\mu$ M) was performed in 50 mM potassium phosphate buffer (pH 7.5) at 20  $^{\circ}$ C. Curves 1–12: fully oxidized,  $-0.110$ ,  $-0.116$ ,  $-0.121$ ,  $-0.126$ ,  $-0.131$ ,  $-0.136$ ,  $-0.140$ ,  $-0.146$ ,  $-0.151$ ,  $-0.155$  V, and fully reduced, respectively. The inset shows a fit of the reduction potential data to a theoretical curve generated from the Nernst equation for one redox center with  $E_m = -0.132$  V ( $n = 2$ ).

The 94-kDa product was subsequently purified and characterized to assess the impact of limited proteolysis on BjPutA function. The 94-kDa product exhibited an FAD UV–visible spectrum with wavelength maxima at 450 and 383 nm and showed no significant changes in PRODH and P5CDH activities or in functional membrane association activity (0.04 U/mg of membrane protein) (see Figure 2). Thus, limited proteolysis by chymotrypsin appears to have no impact on the functional properties of BjPutA. In contrast, limited chymotrypsin proteolysis of reduced EcPutA causes the loss of DNA-binding and functional membrane association activities (17).

**Wild-Type BjPutA FAD Redox Properties and the Effects of Lipids.** A coulometric titration of wild-type BjPutA showed  $<10\%$  semiquinone is stabilized during reduction (data not shown). A potentiometric titration of wild-type BjPutA is shown in Figure 4, and the data are summarized in Table 1. The  $E_m$  value for the FAD/FADH<sub>2</sub> couple in wild-type BjPutA was determined to be around  $-0.132$  V (pH 7.5). A Nernst plot gave a slope of 26 mV which is

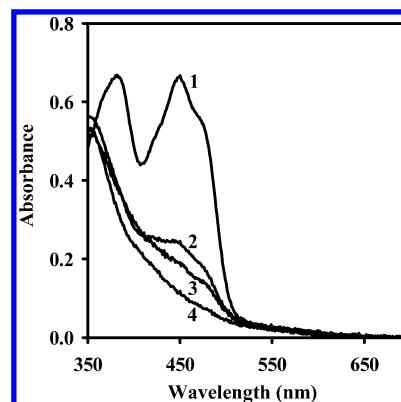


FIGURE 5: Influence of lipids on proline reduction of BjPutA. The UV–visible spectra of BjPutA (48.9  $\mu$ M) were recorded at 20  $^{\circ}$ C under anaerobic conditions in 50 mM potassium phosphate buffer (pH 7.5). (1) Oxidized BjPutA, (2) 30 min after the addition of proline (final concentration 5 mM) to spectrum 1, (3) 30 min after the addition of PC lipids (final concentration 0.2 mg/mL) to spectrum 2, and (4) 30 min after the addition of *E. coli* polar lipids (final concentration 0.2 mg/mL) to a BjPutA–proline mixture identical to that shown in spectrum 2. The spectra have been corrected for effects of dilution.

near the theoretical value of 29 mV for a two-electron-transfer process.

The  $E_m$  value for BjPutA is about 55 mV more negative than the  $E_m$  value previously determined for EcPutA (see Table 1) (26). Consistent with a lower reduction potential for FAD bound to BjPutA, Figure 5 shows that, under anaerobic conditions, proline is not able to fully reduce BjPutA, in contrast to EcPutA, which is fully reduced by proline (Figure 5, curve 2) (28). A limit of  $\sim 71\%$  reduction was observed relative to electrochemical reduction of BjPutA. From the concentration of the oxidized and reduced BjPutA species, a relative potential difference of  $\sim 0.011$  V was estimated between the BjPutA and substrate couples. With the  $E_m$  value for BjPutA being more positive, a conditional reduction potential for the proline/P5C couple is estimated to be around  $-0.143$  V. This reduction potential value for the substrate couple is about 20 mV more negative than previously estimated ( $E_m \sim -0.124$  V) (28).

