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Regulation of flavin dehydrogenase compartmentalization: requirements for PutA–membrane association in *Salmonella typhimurium*

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Abstract

PutA is a multifunctional, peripheral membrane protein which functions both as an autogenous transcriptional repressor and the enzyme which catalyzes the two-step conversion of proline to glutamate in *Salmonella typhimurium* and *Escherichia coli*. To understand how PutA associates with the membrane, we determined the role of FAD redox and membrane components in PutA–membrane association. Reduction of the tightly bound FAD is required for both derepression of the *put* operon and membrane association of PutA. $FADH_2$ alters the conformation of PutA, resulting in an increased hydrophobicity. Previous studies used enzymatic activity as an assay for membrane association and concluded that electron transfer from the reduced FAD in PutA to the membrane is required for the PutA–membrane interaction. However, direct physical assays of PutA association with membrane vesicles from quinone deficient mutants demonstrated that although electron transfer is essential for proline dehydrogenase activity, it is not required for PutA–membrane association per se. Furthermore, PutA efficiently associated with liposomes, indicating that PutA–membrane association does not require interactions with other membrane proteins. PutA enzymatic activity can be efficiently reconstituted with liposomes containing ubiquinone and cytochrome *bo*, confirming that proline dehydrogenase can pass electrons directly to the quinone pool. These results indicate that PutA–membrane association is due strictly to a protein–lipid interaction initiated by reduction of FAD.

Keywords: Peripheral membrane protein; Compartmentalization; Proline utilization; Proline dehydrogenase; Autogenous regulation

1. Introduction

The membrane association of certain types of bacterial dehydrogenases provides a model system for studying protein–lipid interactions. Peripheral membrane-associated flavoenzymes oxidize a large number of substrates in bacteria, and many are dependent on direct interactions with the integral membrane components of the electron transport chain [1]. These flavoproteins seem to interact with the cytoplasmic membrane in two basic ways. Some, such as anaerobic glycerol-3-phosphate dehydrogenase [2] and succinate dehydrogenase [3], bind to specific integral membrane proteins that serve as a membrane 'anchor'. Others, such as pyruvate oxidase [4,5] and malate dehydrogenase [6–8], seem to directly interact

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with membrane lipids and do not appear to have a specific anchor protein. Unlike the flavoenzymes with anchor proteins, this class seems to be adapted for shuttling between the cytoplasm and membrane compartments, depending on the availability of substrate. Previous work suggests that membrane associated dehydrogenases compete for a common membrane binding site [9], implying that activities of these enzymes may be modulated by the competition for available membrane-binding sites.

The PutA protein from *Salmonella typhimurium* and *Escherichia coli* is a multifunctional, 144-kDa flavoprotein which acts both as an enzyme involved in the catabolism of proline and a transcriptional repressor for the *put* operon [10]. The *put* operon encodes two proteins: PutP, the major proline permease, and PutA. Transcription of the *put* operon is autogenously controlled by PutA in response to the cytoplasmic proline concentration. When the intracellular concentration of proline is low, PutA acts as a transcriptional repressor by binding specifically to the *put* operator DNA [11,12]. However, when the intracellular proline concentration increases, PutA peripherally associates with the cytoplasmic membrane [13,14] and catalyzes the two-step oxidation of proline to glutamate (Fig. 1).

The first step catalyzed by PutA is the proline dehydrogenase reaction. Proline dehydrogenase couples the oxidation of proline to pyrroline-5-carboxylate (P5C) with reduction of a tightly associated FAD cofactor [12,15]. Concurrently, membrane association of PutA facilitates transfer of electrons from the reduced FAD to the membrane-associated electron transport chain [16,17]. In the second enzymatic step catalyzed by PutA, P5C dehydrogenase couples the oxidation of P5C to glutamate with reduction of a soluble NAD cofactor [14,15]. Kinetic and substrate competition studies demonstrate that the P5C intermediate is retained by PutA and is passed directly between the proline dehydrogenase and P5C dehydrogenase activities via a NAD-dependent leaky channel [18].

