Flavin Redox State Triggers Conformational Changes in the PutA Protein from *Escherichia coli*†

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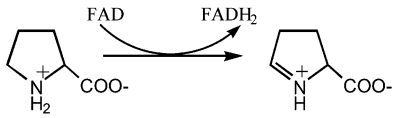
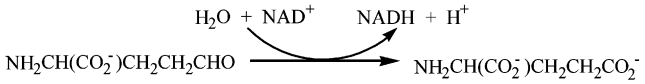
ABSTRACT: The regulation of proline utilization in *Escherichia coli* involves the proline-dependent translocation of the PutA flavoprotein from the cytoplasm to a peripheral position on the membrane. In the cytoplasm, PutA represses transcription of the proline utilization (*put*) genes while membrane-bound PutA catalyzes the oxidation of L-proline to glutamate. The mechanism by which PutA switches from a DNA-binding protein to a membrane-bound enzyme involves a proline-induced conformational change that is characterized by the appearance of a 119-kDa fragment during limited proteolysis of proline-reduced PutA. To establish whether the FAD redox state is responsible for the proline-induced conformational change in PutA, we distinguished the effects that FAD reduction and proline analogue binding have on PutA conformation by limited chymotrypsin proteolysis. Controlled potentiometric proteolysis of PutA demonstrated that the formation of the 119-kDa band occurs at an E_m(conf) value of −0.058 V (pH 7.5), which is within 20 mV of the E_m value for FAD bound to PutA. The manipulation of the E_m(conf) value by reconstitution of PutA with the FAD analogue, 5-deazaFAD, confirmed that the conformational change observed in the presence of proline is solely dependent on the FAD redox state. The proline analogue, L-tetrahydro-2-furoic acid (L-THFA), failed to elicit the formation of the 119-kDa fragment during chymotrypsin cleavage of PutA. Instead, a unique fragment of about 93-kDa was observed, indicating that a distinct PutA conformer is stabilized by L-THFA. Reduction of L-THFA-complexed PutA, however, regenerated the 119-kDa fragment showing that reduction of the FAD cofactor overrides conformational changes induced by L-THFA. Mapping of the protease susceptibility sites in PutA revealed that the conformational changes caused by FAD reduction and L-THFA binding are transmitted to domains outside the proline dehydrogenase active site.

The PutA (proline utilization A) protein is a multifunctional flavoenzyme in bacteria that combines catalytic, membrane binding, and DNA binding activities onto a single polypeptide. From *Escherichia coli*, PutA is a polypeptide of 1320 amino acids that purifies as a dimer of molecular mass 293 kDa, with one noncovalently bound flavin adenine dinucleotide (FAD) per monomer (*1*). PutA catalyzes the two-step oxidation of L-proline to glutamate using discrete proline dehydrogenase (PRODH) and Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH) domains (*1*−*3*). The PRODH domain catalyzes the transfer of two electrons from proline to FAD in the first step to generate Δ^1-pyrroline-5-carboxylate (P5C) and reduced FAD. In a subsequent



oxidative half-reaction, PutA associates peripherally with the membrane where the PRODH domain catalyzes the transfer of electrons from reduced FAD to an acceptor in the respiratory chain to regenerate oxidized FAD (*3*−*5*). After hydrolysis of P5C, the P5CDH active site catalyzes the NAD-dependent oxidation of γ-glutamic acid semialdehyde to glutamate.



In *E. coli*, *Salmonella typhimurium* and soil microbes such as *Pseudomonas putida* PutA is also an autogenous transcriptional repressor of the proline utilization (*put*) regulon (*1*, *6*−*12*). The *put* regulon is composed of the genes *putP* and *putA*, which are transcribed in opposite directions from the *put* control intergenic DNA. The *putP* gene product is a high affinity Na^+/proline transporter (PutP) (*13*). The expression of the *put* genes is dependent on the availability of L-proline and the intracellular location of PutA. In the absence of proline, PutA accumulates in the cytoplasm and

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[1] Abbreviations: FAD, flavin adenine dinucleotide; *put*, proline utilization; NAD^+, nicotinamide adenine dinucleotide; PVDF, poly(vinylidene difluoride); PRODH, proline dehydrogenase; P5CDH, Δ^1-pyrroline-5-carboxylate dehydrogenase; P5C, Δ^1-pyrroline-5-carboxylate; THFA, tetrahydro-2-furoic acid; DCPIP, dichlorophenolindophenol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; E_m, midpoint potential.

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represses transcription of the *put* regulon by binding to promoter sequences in the *put* control intergenic DNA region (*1*, *14*). In the presence of proline, PutA shifts from the cytoplasm to the membrane activating transcription of the *put* genes and proline oxidation (*5*, *10*, *15*, *16*).

Since proline reduces the FAD in PutA, the FAD redox state has been proposed to govern the intracellular location of PutA and thus its function (transcriptional repressor or membrane-bound PRODH) (*5*, *9*, *15*, *16*). Wood (*5*) first proposed a FAD redox mechanism for regulating PutA after observing that reducing conditions were essential for PutA−membrane associations (*5*). In addition, proline reduction of FAD bound to PutA and proline-induced PutA−membrane associations occur at about the same midpoint, 0.1 mM proline (*16*). Surber and Maloy (*15*) reported further evidence for a redox dependent mechanism by showing that the addition of an electron acceptor (2,3-dimethoxy-5-methyl-1,4-benzoquinone) to proline-reduced PutA disrupts PutA−membrane associations presumably through reoxidation of the FAD cofactor (*15*). In contrast to PutA−membrane interactions, the FAD redox state appears to have little influence on PutA−DNA binding affinity. PutA−DNA binding studies have shown that proline elicits just a 2-fold decrease in PutA−DNA binding affinity (*17*). Also, in potentiometric titrations of PutA complexed to the *put* control DNA, only a 2-fold difference in the overall DNA-binding affinity between oxidized and reduced PutA was determined (*18*). Thus, the proline-induced switch in PutA intracellular location and function appears to be primarily directed by changes in PutA−membrane binding affinity. A similar FAD dependent mechanism has been invoked for the transcriptional regulator NifL in *Klebsiella pneumoniae*. NifL is a flavoprotein in *Azotobacter vinelandii* and *K. pneumoniae* that represses the transcription of the nitrogen fixation (*nif*) genes by inhibiting the activity of the transcriptional activator nitrogen fixation regulatory protein A (NifA) through NifL−NifA complex formation (*19*−*21*). The formation of the NifL−NifA complex is favored when the NifL-bound FAD is in the oxidized state (*22*). In recent studies of NifL from *K. pneumoniae*, FAD reduction was proposed to alter the intracellular location of NifL by promoting the binding of NifL to the cytoplasmic membrane, consequently disrupting NifL−NifA interactions and activating the expression of *nif* genes (*23*).