To investigate how lipids enhance the catalytic properties of BjPutA, the effects of lipids on proline reduction of BjPutA and the FAD redox properties were determined. Upon the addition of *E. coli* polar lipid vesicles to the BjPutA–proline mixture, nearly complete reduction ( $\sim 92\%$ ) of the bound FAD was achieved (Figure 5, curve 4). A conditional potential difference of  $\sim 0.031$  V was estimated between the BjPutA and substrate couples. This suggests that the *E. coli* polar lipids cause a change in the  $E_m$  value of the substrate couple or BjPutA-bound FAD. Because lipids are not anticipated to influence the redox potential of the substrate couple, the greater FAD reduction is most likely due to *E. coli* polar lipids perturbing the potential of BjPutA-bound FAD. From the changes in the amount of proline reduced FAD in the absence and presence of the *E. coli* polar lipids, the  $E_m$  value for the bound FAD is anticipated to shift positive by almost 20 mV in the presence of lipids. To test whether negatively charged lipids were required, we also examined overall neutral PC vesicles. The addition of neutral lipids to a BjPutA–proline mixture also enhanced the reduction of the bound FAD but to a lesser

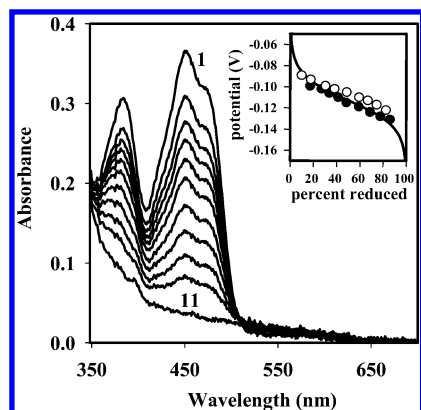


FIGURE 6: Redox properties of BjPutA-bound FAD in the presence of phospholipids. Potentiometric titrations of BjPutA ( $26.7 \mu\text{M}$ ) were performed in the presence of *E. coli* polar lipids ( $0.2 \text{ mg/mL}$ ) in  $50 \text{ mM}$  potassium phosphate buffer ( $\text{pH } 7.5$ ) at  $20^\circ\text{C}$ . Curves 1–11: fully oxidized,  $-0.099$ ,  $-0.102$ ,  $-0.106$ ,  $-0.110$ ,  $-0.115$ ,  $-0.119$ ,  $-0.124$ ,  $-0.128$ ,  $-0.131 \text{ V}$ , and fully reduced. The inset shows a fit of the potential data measured in the reductive direction (solid circles) to a theoretical curve generated from the Nernst equation for one redox center with  $E_m = -0.114 \text{ V}$  ( $n = 2$ ). Potentiometric data obtained from an oxidative titration is also shown (open circles).

extent,  $\sim 80\%$  (see Figure 5, curve 3). Table 1 shows that the steady-state kinetic parameters for PRODH activity in wild-type BjPutA were also less-influenced by neutral lipids.

Figure 6 shows a potentiometric titration of wild-type BjPutA in the presence of *E. coli* polar lipid vesicles. As before, no significant stabilization of semiquinone species was observed during reduction. The  $E_m$  value, however, shifted positive by  $18\text{-mV}$  relative to lipid-free wild-type BjPutA (see Table 1), consistent with the effects of *E. coli* polar lipids on proline reduction of FAD shown in Figure 5. The  $18\text{-mV}$  positive shift in potential corresponds to a Gibbs free energy difference of  $\sim 3.5 \text{ kJ/mol}$ . The redox potential of the FAD was also determined in the oxidative direction from which a potential value of  $-0.107 \text{ V}$  was determined demonstrating a reversible reduction process and a stable BjPutA-lipid complex.