Derepression of the *put* operon requires membrane association; both proline and membranes are required for release of PutA from the operator sites [13]. Under these conditions PutA interacts with the membrane-associated electron transport chain and catalyzes proline degradation. The soluble form of PutA has a K_m for proline of ca. 50 mM and the membrane associated form of PutA has a K_m for proline of ca. 10 mM [17–19]. The low affinity for proline prevents derepression of the *put* operon by the proline pool required for protein synthesis. The decrease in K_m for the proline dehydrogenase activity when PutA associates with the cytoplasmic membrane may ensure that PutA remains membrane-associated as long as a sufficient concentration of proline is available. These observations suggest that compartmentalization of PutA in the membrane is critical for regulation of proline utilization.

Characterization of the PutA–membrane interaction may provide insight into how peripheral membrane proteins interact with membrane lipid and how membrane compartmentalization regulates proline utilization. In this work, we answered two questions about the mechanism of PutA–membrane association. First, what is the role of proline oxidation and FAD redox in driving and stabilizing the PutA–membrane interaction? Second, what membrane components are required for PutA to become membrane-associated and function as an enzyme?

Previous studies demonstrated that reduction of FAD increases the relative hydrophobicity of PutA [20], promotes PutA–membrane association [13,21], and results in release of PutA from *put* operator DNA [20,22]. To determine how proline oxidation drives PutA–membrane association, we quantitated the effect of reduced FAD on the structure, hydrophobicity, and membrane association of PutA. The results demonstrate that although reduced FAD is responsible for only 64% of the relative increase in hydrophobicity, this effect is sufficient for 90% of PutA–membrane association. To determine what membrane components are required for the PutA–membrane interaction, we tested the role of quinones, lipids, and membrane proteins in PutA–membrane association. The results demonstrated that PutA associated with liposomes lacking protein, and proline dehydrogenase activity could be functionally reconstituted with liposomes supporting a minimal electron transport chain. Thus, although quinones are required for proline dehydrogenase activity, they are not required for PutA–membrane association. These results indicate that PutA–membrane association is dependent strictly on the availability of membrane lipid.

2. Materials and methods

2.1. Materials

2,3-Dimethoxy-5-methyl-1,4-benzoquinone (Qo), Tween 20, goat-anti rabbit-horseradish peroxidase (GAR-HRP), and *o*-aminobenzaldehyde (*o*-ab) were purchased from Sigma (St. Louis, MO). 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate was purchased from Kirkegaard and Perry (Gaithersburg, MD). [1-^{14}C]acetate was purchased from American Radiolabeled Chemicals (St. Louis, MO). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (PE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (PG), and 1,1′,2,2′-tetraoleoyl-cardiolipin (CL) were purchased from Avanti Polar Lipids (Alabaster, AL). All other reagents were of reagent grade. Centriprep 3 concentrators were purchased from Amicon (Beverly, MA). The LiposoFast-Basic small volume liposome extrusion apparatus was purchased from Sigma (St. Louis, MO). Immulon 4 flat-bottom 96-well microtiter plates and MR5000 microtiter plate reader were purchased from Dynatech (Chantilly, VA).

S. typhimurium strain MST2616 containing pPC34, an expression vector that places *putA* under the control of P*tac* [20], was used as the source for PutA purification. MST63 has a deletion which removes the entire *put* operon. *E. coli* strain AN384 is a double mutant in both the menaquinone and ubiquinone biosynthetic pathways and AN387 is the wild-type parent of this strain [23]. *E. coli* strain EM1445 contains an *ndh*::Cam mutation that allows preparation of membrane vesicles lacking NADH dehydrogenase. These three *E. coli* strains were generous gifts from J.A. Imlay (Microbiology Department, University of Illinois, Urbana, IL). Pyruvate oxidase (PoxB) and polyclonal rabbit anti-PoxB antibody were generous gifts from Y.Y. Chang (Microbiology Department, University of Illinois, Urbana, IL). Cytochrome *bo* was a generous gift from P. Tsatsos and R.B. Gennis (Biochemistry Department, University of Illinois, Urbana, IL).