The enhanced PutA−membrane associations induced by proline appear to be due to a concomitant conformational change in PutA upon reduction of the FAD. Brown and Wood (*16*) observed that PutA has different protease susceptibilities in the absence and presence of proline, indicating a proline dependent conformational change (*16*). The conformational change caused by proline was shown to be coincident with PutA binding to the membrane and proline reduction of FAD. As a result, it was proposed that the change in PutA conformation observed by protease susceptibility is critical for increasing PutA−membrane associations and is driven by proline reduction of the FAD (*16*).

To further understand the mechanism by which PutA switches from a transcriptional repressor to a membrane-bound PRODH, our efforts are focused on distinguishing the roles that proline binding and FAD reduction have in regulating PutA. Herein, we describe the effects that FAD reduction and substrate analogues have on the conformation of PutA. To test whether the conformational change induced by proline is regulated by FAD reduction, we performed limited proteolysis of PutA by chymotrypsin under different controlled potentiometric conditions. Our controlled potentiometric data explicitly show that the FAD redox state switches PutA between two different conformers. Unexpectedly, a new conformational change was induced by complexation of PutA to the proline analogue, L-tetrahydro-2-furoic acid (L-THFA), demonstrating that substrate binding also influences PutA conformation. However, electrochemical reduction of L-THFA-complexed PutA showed that the FAD redox state is the central modulator of PutA conformational changes. Mapping of the chymotrypsin cleavage sites revealed that FAD reduction exposes a region outside the PRODH domain to proteolysis.

MATERIALS AND METHODS

Enzymes and Chemicals. All chemicals and buffers were purchased from Fisher and Sigma-Aldrich Inc. Chymotrypsin (type VII) was purchased from Sigma-Aldrich. 5-DeazaFAD was synthesized in the dark from 5-deazariboflavin using FAD synthetase as described except that sodium dithionite and sodium fluoride were omitted (*24*). 5-Deazariboflavin was a generous gift from M. Stankovich (University of Minnesota, Minneapolis, Minnesota) and FAD synthetase was purified from *Corynebacterium ammoniagenes* as published (*25*). 5-DeazaFAD was separated from 5-deazariboflavin and 5-deazaflavin mononucleotide in the reaction mixture on a Hamilton C-18 HPLC column using a linear gradient of 5−95% methanol in 5 mM ammonium acetate, pH 3.0−3.5, at a flow rate of 2 mL/min for 60 min. The efficiency of the enzymatic conversion of 5-deazariboflavin to 5-deazaFAD was >80%. The 5-deazaFAD fraction was lyophilized and stored at −20 °C. Pyocyanine was prepared by photooxidation of phenazine methosulfate. All experiments utilized NanoPure water.

PutA Preparation. PutA holoprotein was expressed and purified with a C-terminal hexahistidine tag by Ni^{2+} NTA affinity chromatography and anion exchange chromatography as reported previously (*26*). The C-terminal hexhistidine tag was retained after purification. Purified PutA holoprotein was stored at −70 °C in 50 mM potassium phosphate (pH 7.5) containing 10% (v/v) glycerol at 10 mg/mL. The concentrations of oxidized PutA were determined spectrophotometrically using an extinction coefficient of 12700 M^{-1} cm^{-1} at 451 nm and by the BCA method (Pierce) with bovine serum albumin as the standard (*16*).

Reconstitution of PutA with 5-DeazaFAD. PutA apoprotein was prepared according to a method reported previously (*16*). A 2 mL sample of purified PutA holoprotein was dialyzed into 1 L of 100 mM sodium phosphate (pH 7.5) containing 1 M potassium bromide for 24 h at 4 °C with three exchanges of buffer. The PutA sample was then dialyzed into 1 L of 50 mM potassium phosphate (pH 7.5) containing 10% (v/v) glycerol for 24 h at 4 °C with three exchanges of buffer. The resulting PutA apoprotein was stored at −70 °C. In preparation for the reconstitution of the PutA apoprotein, lyophilized 5-deazaFAD was first resuspended in 50 mM potassium phosphate (pH 7.5), and the concentration of the 5-deazaFAD solution was determined spectrophotometrically using a molar extinction coefficient of 11500 M^{-1} cm^{-1} at 399 nm (*27*). PutA apoprotein was then reconstituted with

5-deazaFAD by incubating the apoprotein with a 10-molar excess of 5-deazaFAD at 4 °C for 24 h followed by overnight dialysis in 50 mM potassium phosphate (pH 7.5) containing 10% (v/v) glycerol. Unbound 5-deazaFAD was then removed by further dialysis with repeated exchanges of the same buffer. The concentration of the PutA apoprotein was determined by the BCA method. The concentrations of oxidized PutA reconstituted with 5-deazaFAD were estimated spectrophotometrically using a molar extinction coefficient of 12870 M^{-1} cm^{-1} at 401 nm. The molar extinction coefficient of 5-deazaFAD bound to PutA was estimated by denaturing reconstituted PutA and determining the concentration of free 5-deazaFAD using the molar extinction coefficient of 11500 M^{-1} cm^{-1} at 399 nm (*27*). The molar extinction coefficient reported for 5-deazaFAD bound to PutA is an average value from two separate determinations in which PutA was denatured in 6.5 M guanidinium chloride or 0.08% SDS. To estimate the molar ratio of 5-deazaFAD to PutA polypeptide, reconstituted PutA was denatured in 6.5 M guanidinium chloride (pH 6.5, 40 mM potassium phosphate) and the spectrum was recorded from 600 to 250 nm. After subtracting the contribution of free 5-deazaFAD from the spectrum, the total amount of PutA polypeptide was determined using the predicted molar extinction coefficient for denatured PutA of 122562 M^{-1} cm^{-1} at 278 nm in 6.5 M guanidinium chloride. The amount of 5-deazaFAD was determined using the molar extinction coefficient of 11500 M^{-1} cm^{-1} at 399 nm for free 5-deazaFAD. Titration of 5-deazaFAD−PutA with proline was performed and analyzed as previously described (*18*). The UV−visible spectra of 5-deazaFAD−PutA in the presence of L-THFA, proline, and sodium dithionite were recorded after a 10 min incubation of the reconstituted enzyme with each reagent (20 mM). Further decreases in the absorbance spectra of 5-deazaFAD−PutA were not observed with proline or sodium dithionite after 10 min of incubation.