To determine whether these observations require negatively charged lipids, potentiometric titrations of BjPutA in the presence PC lipids were performed. The  $E_m$  value for BjPutA in the presence of neutral PC lipids was not significantly perturbed (see Table 1) consistent with only a small increase ( $\sim 9\%$ ) in the reduction of the FAD cofactor by proline in the presence of neutral PC lipids (Figure 5, curve 3). The smaller shift in the  $E_m$  value relative to *E. coli* polar lipids suggests the effect of lipid vesicles on the FAD/FADH<sub>2</sub> redox couple is preferential for negatively charged lipids. Although an overall acidic protein, BjPutA may have a positively charged surface near the FAD active site that interacts favorably with negatively charged lipids. As a final test, we also examined the effects of lipids on EcPutA. Recently, surface plasmon resonance studies showed that oxidized PutA does not bind to the membrane, while reduced PutA forms a tight complex with lipids ( $K_d < 0.01 \text{ nM}$ ). Unlike BjPutA, the *E. coli* polar lipids did not cause a positive shift in the reduction potential of the EcPutA-bound FAD (see Table 1). Thus, the influence of lipids on the redox properties of bound FAD appears to be unique for BjPutA.

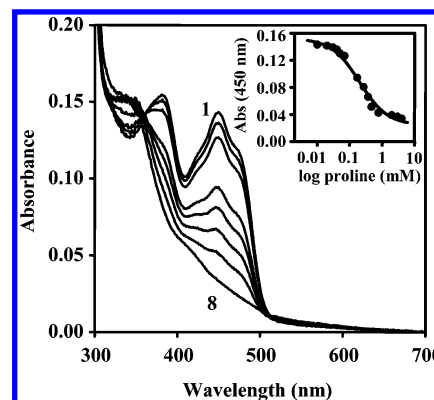


FIGURE 7: Proline titration of BjPutA A310V. UV-visible spectra of BjPutA A310V ( $10.5 \mu\text{M}$ ) were recorded 5 min after each proline addition at  $20^\circ\text{C}$  in  $50 \text{ mM}$  potassium phosphate buffer ( $\text{pH } 7.5$ ). Curves 1–8 are selected spectra from the titration in the presence of  $0$ ,  $0.04$ ,  $0.07$ ,  $0.17$ ,  $0.27$ ,  $0.37$ ,  $0.47$ , and  $3.97 \text{ mM}$  proline, respectively. The inset shows the best-fit analysis of the proline titration data assuming the formation of a reduced PutA–P5C complex from which a  $K_{eq}$  of  $5 \pm 0.8 \text{ mM}^{-1}$  proline was estimated (28).

**BjPutA Mutant A310V Properties.** In the three-dimensional structure of the EcPutA PRODH domain, 35 residues are observed to interact with the FAD through electrostatic or nonpolar interactions (33). Substrate binding and electron transfer occur on the *si* face of the flavin while the *re* face is supported by nonpolar interactions (33). Amino acid sequence alignment of BjPutA and EcPutA (see Figure 1) indicates that the two enzymes share all of the 35 residues that interact with FAD in the EcPutA PRODH domain structure except for EcPutA residues Val402 and Thr564 which are replaced by Ala310 and Ser465 at the corresponding positions in BjPutA, respectively (33). Thr564 forms a water-mediated hydrogen bond to the adenine and is not near the isoalloxazine ring; thus, it is unlikely that substitution of Ser465 in BjPutA would perturb the FAD redox properties (33). Val402 forms nonpolar contacts on the *re* face of the isoalloxazine near the C(4) and C(4a) positions of the pyrimidine ring which experience significant increases in electron density upon flavin reduction (33). The lack of the alkyl group of Val402 near the C(4) and C(4a) positions could affect the binding and orientation of the FAD cofactor in the active site and thus the redox potential of FAD.