2.2. Preparation of inverted membrane vesicles

For inverted membrane vesicles that lack PutA protein, an 800 ml culture of MST63 (Δ*put*) was grown aerobically to mid-exponential phase in minimal E medium [24] supplemented with 0.6% succinate. For wild-type and ubiquinone/menaquinone deficient inverted membrane vesicles, 800 ml cultures of AN387 and AN384 were grown anaerobically to mid-exponential phase in the minimal E medium supplemented with 0.6% D-glucose, 50 μM thiamine, and 50 μM uracil. Cells were harvested by centrifugation, washed twice with 1 vol. of 0.85% NaCl, resuspended in 20 ml Buffer 1 (10 mM cacadylic acid, pH 6.8), and ruptured in a French pressure cell. Unbroken cells were removed by centrifugation at 6000×*g*. Membranes were isolated by centrifugation at 110 000×*g* for 30 min. The membranes were resuspended in 2 ml Buffer 1 with 5% glycerol, and stored at −70°C until use. EM1445 inverted membrane vesicles lacking NADH dehydrogenase were prepared as described previously [17]. For EM1445 membrane vesicle lipid content, lipids were first extracted by the Bligh and Dyer technique [25] and fatty acid esters were quantitated by a colorimetric assay [26].

2.3. Preparation of synthetic liposomes

To prepare liposomes with a phospholipid composition similar to natural membranes, a 70:25:5 (PE:PG:CL) molar ratio of lipid desiccate was first combined in chloroform and then dried under a stream of nitrogen. Liposome vesicles were formed by first reconstituting the dried lipid mixture in Buffer 1 to 2.8 mM. The dissolved mixture was then passed 19 times through a 400-nm pore size filter using a LiposoFast-Basic liposome extrusion instrument [27]. The resulting liposomes sediment as a tight band just below the interface between 5 and 20% (w/w) sucrose in Buffer 1. These liposome vesicles were used directly for PutA– and PoxB–liposome association assays. For ubiquinone and/or cytochrome *bo* reconstituted liposome vesicles we followed a modification of the protocol described previously [5]. For liposomes including quinone, chloroform solubilized ubiquinone 10 (81 nmol/mg lipid) was added to the original organic solubilized lipid mixture prior to drying. For liposomes including cytochrome *bo*, solubilized complex (50 mM KH_2PO_4, pH 8.3, 1 mM EDTA, 0.05% dodecyl maltoside) was added to the liposome preparation fol-

lowing filtration. Prior to enzyme assays, vesicles containing quinone, terminal oxidase, or both, were dialyzed versus three changes of Buffer 1 for 8 h each. For each of these conditions, controls were performed to ensure that the liposomes were functional.

2.4. PutA purification and kinetic assays

PutA was purified as described [21]. All kinetic assays were performed in Buffer 1. Unless otherwise noted, kinetic assays were conducted with 0.5 μg PutA, and proline dehydrogenase activity was determined by measuring the production of P5C in the presence of synthetic liposomes or inverted membrane vesicles (20 nmol total lipid). The concentration of P5C was determined using the colorimetric *o*-amino-benzaldehyde (*o*-ab) assay [28]. Initial rates were measured in all the assays performed to determine the kinetic parameters of the proline dehydrogenase reaction, with substrate depletion limited to less than 1% of the initial concentration. All proline dehydrogenase reactions were corrected for a control lacking PutA.

2.5. Membrane association and competition assays

The association of PutA with inverted membrane vesicles was measured in vitro using sucrose step-gradients. All stock solutions were made fresh in Buffer 1. Reaction mixtures were prepared with 10 mM $MgCl_2$ to a final volume of 100 μl. All reagent concentrations were optimized to reduce substrate and cofactor depletion to less than 1%. Following the final incubation, the membrane and soluble fractions were separated by centrifugation through a sucrose step-gradient containing two layers of 35% (w/w) and 65% (w/w) for 1 h at 140 000 × *g* in a Beckman SW-55 Ti rotor. Material collected from the cushion on top of the 35% sucrose layer contained the soluble PutA fraction and the material collected between the layers contained the membrane fraction. A 24-μl portion of each fraction was then diluted in 216 μl borate buffer (80 mM boric acid, and 50 mM sodium borate, pH 9.5) for ELISA. Ninety-six-well microtiter plates were coated with 100 μl volumes in duplicate and incubated for 1 h. The plates were then blocked for 1 h with masking buffer (10 mM Tris base, 50 mM NaCl, 1 mM EDTA, 0.05% Tween 20, 0.2% gelatin, pH 8.0). After blocking, the plates were incubated in masking buffer with primary antibody, and then in masking buffer with secondary antibody. Reactions were developed with TMB and stopped with an equal volume of 0.2 N HCl. All incubations were performed at 37°C and followed by three washes with wash buffer (10 mM Tris base, 50 mM NaCl, 1 mM EDTA, 0.05% Tween 20, pH 8.0). The reaction was then quantitated on a microtiter plate reader at 450 nm. Optical densities were averaged and corrected with a membrane only control.