Limited Proteolysis. Limited proteolysis of PutA by chymotrypsin was performed according to the method of Brown and Wood with slight modifications (*16*). In all digests, PutA was first incubated with proline, L-THFA, L-lactate, sodium sulfite, or sodium dithionite for 5 min prior to adding chymotrypsin. PutA (1 mg/mL) was then reacted with chymotrypsin (10 μg/mL) for 1 h at 23 °C in 50 mM potassium phosphate (pH 7.5) containing 10% glycerol. The digests in the presence of sodium sulfite and sodium dithionite were performed under an argon atmosphere after all the solutions were first degassed separately with cycled argon/vacuum. Sodium dithionite solutions were made fresh before each experiment in 0.1 M sodium pyrophosphate buffer (pH 9.0). Proline, L-THFA, L-lactate and sodium sulfite solutions were made in 50 mM potassium phosphate (pH 7.5). The reactions were stopped by adding phenylmethylsulfonyl fluoride (PMSF) (2 mM final concentration) and hot SDS buffer at a 3:2 (v/v) ratio of SDS buffer/sample and then frozen until needed for SDS−PAGE analysis. The samples were analyzed by SDS−PAGE using 7.5% or 15% acrylamide separating gels. Products from the proteolysis of PutA were visualized with Coomassie Blue G-250 and imaged using a Kodak Imaging Station 440CF. The molecular weight of the major protease products was estimated from a plot of log molecular weight versus relative migration distance of the standard proteins.

Controlled Pontentiometry. Controlled potentiometric measurements of PutA were performed under an argon atmosphere using a spectroelectrochemical cell with a 1.0 mL working volume and equipped with a gold working electrode, silver/silver chloride reference, and silver wire auxiliary electrodes. Potential measurements were recorded at 23 °C with PutA in 50 mM potassium phosphate (pH 7.5) containing 10% glycerol and 5 mM EDTA. All potential values are reported relative to the normal hydrogen electrode and were determined in the reductive direction. Methyl viologen (0.1 mM) and ferrocyanide (0.1 mM) were used as mediator dyes, while pyocyanine ($E_m = -0.04$ V, pH 7.5) (5 μM) and indigo disulfonate ($E_m = -0.109$ V, pH 7.5) (2−3 μM) were used as indicator dyes. PutA concentrations were around 7 μM (1 mg/mL). In experiments with 5-deazaFAD−PutA, mediators benzyl viologen ($E_m = -0.362$ V, pH 7.0) (10 μM), phenosafranin ($E_m = -0.252$ V, pH 7.0) (10 μM), and anthraquinone-2,6-disulfonate ($E_m = -0.184$ V, pH 7.0) (10 μM) were also used. To monitor the progress of the reduction, the UV−visible spectra of FAD in PutA were recorded on a Cary 100 spectrophotometer in each experiment. The absorbance at 451 and 476 nm (isobestic point for indigo disulfonate) were used to monitor the amount of oxidized and reduced FAD and to help decide at which point to equilibrate the cell potential. The equilibrium of the system in the potentiometric measurements was considered to be achieved when the measured potential drift was less than 1 mV in 3 min; this was typically around 1 h. Once the potential was poised, chymotrypsin was tipped into the solution and reacted with PutA for 1 h. The digests were terminated by opening the cell and adding PMSF and hot SDS buffer as described above. The denatured samples from the controlled potentiometric limited proteolysis were frozen at −20 °C until needed for SDS−PAGE analysis. The measured potential (E) for each limited proteolysis was an average of the potentials recorded before and after the digestion. The measured potential drift was typically 20 mV during the 1 h chymotrypsin digest. The midpoint potential (E_m) and n values were calculated from the Nernst equation (eq 1)

$$E = E_m + (0.059/n)\log([ox]/[red]) \quad (1)$$

where E is the measured (averaged) potential from each digest and n is the number of electrons transferred. The 119-kDa band was quantitated at each potential using the Kodak Imaging Station 440CF to estimate the amount of oxidized and reduced forms of PutA. Reoxidized PutA samples were prepared by first fully reducing PutA then completely reoxidizing PutA by switching the applied potential from −0.8 V to +0.8 V and using ferrocyanide (0.1 mM) as a mediator. Reoxidized PutA was then reacted with chymotrypsin for 1 h as described above.

Enzyme and Membrane Association Assays. PRODH activity of PutA was measured at 25 °C using dichlorophenolindophenol (DCPIP) as the terminal electron acceptor (*1*). One unit of PRODH activity is the quantity of enzyme that transfers electrons from 1 μmol of proline to DCPIP/min at 25 °C. Functional membrane association activity of PutA was measured as essentially described at 25 °C by following the formation of a yellow complex between P5C and *o*-aminobenzaldehdye at 443 nm ($\epsilon = 2590$ M^{-1} cm^{-1})

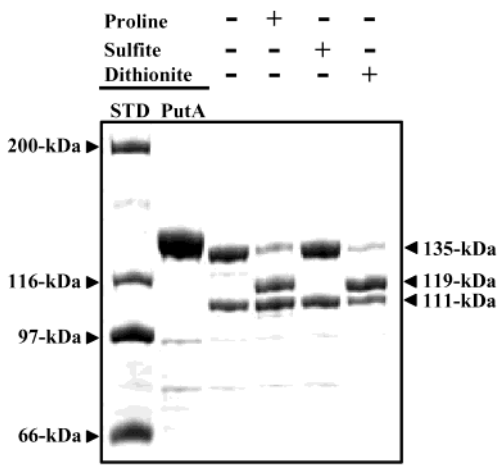

FIGURE 1: Limited proteolysis of PutA by chymotrypsin. Purified PutA (1 mg/mL) was incubated for 5 min each with proline (5 mM), sodium dithionite (10 mM), and sodium sulfite (10 mM) in 50 mM potassium phosphate (pH 7.5) followed by digestion with chymotrypsin (10 μg/mL) for 1 h at 23 °C. The reactions were quenched with PMSF and hot SDS buffer after 1 h. After complete denaturation in SDS buffer, 10 μg of each digestion was loaded onto a 7.5% polyacrylamide denaturing gel for analysis. The gel was stained with Coomassie Blue to visualize the major products. A molecular mass standard (STD) and purified PutA without protease treatment are shown in the two left-hand lanes.

in a 1 mL reaction volume (*3*, *4*). One unit of activity is the quantity of membrane vesicles that generates 1 μmol of the chromogenic complex/min. Inverted *E. coli* strain JT31 *putA*$^-$ membrane vesicles were prepared as reported by Abrahamson et al., frozen in liquid N_2, and stored at − 70 °C until needed (*3*). *E. coli* strain JT31 *putA*$^-$ was a generous gift from J. Wood (University of Guelph, Guelph, Ontario, Canada).