To test whether the Ala substitution in BjPutA contributes to the more negative FAD reduction potential relative to EcPutA, the BjPutA mutant A310V was purified and characterized by spectroelectrochemistry. The steady-state kinetic properties of the BjPutA A310V mutant are similar to wild-type BjPutA with a slight increase in the turnover number for PRODH activity (Table 1). The UV-visible spectrum of BjPutA mutant A310V displays FAD absorbance peaks at  $381$  and  $450 \text{ nm}$  with a FAD/polypeptide ratio of  $0.6 \text{ mol}$  (Figure 7). The intensity of the transition at  $381 \text{ nm}$  is slightly higher than the  $450 \text{ nm}$  transition for FAD bound to the BjPutA mutant A310V. Unlike wild-type BjPutA, Figure 7 shows that the BjPutA mutant A310V is fully reduced by proline. Consistent with enhanced proline reduction, potentiometric titrations of the BjPutA mutant A310V shown in Figure 8 indicate a  $>40\text{-mV}$  positive shift in the  $E_m$  value of the bound FAD (Nernst slope  $24 \text{ mV}$ ) compared to wild-type BjPutA (see Table 1). Analogous to wild-type BjPutA,



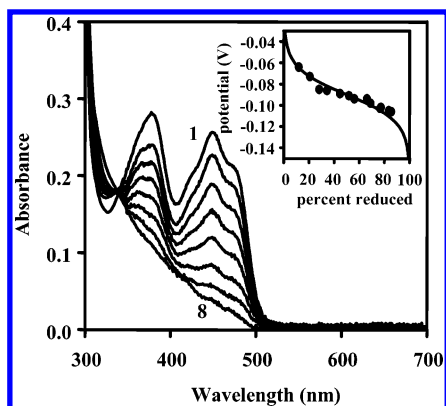


FIGURE 8: Potentiometric titration of BjPutA A310V mutant (19.4  $\mu$ M) in 50 mM potassium phosphate buffer (pH 7.5) at 20  $^{\circ}$ C. Curves 1–8: fully oxidized,  $-0.083$ ,  $-0.086$ ,  $-0.091$ ,  $-0.094$ ,  $-0.097$ ,  $-0.101$ , and fully reduced. The inset shows a fit of the potential data to a theoretical curve generated from the Nernst equation for one redox center with  $E_m = -0.09$  V ( $n = 2$ ).

little semiquinone was stabilized during reduction of the bound FAD in A310V.

## DISCUSSION

This is the first electrochemical and functional characterization of a PutA family member that lacks DNA binding activity. Although BjPutA and EcPutA have similar catalytic and membrane association activities, notable differences between the two enzymes were identified in FAD redox properties, lipid interactions, and redox-dependent conformational changes. The redox potential of BjPutA-bound FAD is more negative (55 mV) than EcPutA which may be related to the microaerobic conditions where *B. japonicum* exists in the symbiotic form. Amino acid sequence alignment of BjPutA and EcPutA (see Figure 1) indicates that only two residues are different in the FAD binding site with V402 and T564 in EcPutA replaced by A310 and S465 in BjPutA, respectively. A three-dimensional model of the FAD active site in BjPutA was generated by comparative modeling using the solved structure of the PRODH domain in EcPutA (7, 33, 34). The model displayed in Figure 9 shows that A310 is positioned on the *re* face (nonsubstrate binding face) of the isoalloxazine ring of FAD and is surrounded by residues that make important hydrogen-bond interactions with FAD such as R339, Q312, and the backbone amide of A279. The corresponding residues in EcPutA are R431, Q404, and A371. In the  $\beta_8\alpha_8$  barrel structure of the EcPutA PRODH active site, the *re* face of FAD is supported by  $\beta 4$ – $6$  with Q404 and V402 on  $\beta 4$ , Ala371 as part of loop that connects  $\beta 3$  and  $\alpha 3$ , and R431 located on  $\beta 5$  (7, 33). The polypeptide backbone carbonyl and main-chain nitrogen groups of A371 hydrogen-bond to N(3)H and O(4) of FAD, Q404 hydrogen-bonds to O(2) of FAD, and R431 hydrogen-bonds to the FAD N(5) (33). V402 makes nonpolar contacts with the C(4) and C(4a) positions of FAD (33). Because V402 contacts the isoalloxazine ring and is positioned between residues that make key hydrogen-bond interactions with FAD, the replacement of Val by Ala in wild-type BjPutA could change the orientation of the isoalloxazine ring in the active site and perturb the aforementioned hydrogen bonds. In addition, the absence of two methyl groups caused by A310 in the active site of BjPutA relative to Val402 in EcPutA may