2.6. Measurement of relative hydrophobicity

The relative hydrophobicity of PutA was assayed by partitioning into the detergent Triton X-114. This procedure was based on the methods of Bordier [29] and Pryde [30]. Triton X-114 was pre-equilibrated in Buffer 1 with 10 mM $MgCl_2$ as described. Reaction mixtures (200 μl) were incubated at 37°C for 10 min with 50 μl equilibrated Triton X-114. The reactions were then incubated on ice for 25 min, mixed, and incubated at 37°C for 15 additional minutes. A 50-μl amount was then removed from the aqueous phase and interface between the two phases. These fractions were then diluted with 200 μl borate buffer and developed in duplicate by ELISA. Optical densities were averaged and corrected for a control lacking PutA.

2.7. Acylation of PutA

To determine if PutA is acylated in vivo, we constructed an *E. coli* strain that targets incorporated acetate into growing fatty acyl chains. EM1573 (*fadE ΔaceEF aceA1*) was grown overnight in minimal NCE media [31] supplemented with 0.2% succinate, 0.2% lactate, 1 mM $MgSO_4$, and 20 mM ammonium acetate, pH 7.5. Cells were subcultured into 100 ml of the same medium, grown to early log phase, and supplemented with 100 μCi [^{14}C]acetate (55 mCi/mmol) in the presence or absence of 30 mM L-proline. Cultures were labeled for 30-min intervals up to 1.5 h. Labeled cells were washed with saline, resuspended in 2 ml G-buffer [21], and sonicated in the presence of 0.5% Tween 20. Lysates were centri-