N-Terminal Sequencing and Western analysis. The major products from chymotrypsin cleavage of PutA were separated by SDS−PAGE and then transferred onto a Sequi-Blot poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad, 0.2 μm pore size) using a EBU-4000 Semi-Dry Electrophoretic Blotting System (CBS:Scientific Company, Inc., Der Mar, CA) according to the method of Towbin et al. (*28*). N-Terminal amino acid sequencing of the immobilized fragments on the PVDF membrane was then performed at the Protein Core Facility at Washington University (St. Louis, MO). For Western analysis, PutA protease products were separated by SDS−PAGE and electroblotted from the gel onto an Immun-Blot PVDF membrane (Bio-Rad, 0.2 μm pore size). Colorimetric detection of C-terminal hexahistidine tags in the fragments was then performed using a His-Tag Monoclonal Antibody Kit (Novagen) according to the manufacturers recommendation.

RESULTS

Redox Dependent Conformational Changes. Brown and Wood (*16*) showed that oxidized and proline-reduced PutA have different chymotrypsin protease susceptibilities, indicating the presence of two PutA conformers (*16*). The major products of limited chymotrypsin proteolysis of oxidized PutA are 135-kDa and 111-kDa fragments (Figure 1). Digestion of proline-reduced PutA yields major products of 119-kDa, 111-kDa, and a 135-kDa remnant (Figure 1). Because the formation of the 119-kDa band is coincident

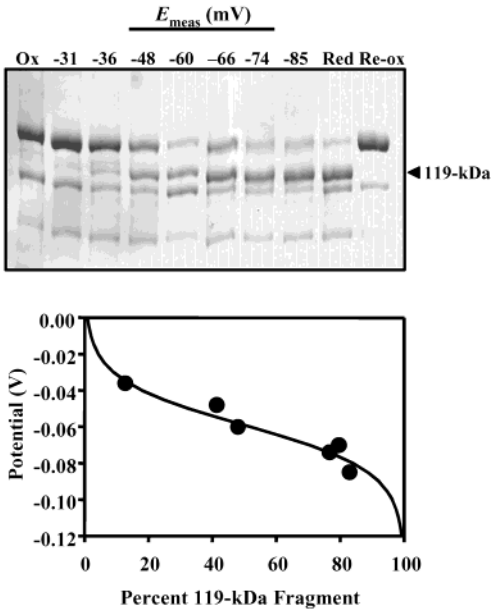

FIGURE 2: Cleavage of PutA by chymotrypsin under controlled potentiometric conditions. (Top) PutA (1 mg/mL) was reacted with chymotrypsin (10 μg/mL) for 1 h at 23 °C in 50 mM potassium phosphate (pH 7.5) under fully oxidizing (Ox), reducing (Red), and reoxidizing (Re-ox) conditions and under different poised redox potentials. About 10 μg from each limited chymotrypsin digest was analyzed by denaturing gel electrophoresis (7.5% polyacrylamide) and visualized with Coomassie Blue. (Bottom) A plot of the percentage of the 119-kDa fragment observed during limited chymotrypsin digests of PutA at various measured redox potentials relative to the amount of the 119-kDa product formed under fully reducing conditions. The data are fit to a theoretical curve generated from the Nernst equation for one redox center with a midpoint potential of −0.058 V ($n = 2$).

with PutA−membrane binding, it is thought that the 119-kDa band is a signature of a functionally important conformational transition during proline reduction of FAD (*16*). To initially test whether reduction of the FAD alone can induce the formation of the 119-kDa band, PutA was digested with chymotrypsin in the presence of sodium dithionite. The major fragments generated in the presence of sodium dithionite match the pattern observed in the presence of proline (Figure 1). As a control, we also performed a chymotrypsin digest of PutA in the presence of sodium sulfite since oxidation of dithionite generates bisulfite. Figure 1 shows sodium sulfite did not cause the formation of the 119-kDa band and that the protease fragments match those generated with oxidized PutA. Limited proteolysis of PutA in the presence of dithiothreitol (1 mM) also generated the 135-kDa and 111-kDa fragments demonstrating that the appearance of the 119-kDa band is not due to disulfide bond reduction in PutA (data not shown).

To more carefully test the role of the FAD redox state in influencing conformational changes in PutA, we performed limited proteolysis of PutA by chymotrypsin at various poised redox potentials. A midpoint potential value (E_m(E-FAD/E-FADH$^-$)) of −0.077 V (pH 7.5) for the reduction of FAD bound to PutA was determined previously from potentiometric titrations (*26*). Accordingly, limited proteolysis of PutA was performed at redox potentials poised near the E_m(E-FAD/E-FADH$^-$) value of PutA. Figure 2 (top) shows the results of limited proteolysis of PutA by chymotrypsin at controlled redox potentials ranging from −31 to

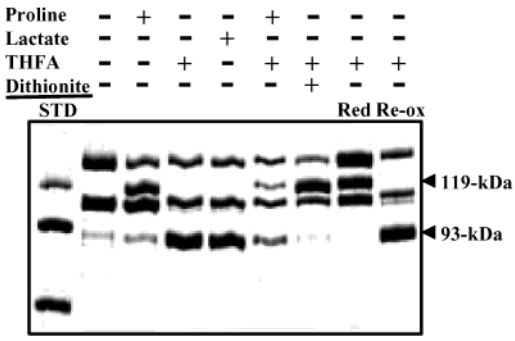

FIGURE 3: Effects of proline analogue binding on the protease susceptibility of PutA. Purified PutA (1 mg/mL) was digested with chymotrypsin (10 μg/mL) in the presence L-THFA (5 mM) and L-lactate (5 mM) in 50 mM potassium phosphate (pH 7.5) for 1 h at 23 °C. PutA was also treated with chymotrypsin in reactions containing 5 mM L-THFA mixed with 5 mM L-proline and 5 mM sodium dithionite. Chymotrypsin cleavage of fully reduced (Red) and reoxidized (Re-ox) PutA in the presence of L-THFA was performed under controlled potentiometric conditions. The reaction products from the different digests were analyzed by denaturing gel electrophoresis (7.5% acrylamide), and the gels were stained with Coomassie Blue. The left-hand lane shows the migration of molecular mass standards (STD) of 116 kDa, 97 kDa, and 66 kDa.

−85 mV. The appearance of the 119-kDa is noticeable at −36 mV and becomes significant at −48 mV. At −85 mV, an intense 119-kDa band is observed indicating nearly complete formation. A midpoint potential value (E_m(conf)) of −0.058 V (pH 7.5) was estimated for the formation of the 119-kDa band by monitoring the intensity of the 119-kDa band at the different measured potentials (Figure 2, bottom). A Nernst plot gave a 28 mV slope for the potentiometric measurement, which is near the theoretical value of 29 mV for a two-electron transfer. The E_m(conf) value is about 20 mV positive of the E_m(E-FAD/E-FADH$^-$) value for PutA. The PutA conformational transition observed during reduction of FAD was shown to be reversible by electrochemical reoxidation of fully reduced PutA followed by limited proteolysis. Figure 2 (Top) shows that the 135-kDa and 111-kDa bands indicative of oxidized PutA were also the major products of a limited chymotrypsin digest of reoxidized PutA.