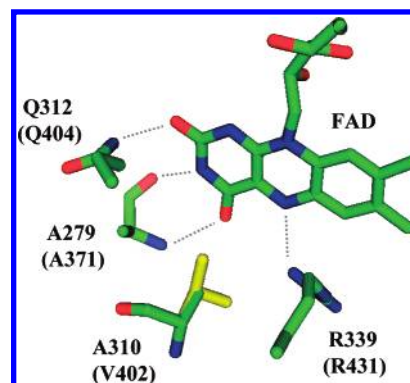


FIGURE 9: Modeling of the FAD binding site in BjPutA. A three-dimensional model of BjPutA was generated using the automated protein homology-modeling server (SWISS-MODEL) and the PRODH active site structure of EcPutA (1TIW). Shown is the isoalloxazine ring of the FAD, A310, and nearby residues that may be affected by the A310V mutation in BjPutA. In parentheses are the corresponding residues in EcPutA with V402 shown in yellow to highlight the additional methyl groups. Hydrogen-bonding is indicated by broken gray lines. The illustration was drawn using PyMOL.

induce subtle changes in the configuration of the loop supporting A279 (EcPutA A371) and the side chain of nearby R339 (EcPutA R431). Most likely, the Ala substitution in wild-type BjPutA does not disrupt main-chain configurations in  $\beta 4$  and the orientation of the nearby Q312 (EcPutA Q404). Extensive mutagenesis studies on flavodoxin and model studies with flavin analogues have shown that hydrogen-bond interactions with the O(2), O(4), N(3), and N(5) positions of the isoalloxazine ring strongly influence flavin binding and its redox properties (35–38). Charged residues, such as Arg that are near the flavin, have also been shown to influence reduction potentials in flavoproteins (39, 40). In the BjPutA mutant A310V, the reduction potential of the bound FAD shifts  $>40$ -mV (7.7 kJ/mol) positive to  $-0.09$  V, an  $E_m$  value that is more analogous to EcPutA. Thus, the A310V mutation in BjPutA most likely increases hydrogen-bond interactions with FAD that stabilize reduction such as with the O(2) and O(4) positions of the isoalloxazine ring, which would explain the more positive reduction potential of FAD in EcPutA relative to wild-type BjPutA (38). The anticipated X-ray crystal structure of BjPutA will provide insights into how Ala310 impacts the hydrogen-bond interactions and the flavin environment in BjPutA.

Interestingly, primary structure comparison of PutA family members from all the proteobacteria subclasses reveals that Ala replacement of the corresponding Val402 in EcPutA occurs only in bifunctional PutA proteins, while all of the predicted trifunctional PutA members share the V402 residue of EcPutA. A significance of the higher reduction potential of FAD in EcPutA is that proline can fully reduce the FAD cofactor and effectively regulate the intracellular location and function of EcPutA. In *B. japonicum*, proline regulation of BjPutA is not only unnecessary because BjPutA lacks a DNA-binding function but would also be less effective because proline only partially reduces the FAD. The lower redox potential of BjPutA in the absence of lipids shows BjPutA is not amenable for regulation by proline which is consistent with BjPutA being only responsible for catalyzing proline oxidation and not having a role in proline-dependent

regulation of *put* gene expression. It appears that the Val residue at position 402 in EcPutA and at the corresponding position in other trifunctional PutA family members (~20) has an important role in poising the reduction potential of FAD for favorable proline reduction thereby enabling PutA to respond efficiently to changes in intracellular proline levels.