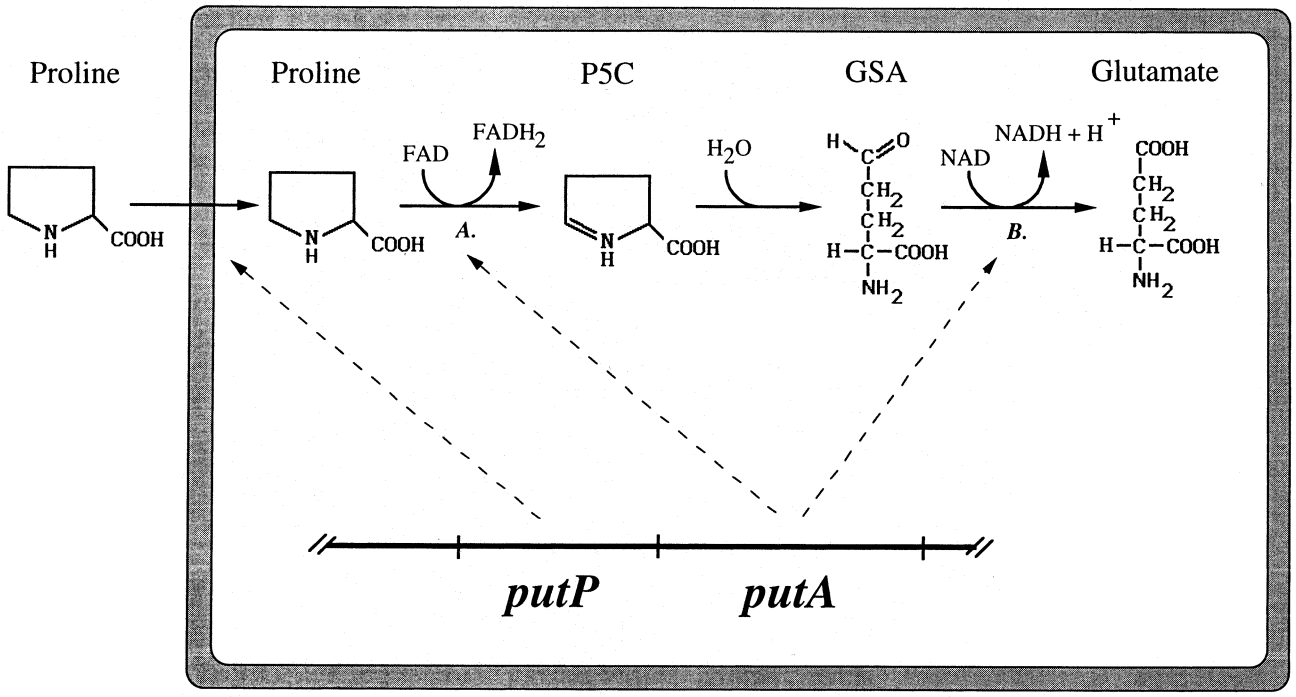


Fig. 1. PutA enzymatic reactions. Proline dehydrogenase (A) catalyzes the oxidation of proline to pyrroline-5-carboxylate (P5C) coupled with reduction of a tightly associated FAD. Reoxidation of the $FADH_2$ requires PutA to interact with the membrane-associated electron transport chain. P5C is then oxidized to glutamate by P5C dehydrogenase. P5C is first hydrolyzed to glutamate semialdehyde (GSA) and the oxidation of GSA to glutamate is coupled with the reduction of soluble NAD.

fuged at 110 000 × *g* for 30 min, and PutA was visualized by separating 50 μl supernatant on a 8% SDS-polyacylamide gel. Following Commassie staining, the gels were dried and ^{14}C incorporated was measured with a phosphorimager.

2.8. PutA- and PoxB–liposomal association assays

Association of PutA or PoxB to liposomes was measured as described for inverted-membrane vesicles except for the use of a modified sucrose step-gradient. For PutA–liposomal interactions, the liposome and soluble fractions were separated by centrifugation through a sucrose step-gradient containing three layers of 5% (w/w), 20% (w/w), and 35% (w/w) for 4 h at 140 000 × *g*. PoxB–liposomal interactions were separated on the same gradient for 18 h at 140 000 × *g*. The centrifugation times account for the different sedimentation rates of PutA and PoxB. All reactions were performed in Buffer 1 with 10 mM $MgCl_2$. Following centrifugation, fractions were collected and the location of PutA and PoxB were determined by ELISA using polyclonal rabbit anti-PutA and anti-PoxB, respectively. Optical densities were averaged and corrected for a control lacking protein. The location of liposomes was determined visually.

For liposome–membrane vesicle competition assays, equal molar amounts of synthetic liposome and AN384 inverted membrane vesicle lipid was used. To determine which vesicle PutA preferred, reactions were separated on the sucrose gradient described above with addition of a 65% (w/w) lower layer. Following centrifugation, fractions were collected and both the location of PutA and moles lipid present in liposome and AN384 membrane vesicle fractions was determined. The amount of PutA in each fraction was corrected for lipid content.

3. Results

3.1. $FADH_2$ promotes PutA–membrane association

Previous studies indicate that reduction of FAD is at least partially responsible for release of PutA from

Table 1
Effect of substrate and cofactors on PutA–membrane association

Reaction mix prior to membrane addition[a]	Fraction[b]	PutA[c]	% of Total	% Substrate-induced membrane association	% Decrease in membrane association
(1) PutA	S	1.30	69	–	–
	M	0.58	31[d]		
(2) PutA, Proline	S	0.47	29	40	–
	M	1.14	71		
(3) PutA, DHP	S	0.28	18	51	–
	M	1.29	82		
(4) PutA, Qo	S	1.34	68	–	–
	M	0.63	32		
(5) PutA, Proline, Qo	S	1.14	64	4	90
	M	0.65	36		
(6) PutA, DHP, Qo	S	1.00	52	16	69
	M	0.93	48		

[a]All reactions were performed in Buffer 1 with 1 μg PutA and 10 mM $MgCl_2$. Where indicated, 20 mM L-proline or 20 mM L-dehydroproline (DHP) was included and reactions were incubated for 5 min at 37°C. Where appropriate, 1 mM Qo was then added and the reaction was incubated another 5 min at 37°C. Inverted membrane vesicles isolated from MST63 (*S. typhimurium* Δ(*putPA*)*559*; 200 μg total membrane protein) were then added to each reaction mixture and incubated for an additional 5 min at 37°C prior to loading a sucrose step-gradient.