Proline Analogue Binding. The proline analogue, L-THFA, was used to test the role of proline binding on conformational changes in PutA. L-THFA is a competitive inhibitor of PRODH activity in PutA (K_i = 0.2 mM) that does not undergo turnover and significantly perturbs the FAD absorbance spectrum upon binding to PutA (*26*). The dissociation constants for the binding of L-THFA to the oxidized and reduced forms of PutA are 0.24 and 0.55 mM, respectively. The main products of limited proteolysis of PutA by chymotrypsin in the presence of L-THFA (5 mM) are shown in Figure 3. Surprisingly, a ~93-kDa fragment appeared that was not observed in digests with oxidized and reduced PutA. L-lactate, another competitive inhibitor of PutA (K_i = 1.4 mM), also induced the formation of the distinct 93-kDa band. Thus, inhibitor binding to oxidized PutA promotes a new PutA conformation. When PutA was subjected to limited proteolysis in a 1:1 mixture of proline and L-THFA, both the 119-kDa and 93-kDa bands were observed, demonstrating competition between proline and L-THFA for the PRODH active site (Figure 3). More interesting, however, is that the 93-kDa band disappears upon reduction of the FAD. Figure 3 shows that limited proteolysis of L-THFA-complexed PutA under a reducing environment generates only the 119-kDa band with little evidence of the 93-kDa band. The formation of the 119-kDa band upon electrochemical reduction of L-THFA-complexed PutA indicates that FAD reduction overrides the conformational change induced by L-THFA binding. The conformational change caused by electrochemical reduction of L-THFA-complexed PutA was shown to be reversible by electrochemical reoxidation of reduced L-THFA-complexed PutA followed by limited proteolysis, which yielded the 93-kDa band.

5-DeazaFAD Reconstituted PutA. To confirm that the FAD redox state controls the proline-induced conformational change in PutA, PutA apoprotein was prepared for reconstitution studies with 5-deazaFAD. Similar to that reported earlier, PutA apoprotein exhibited <3% of the PutA holoprotein PRODH activity, and after incubation with FAD, activity was fully restored (data not shown) (*16*). Upon reconstitution of PutA apoprotein with 5-deazaFAD, the PRODH activity increased to ~5% of that of PutA holoprotein. Therefore, the PRODH activity of 5-deazaFAD−PutA is estimated to be ~2%. Figure 4a shows the UV−visible spectra of FAD-PutA, PutA apoprotein, and PutA reconstituted with 5-deazaFAD. The absorption maxima for 5-deazaFAD−PutA are at 343 and 401 nm compared to that of 381 and 451 nm for FAD-PutA. A slight shoulder around 415 nm is observed for 5-deazaFAD bound to PutA that disappears upon denaturing PutA and subsequent release of 5-deazaFAD (Figure 4a). The molar extinction coefficient for 5-deazaFAD bound to PutA was determined to be 12870 M^{-1} cm^{-1}, which is slightly higher than the molar extinction coefficient for free 5-deazaFAD (11500 M^{-1} cm^{-1}). On the basis of the denatured protein absorbance at 278 nm and the amount of released 5-deazaFAD, reconstituted PutA contains >0.9 mol of 5-deazaFAD per mole of polypeptide.

Figure 4b shows the results of chymotrypsin digests of PutA reconstituted with 5-deazaFAD. In the absence of proline, the 135-kDa and 111-kDa are the principal products. In the presence of proline or sodium dithionite, the 119-kDa band is observed, indicating that proline and sodium dithionite are both capable of reducing the 5-deazaFAD bound to PutA. Figure 4a shows the spectra of 5-deazaFAD−PutA in the presence of proline and sodium dithionite, confirming that 5-deazaFAD is reduced. From the absorbance spectrum, sodium dithionite generates a 10% further reduction of 5-deazaFAD−PutA relative to proline. An equilibrium constant of ~2 mM^{-1} for the reduction of 5-deazaFAD−PutA by proline was determined which is almost 20-fold lower than the equilibrium constant (36 mM^{-1}) for the proline reduction of native PutA (*18*). This corresponds to a Gibbs free energy difference of ~1.7 kcal mol^{-1}. Upon complexation of L-THFA to 5-deazaFAD−PutA, the 93-kDa band appears as a product showing that PutA reconstituted with 5-deazaFAD binds L-THFA similar to PutA holoprotein. The binding of L-THFA to 5-deazaFAD−PutA is also evident by the increased resolution in the visible spectrum of 5-deazaFAD and the appearance of a shoulder at 421 nm. A more resolved flavin absorbance spectrum and a prominent shoulder at 474 nm are also observed in FAD-PutA upon binding L-THFA (*26*).

Because the midpoint potential of free deazariboflavin is about 100 mV more negative than the midpoint potential