The incomplete reduction of soluble BjPutA is overcome by lipid interactions that are distinct from EcPutA. Lipids were shown to influence the reduction potential of FAD in BjPutA but had no effect on EcPutA-bound FAD. This implies that lipids are most likely interacting with BjPutA near the FAD binding domain. So far, the membrane binding domains in BjPutA and EcPutA have not been identified. The 18-mV positive shift in  $E_m$  is consistent with the lipid-enhanced proline reduction of FAD and demonstrates that changes in the thermodynamic properties of bound FAD contribute to the ~10-fold increase in  $k_{cat}/K_m$  in the presence of phospholipids. Thus, even though the lower redox potential of FAD in BjPutA slightly diminishes the turnover rate of the soluble enzyme, improved catalytic efficiency is achieved in the presence of lipids demonstrating that membrane binding enhances the catalytic function of BjPutA and thus proline catabolic rates *in vivo*. The binding of BjPutA to the membrane is critical for its physiological function of transferring reducing equivalents from proline to the terminal electron transport system of *B. japonicum* and ultimately for satisfying the energy demands of  $N_2$  fixation in the bacteroid. Because proline completely reduces FAD in EcPutA, modulation of the flavin thermodynamic properties through lipid interactions is apparently not necessary. However, EcPutA does exhibit an improved  $K_m$  for proline in the presence of lipids suggesting that PutA enzymes generally become more catalytically efficient when membrane-associated (32). A well-characterized example of a flavoenzyme that is activated by phospholipids is pyruvate oxidase, a peripheral membrane-associated flavoenzyme from *E. coli* that catalyzes the oxidative decarboxylation of pyruvate (41). Upon binding the substrate pyruvate and reduction of the flavin cofactor, a conformational change occurs in pyruvate oxidase that exposes a lipid binding site on the enzyme (41, 42). The catalytic efficiency of lipid-activated pyruvate oxidase is increased by 250-fold relative to the soluble form of the enzyme (42). A primary contributor to the enhanced turnover of the activated enzyme is the nearly 150-fold increase in the pseudo-first-order rate of flavin reduction by the intermediate hydroxyethylthiamin pyrophosphate during the catalytic cycle (43).

Last, no redox-dependent conformational changes were detected in BjPutA by limited chymotrypsin proteolysis. These observations support the notion that only trifunctional PutA proteins that are transcriptional repressors have a redox-dependent regulatory system to control *put* gene expression. In EcPutA, FAD reduction induces protease susceptibility in a flexible region (residues 215–235) near the PRODH domain (17). Protease susceptibility was not observed in the corresponding region of BjPutA (residues 124–143), and coincidentally BjPutA shares only one residue (R142) with EcPutA (R234) in this region. It appears EcPutA has extensions/insertions that are necessary for the redox control of EcPutA conformation and function such as N-terminal

residues 1–90 that include the RHH motif and a linker domain.

Further insights into the regulation of EcPutA and structure–function relationships in PutA proteins will be gained from the ongoing functional and structural characterization of BjPutA. The discovery of how BjPutA forms a stable dimer even though it lacks the dimerization domain of EcPutA will contribute to the understanding of PutA oligomeric structures. Work is in progress to characterize BjPutA–lipid interactions to address differences in membrane binding between BjPutA and EcPutA. Further characterization of BjPutA will also generate insights into proline catabolism in *B. japonicum* which may help strategies for enhancing nitrogen fixation with its soybean host (*Glycine max*) (44). Studies have shown that proline can be used as a carbon and energy source in bacterioids which may be especially critical during environmental stress and when energy sources are limited (44, 45). Increased levels of proline catabolism in bacterioids have been correlated with higher  $N_2$  fixation rates and seed yields (44). van Dillewijn et al. also showed increased nodulation efficiency of a *Sinorhizobium meliloti* strain that contained an additional plasmid-encoded *putA* gene (46).

## ACKNOWLEDGMENT

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