[b]Fractions were taken from both the top of the 35% sucrose (soluble PutA, S) and top of the 65% sucrose gradient (membrane-bound PutA, M).

[c]The OD_{450} from ELISA using polyclonal rabbit anti-PutA antibody with goat anti-rabbit horseradish peroxidase secondary antibody. Data based on an average of four independent trials with errors less than OD 0.15.

[d]The degree of non-specific PutA–membrane association seen in all trials and when the soluble fraction was added back to fresh membrane vesicles.

put operator DNA and partitioning into the membrane [13,20,22]. Furthermore, reduction of the FAD cofactor has been correlated with a conformational alteration in PutA [21]. However, these studies either did not differentiate between membrane-associated versus aggregated PutA or they measured membrane association by an indirect assay [21]. Therefore, we used a more sensitive, direct assay of PutA–membrane association to determine the effect of FAD redox and other factors on PutA–membrane association. PutA–membrane association was assayed using sucrose step-gradients that separated soluble PutA from membrane-associated PutA.

Oxidation of proline results in reduction of FAD and concurrent membrane association (Table 1, row 2). The role of reduced FAD in PutA–membrane association was determined by addition of Qo prior to adding membrane vesicles. Qo rapidly reoxidizes the $FADH_2$ to FAD. The addition of Qo decreased proline-induced PutA–membrane association by ~90% (Table 1, row 5), but had no effect in the absence of proline, indicating that this effect was specifically due to FAD oxidation. These results indicate that the reduction of FAD promotes PutA–membrane association, in agreement with previous studies [13,21]. Addition of Qo inhibited PutA–membrane association, but subsequent dialysis and addition of proline restored the interaction, indicating that PutA–membrane association is a reversible event.

To further demonstrate that these results are due to the proline dehydrogenase reaction and not the second enzymatic reaction catalyzed by P5C dehydrogenase, we tested the effect of dehydroproline (DHP) on PutA–membrane association. DHP is a proline analog which is readily oxidized by proline

Table 2
Effect of quinones on PutA–membrane association

Reaction mix prior to membrane addition[a]	Strain[b]	Membrane phenotype	Fraction[c]	PutA[d]	% of Total	% Proline-induced membrane association
PutA	MST63	Δ*put*	S	0.328	70	–
			M	0.142	30	
PutA, proline	MST63	Δ*put*	S	0.135	30	40
			M	0.309	70	
PutA, KCN	MST63	Δ*put*	S	0.216	72	–
			M	0.080	28	
PutA, proline, KCN	MST63	Δ*put*	S	0.090	30	42
			M	0.215	70	
PutA	AN387	Quinone$^+$	S	0.352	67	–
			M	0.173	33	
PutA, proline	AN387	Quinone$^+$	S	0.158	30	37
			M	0.374	70	
PutA	AN384	Quinone$^-$	S	0.340	79	–
			M	0.091	21	
PutA, proline	AN384	Quinone$^-$	S	0.148	27	52
			M	0.393	73	

[a]All reactions were performed in Buffer 1 and contained 1 μg PutA and 10 mM $MgCl_2$. Where appropriate, 100 mM L-proline and/or 1 mM KCN was included and reactions were incubated for 5 min at 37°C. Appropriate inverted membrane vesicles (200 μg total membrane protein) were then added to the reaction mixture and incubated for an additional 5 min at 37°C prior to loading the sucrose step-gradient.
[b]MST63 (*S. typhimurium* Δ(*putPA*)559); AN387 (*E. coli rpsL gal*); and AN384 (*E. coli ubiA420 menA401 rpsL gal*).
[c]Fractions were taken from the top of the 35% sucrose (soluble PutA, S) and top of the 65% sucrose gradient (membrane-bound PutA, M).
[d]OD_{450} from ELISA using polyclonal rabbit anti-PutA antibody with goat anti-rabbit horseradish peroxidase secondary antibody. Data based on an average of two independent trials with errors less than OD 0.05.