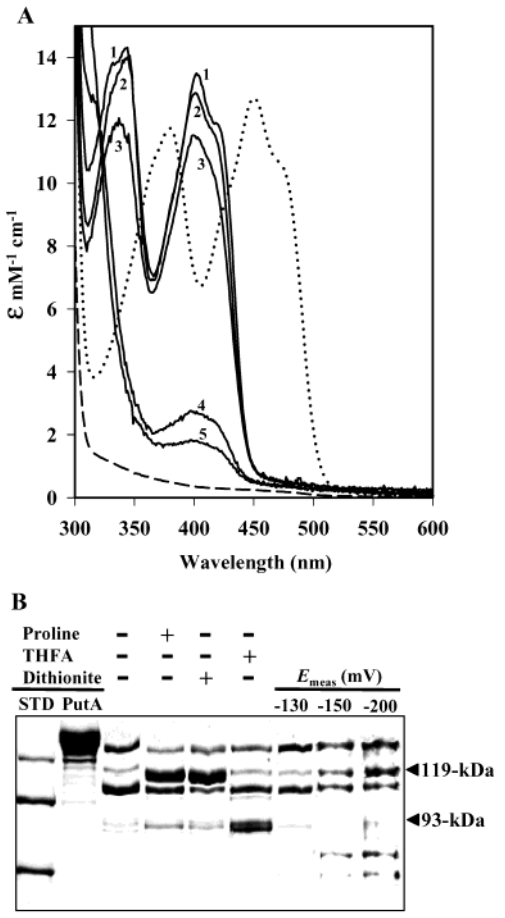

FIGURE 4: Reconstitution of PutA with 5-deazaFAD. Panel A: UV−visible spectra of 5-deazaFAD−PutA under the conditions used for chymotrypsin digestion. (1) 5-deazaFAD−PutA (5.8 μM) in the presence of L-THFA (20 mM); (2) 5-deazaFAD−PutA (5.43 μM); (3) 5-deazaFAD (5.43 μM) released after reconstituted PutA was denatured with 0.08% SDS; (4) 5-deazaFAD−PutA (6.5 μM) in the presence of L-proline (20 mM); and (5) 5-deazaFAD−PutA (11.9 μM) in the presence of sodium dithionite (20 mM). The UV−visible spectra of (···) FAD-PutA (20.2 μM) and (- - -) PutA apoprotein (69 μM) are also shown. Panel B: Analysis of products from limited chymotrypsin (10 μg/mL) digests of PutA (1 mg/mL) reconstituted with 5-deazaFAD by denaturing gel electrophoresis. Limited digests of 5-deazaFAD−PutA were performed in 50 mM potassium phosphate buffer (pH 7.5) at 23 °C in the presence of 20 mM proline, 20 mM sodium dithionite, and 20 mM L-THFA and under different controlled redox potentials. After quenching the digests, approximately 10 μg of protein from each reaction was loaded onto the denaturing gel (7.5% acrylamide). The two left-hand lanes show the migration of molecular mass standards (STD) of 116 kDa, 97 kDa, and 66 kDa and 5-deazaFAD−PutA without chymotrypsin cleavage.

of free riboflavin, we anticipated that a corresponding shift in the midpoint potential for the conformational change (E_m(conf) = −0.058 V) would occur in PutA reconstituted with 5-deazaFAD (*29*, *30*). This was tested by limited proteolysis of 5-deazaFAD−PutA at potentials poised approximately 100 mV more negative than the E_m(conf) for FAD-PutA. Figure 4b shows that at −130 mV, the 119-kDa band is observed, but the oxidized pattern of the 135-kDa and 111-kDa bands predominates. This is in contrast to limited proteolysis of FAD-PutA, which at −85 mV, the formation of the 119-kDa is nearly complete (see Figure 2). Limited proteolysis of 5-deazaFAD−PutA at −150 mV and −200 mV significantly increases the production of the 119-kDa fragment, demonstrating that the transition from the

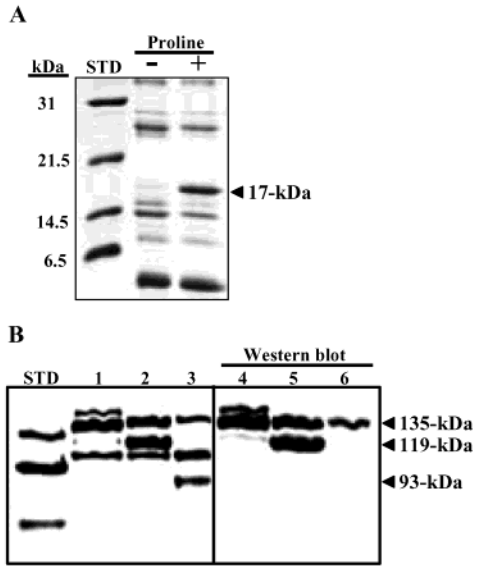

FIGURE 5: Separation and Western blot analysis of polypeptides from chymotrypsin cleavage of PutA. (A) PutA (2 mg/mL) was treated with chymotrypsin (10 μg/mL) in the absence and presence of 5 mM proline for 1 h at 23 °C. The reaction mixtures were loaded onto a denaturing gel (15% acrylamide) and visualized by staining with Coomassie Blue. A molecular mass standard (STD) is shown in the left-hand lane. (B) Limited digests of PutA (1 mg/mL) were performed with oxidized PutA (lanes 1 and 4), proline-reduced PutA (lanes 2 and 5), and L-THFA-complexed PutA (lanes 3 and 6) and separated by denaturing polyacrylamide gel electrophoresis. The molecular mass standards (STD) of 116 kDa, 97, kDa, and 66 kDa and lanes 1−3 were stained with Coomassie Blue to visualize the polypeptide fragments. Lanes 4−6 were electrotransferred onto a PVDF membrane, immunoblotted with a hexahistidine-tag monoclonal antibody, and visualized using a colorimetric assay.

oxidized to the reduced PutA conformer is occurring at a lower potential than that observed for FAD-PutA (Figure 2). From the results in Figure 4, it is clear that the E_m(conf) has decreased from −58 mV to a value of <-130 mV in 5-deazaFAD−PutA, validating that the formation of the 119-kDa band observed in the presence of proline is controlled by the FAD redox state. Thus, the reduction of the FAD is the chief determinant of PutA conformational changes.

Mapping of Protease Cleavage Sites. To identify the regions of the PutA polypeptide that are involved in conformational transitions, we obtained the N-terminal amino acid sequence of the major products of limited proteolysis by chymotrypsin. The N-terminal amino acid sequences were compared to the known sequence of PutA to establish the origin of the fragments and the location of chymotrypsin cleavage. We first identified the fragment that is cleaved by chymotrypsin in the presence of proline, which results in the disappearance of the 135-kDa band and the formation of the 119-kDa band. PutA was subjected to limited proteolysis by chymotrypsin in the absence and presence of proline, and the products were analyzed by 15% SDS−PAGE. A band at ∼17 kDa was generated in the presence of proline that was not observed in the absence of proline (Figure 5a). Subsequent isolation and amino acid sequencing of this band revealed a N-terminal sequence of SGAANES-DE, locating its origin at Ser58 in PutA, and a chymotrypsin cleavage site at Leu57. An N-terminal sequence of RIIGKS-GEPLIR was obtained for the 119-kDa band, demonstrating that it begins at Arg234 in PutA. Thus, the fragment that is lost during limited proteolysis in the presence of proline

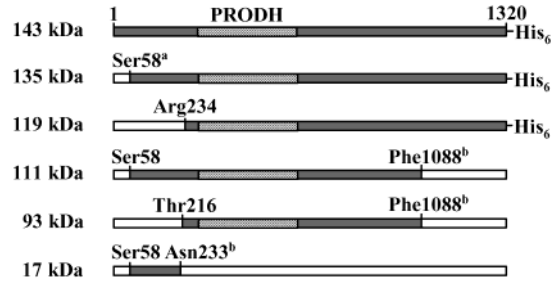

FIGURE 6: Map of polypeptides generated by chymotrypsin cleavage of PutA. Polypeptides are shown in order of estimated molecular weight by SDS−PAGE and are shaded in gray with the boundaries marked by N-terminal and C-terminal amino acids. The box with the diagonal lines shows the location of the PRODH domain (261−612). The identities of the N-terminus for the 119, 111, 93, and 17 kDa fragments were determined by N-terminal amino acid sequencing. The C-terminals of the 135 and 119 kDa fragments were determined by Western analysis. (a) N-terminal residue is predicted from the N-terminal amino acid sequence of the 17-kDa peptide. (b) C-terminal residues are predicted from the N-terminal amino acid sequence and the size of the polypeptides estimated by SDS−PAGE analysis.

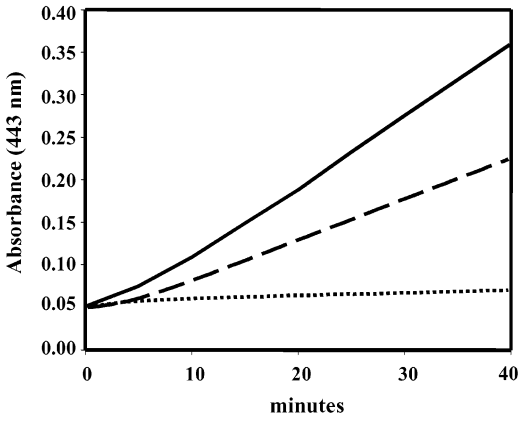

FIGURE 7: Functional membrane association assays. PutA (1 mg/mL) was digested with chymotrypsin (10 μg/mL) in the absence and presence of proline (5 mM) for 1 h at 23 °C. Samples of protease-treated PutA (100 μg) were then incubated with 60 mM proline, 4 mM *o*-aminobenzaldehyde, and inverted membrane vesicles from *E. coli* strain JT31 *putA*$^-$ (100 μg membrane protein) in 20 mM Mops buffer (pH 7.5) at 25 °C. The time course for the reactions were monitored at 443 nm to determine the membrane-associated PRODH activity of untreated PutA (−), oxidized PutA treated with chymotrypsin (- - -), and proline-reduced PutA treated with chymotrypsin (···). The calculated specific PRODH activities of PutA and protease-treated oxidized and proline-reduced PutA in the above assays were 0.08, 0.05, and 0.003 units/mg of membrane protein, respectively.

begins at Ser58 and ends near Asn233. The predicted molecular size of a Ser58-Asn233 fragment is 19.1 kDa, which is close to the estimated size of 17-kDa by SDS−PAGE analysis. Because the 17-kDa peptide is generated from the 135-kDa fragment, the 135-kDa fragment most likely has the same N-terminus beginning at Ser58 in PutA. The 111-kDa fragment was determined to have an N-terminal sequence of SGAANESDE positioned also at Ser58 in PutA. The 93-kDa product generated by limited proteolysis of L-THFA-complexed PutA was found to have an N-terminal sequence of TGKLVSTHN originating at Thr216 with a chymotrypsin cleavage site at Phe215. On the basis of the N-terminal sequences of the 111-kDa and 93-kDa fragments, the calculated molecular mass value of a Ser58-Phe215 fragment is 17.2 kDa, which is similar to the estimated difference of 18 kDa between the 111-kDa and 93-kDa fragments.

Next, Western blot analysis of the protease products was performed with hexahistidine tag antibodies to assess which fragments had an intact C-terminus (Figure 5b). The 135-kDa and 119-kDa bands were found to retain the C-terminal hexahistidine tag while the 111-kDa and 93-kDa bands lacked the C-terminal end. Thus, the FAD redox dependent conformational change is occurring at the N-terminus of PutA while L-THFA binding induces changes at both the N-terminus and C-terminus of PutA. Since the 111-kDa and 93-kDa fragments vary in molecular weight according to differences at the N-terminus and are both missing the C-terminal hexahistidine tag, the 111-kDa and 93-kDa bands most likely are cut in the same region at the C-terminus of PutA. On the basis of N-terminal amino acid sequencing and estimated molecular weights, the 111-kDa and 93-kDa fragments are both predicted to have a C-terminus ending in the region of 1080−1100 of PutA. A plausible residue at which chymotrypsin may cleave PutA in this region is Phe1088. The calculated molecular weights of Ser58-Phe1088 and Thr216-Phe1088 fragments are 112.5 and 95.5 kDa, respectively. The major products generated by chymotrypsin cleavage of PutA are diagrammed in Figure 6.

PutA−Membrane Associations. The effect that chymotrypsin cleavage has on the PRODH and membrane functional association activities of PutA was determined to ascertain whether the removal of the region, Ser58-Asn233, has any functional consequence. PRODH and membrane functional association assays were performed with PutA samples that had been treated with chymotrypsin in the absence and presence of proline. PutA treated with chymotrypsin in the absence of proline was assumed to be a mixture of the 135-kDa and 111-kDa products while PutA treated with chymotrypsin in the presence of proline was assumed to a mixture of the 119-kDa and 111-kDa products. Thus, the main difference between the protease-treated PutA samples was the loss of the Ser58-Asn233 fragment in the presence of proline (see Figure 6). The decrease in PRODH activity of protease-treated oxidized and reduced PutA were about 1.5-fold and 2-fold, respectively, relative to PutA with no protease treatment. Since the protease treatment of oxidized and reduced PutA diminishes PRODH activity similarly, the removal of the Ser58-Asn233 fragment from PutA does not appear to affect PRODH activity. The Ser58-Asn233 region, however, was found to significantly influence PutA membrane association activity. Figure 7 shows that the membrane functional association activity of protease-treated reduced PutA is decreased by ∼25-fold compared to untreated PutA while cleavage of oxidized PutA by chymotrypsin decreases the membrane association by only about 2-fold. Thus, it appears that cleavage of PutA near Asn233 destroys the ability of PutA to functionally associate with the membrane and that the Ser58-Arg233 region of PutA is critical for productive membrane binding. To evaluate whether the chymotrypsin cleavage sites in PutA are in regions that contact the membrane, limited proteolysis of PutA was performed in the presence of membrane vesicles (5 mg/mL membrane protein). The membrane vesicles did not occlude chymotrypsin from the cleavage sites in oxidized, proline reduced, or L-THFA-bound PutA since the same protease products, namely the 119-kDa and 93-kDa bands,

were observed (data not shown). Apparently, the cleavage sites that become accessible to chymotrypsin do not intimately associate with the membrane.