dehydrogenase, reducing the FAD cofactor and forming an intermediate which is not a substrate for P5C dehydrogenase. The degree of PutA–membrane association induced by DHP and proline were similar (Table 1, row 3). Moreover, selective oxidation of $FADH_2$ by Qo addition resulted in a decrease in DHP-induced PutA–membrane association comparable to that seen with proline-induced PutA–membrane association (Table 1, row 6). These results indicate that reduction of FAD in the proline dehydrogenase reaction is necessary and sufficient to promote PutA–membrane association.

3.2. The membrane-associated quinone pool is not required for PutA–membrane association

The membrane associated respiratory chain removes electrons from $FADH_2$ within PutA [16]. This interaction is essential for enzymatic activity in vivo, suggesting that the quinone pool may directly interact with the reduced form of PutA, and may stabilize the PutA–membrane interaction. Furthermore, using proline dehydrogenase activity as a measure of membrane association, it has been suggested that enzymatic activity and electron transfer from PutA to the electron transport chain is required for PutA to associate with the membrane in *E. coli* [14]. We tested this hypothesis by assaying the association of PutA with inverted membrane-vesicles isolated from a double mutant defective for both ubiquinone and menaquinone biosynthesis (Table 2). As expected, these vesicles do not permit PutA enzymatic activity (Table 3). Nevertheless, in the presence of proline PutA efficiently associated with these quinone-less vesicles, indicating that membrane association of PutA does not require quinones. Moreover,