DISCUSSION

Brown and Wood (*16*) proposed that the different protease susceptibilities of PutA in the absence and presence of proline are controlled by the FAD redox state (*16*). Two key results from our work support this model and show unambiguously that the FAD redox state switches PutA between two conformers. First, the E_m(conf) value (−0.058 V) determined by the appearance of the 119-kDa product during controlled potentiometric limited proteolysis varies from the E_m value (−0.077 V) for FAD bound to PutA by only about 20 mV, thus linking the conformational change in PutA with FAD reduction. The conformational change caused by FAD reduction was also observed to be reversible, demonstrating that PutA is capable of switching between two different conformers. Second, PutA reconstituted with 5-deazaFAD, a FAD analogue with a midpoint potential more negative than FAD by about 100 mV, requires a reduction potential of <-130 mV to generate the 119-kDa fragment. This manipulation of the E_m(conf) by altering the redox properties of FAD bound to PutA confirms that the PutA conformational transition is controlled by the FAD cofactor.

Surprisingly, the nonreducing proline analogue, L-THFA, elicits a new major 93-kDa product during limited proteolysis by chymotrypsin. Thus, PutA complexed to proline analogues is in a form that is distinct from the previously discovered oxidized and proline-reduced PutA conformers. However, limited proteolysis of L-THFA-complexed PutA by chymotrypsin under reducing conditions regenerated the 119-kDa band, demonstrating that FAD reduction is the principal modulator of PutA conformation. Because Brown and Wood (*16*) have shown that limited proteolysis of the PutA apoprotein in the presence of proline yields the 135-kDa and 111-kDa fragments, the conformational change induced by L-THFA complexation to PutA requires FAD in the PRODH active site (*16*). An FAD analogue in the PRODH active site is also adequate since the 93-kDa product was observed by limited proteolysis of 5-deazaFAD−PutA in the presence of L-THFA. At this time it is unclear what the functional significance is of the PutA conformation caused by proline analogue or inhibitor binding. Gel-mobility shift assays have shown that L-lactate and L-THFA do not disrupt PutA-DNA binding, similar to that observed previously with proline or electrochemical reduction of FAD (data not shown) (*1*, *17*, *18*). Work is in progress to determine whether L-lactate or L-THFA promote PutA−membrane binding.

Chymotrypsin cleavage of PutA in the presence of proline nearly abolishes PutA functional membrane association activity, suggesting that residues located in the Ser58-Asn233 peptide are critical for membrane binding. However, on the basis of previous work it seems unlikely that Ser58-Asn233 is the only region in PutA that contains residues necessary for membrane binding. PutA669, a truncated PutA protein containing residues 1−669, was shown to have PRODH and DNA binding properties comparable to those of PutA but lacks P5CDH and membrane binding activity (*31*). Ling et al. (*32*) in their primary structure analysis of PutA identified three hydrophobic regions 160−170, 770−820, and 1205−1220 as plausible membrane binding domains (*32*). Thus, we propose that two regions in PutA are critical for membrane binding with one region contained in Ser58-Asn233 and a second region located toward the C-terminus of PutA. Attempts to purify and characterize a PutA protein that lacks residues 58−233 (e.g., PutA260−1320) or residues 1200−1320 (e.g., PutA1−1200) have so far been unsuccessful due to the insolubility of the engineered truncated PutA proteins.

Mapping of the chymotrypsin cleavage sites reveals that both the reduction of FAD and L-THFA binding induce protease susceptibility in the region of 215−235 in PutA. Structural insights into this region in PutA can be gained from recent X-ray crystallographic studies of PutA669. PutA669 was crystallized with L-lactate in the active site, and its three-dimensional structure was solved to 2.0 Å resolution. PutA669 was observed to be an interlocked dimer with each subunit containing a dimerization domain (residues 87−138), a helix-turn-helix (HTH) motif (residues 137−258), and a $\beta_8\alpha_8$-barrel which comprises the FAD binding and PRODH active sites (residues 261−612) (*33*, *34*). A PutA mutant containing only the first 261 N-terminal residues (PutA261) was shown to bind specifically to the *put* control DNA, demonstrating that the DNA binding domain can function separately from the PRODH domain. In the 137−258 region, 15 of the 120 residues were disordered indicating possible sites of mobility. It is known that protease cleavage sites are often associated with enhanced flexibility or local unfolding of the protein and that limited proteolysis is difficult in regions that have a more rigid structure (*35*). Thus, both X-ray crystallography and limited proteolysis suggest that the region around 215−235 is flexible and likely to be involved in conformational transitions. Because residues 215−235 are not part of the PRODH domain, both FAD reduction and substrate analogue binding initiate signals that evoke a conformational change outside the FAD active site. From the X-ray crystal structure of PutA669 and the functional characterization of PutA261, it also appears that the proline-induced conformational change involves residues in the DNA binding domain. The fact that L-THFA binding exposes a protease site in a second domain near the C-terminus of PutA further demonstrates that perturbations in the FAD environment are capable of inducing global conformational changes that affect other functional domains in PutA. The nature of the conformational change induced by FAD reduction in PutA has been shown to involve an increase in the overall hydrophobicity of PutA (*9*).

Future investigations into how FAD reduction and substrate analogue binding induce conformational changes in PutA will focus on active site residues implicated in substrate binding. Because L-THFA and L-lactate cause the same conformational change in PutA, analysis of the PRODH active site complexed to L-lactate should reveal important interactions that initiate conformational changes in PutA. Three basic active site residues, Lys329, Arg555, and Arg556, were found in the three-dimensional structure of PutA669 to bind the carboxylate group of L-lactate (*34*). These basic residues are conserved in PutA from other organisms such as the soil microbe, *Bradyrhizobium japonicum*, and are most likely critical for proline binding (*32*, *34*, *36*). The importance of Arg556 is evident from a Arg556His PutA mutant isolated by random mutagenesis from *S.*

typhimurium which was shown to have <0.25% PRODH activity relative to native PutA and was severely defective in membrane binding (*37*). Residues Arg555 and Arg556 are part of helix α8 of the $\beta_8\alpha_8$-barrel in the PRODH domain. Interestingly, helix α8 contacts the 137−258 region where the conformational change occurs suggesting that helix α8 may be involved in transmitting signals out of the FAD active site. The roles that Arg555 and Arg556 have in PutA conformational changes will be explored to test this possibility.

In summary, this work underscores the key role of the FAD cofactor in regulating PutA conformation. We have shown explicitly that the FAD redox state is the chief determinant of conformational changes in PutA. The discovery of a new conformation induced by L-THFA and L-lactate shows PutA has three different conformers: oxidized, reduced, and substrate analogue bound. Because the protease susceptibility of proline and electrochemically reduced PutA is identical, FAD reduction appears to be responsible for the proline-induced binding of PutA to the membrane as initially proposed (*5*, *16*). Future work will be directed at investigating the influence of FAD reduction and proline analogue binding on PutA membrane associations.

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