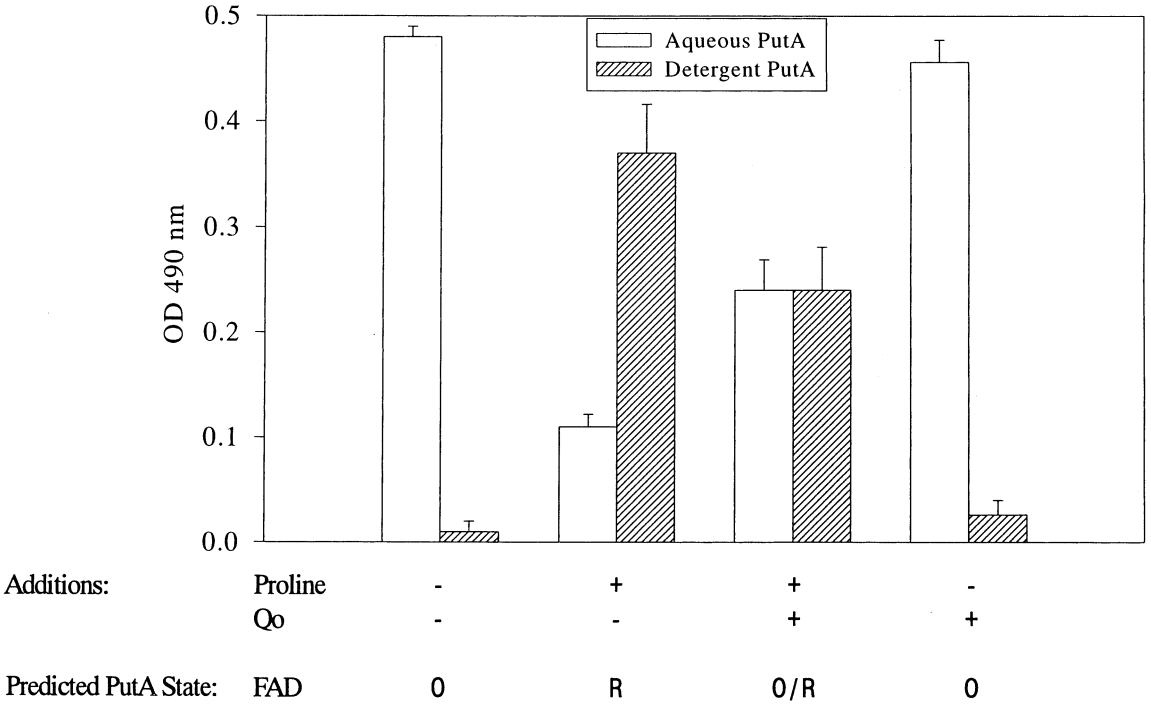


Fig. 2. PutA partitioning into Triton X-114. In triplicate reactions, 1 μg PutA was incubated in Buffer 1 with pre-equilibrated Triton X-114. All reactions contained 10 mM $MgCl_2$. Where appropriate, reactions included combinations of the following: 20 mM L-proline, and/or 1 mM Qo. Following incubation, aqueous and aqueous-detergent interface fractions were removed and PutA was located by ELISA. Predicted redox states of FAD: oxidized (O), reduced (R), and turnover (O/R).

although 1 mM KCN inhibits enzymatic activity (Table 3), it does not prevent the membrane association of PutA (Table 2).

3.3. The relative hydrophobicity of PutA is increased by $FADH_2$

The role of $FADH_2$ in promoting PutA–membrane association suggests that $FADH_2$ may induce a conformational changes in PutA. Partial proteolysis ([21]; B. Gigliotti, and S. Maloy, unpublished results) and circular dichroism (data not shown) studies indicate that the conformation of PutA is altered when FAD is reduced. Reduction of FAD has been correlated with an increase in the hydrophobicity of PutA [20]. However, the effect of quinones was not previously tested and the conditions used previously did not equal the conditions used for membrane association. Therefore, we assayed the relative hydrophobicity of PutA by measuring the par-

Table 3
Requirement for a functional electron transport chain for PutA proline dehydrogenase activity[a]

Membrane vesicle strain[b]	Membrane phenotype	KCN (mM)	$K_{m\ L\text{-proline}}$ (mM)	V_{max} (nmol min^{-1} mg^{-1})	K_{cat} (s^{-1})
AN387	$Quinone^+$	0	4	260	623
		1	nd[c]	nd	nd
AN384	$Quinone^-$	0	nd	nd	nd

[a]Proline dehydrogenase activity was determined in the presence of inverted membrane vesicles (4 μg total membrane protein) by measuring the proline-dependent formation of a yellow colored complex between P5C and *o*-aminobenzaldehyde (*o*-ab).
[b]AN387 (*E. coli rpsL gal*), and AN384 (*E. coli ubiA420 menA401 rpsL gal*).
[c]Below detectable limits.

titioning of PutA into Triton X-114 under conditions used for the membrane association assay (Fig. 2). These results confirm previous studies on the effect of $FADH_2$ [20], and demonstrate that oxidation of $FADH_2$ partially restores the hydrophilicity of PutA (Fig. 2).

3.4. PutA–membrane association is mediated by protein–lipid interactions

PutA could potentially interact with the cytoplasmic membrane in three different ways. (1) PutA could become anchored to the membrane via a hydrophobic, fatty acyl modification. It appears unlikely that PutA is acylated because it is not labeled using ^{14}C-acetate in a *fadE ΔaceEF aceA1* strain (EM1573). (2) PutA could associate with the membrane via a strict hydrophobic interaction. The Triton X-114 results indicate that hydrophobicity of PutA may play a role in membrane binding, but does not prove that the association is solely via a protein–lipid interaction. (3) PutA could interact with an integral membrane protein. Despite an extensive search, no extragenic transposon mutations have yet been isolated which affect proline utilization. Nevertheless, it is possible that such mutations would be lethal, either because the gene itself or a gene product encoded downstream is essential.

To determine if PutA associates with the cytoplasmic membrane via a protein–lipid or protein–protein interaction, we assayed the association of PutA with synthetic liposomes lacking protein. Membrane association was monitored on a sucrose step-gradient that separates soluble protein from liposome-associated protein. To confirm that the liposomes were functional, we measured the association of another purified peripheral membrane protein, PoxB. In agreement with previous studies [32], PoxB demonstrated pyruvate-dependent binding to the liposomes (Table 4). Similarly, PutA demonstrated proline-dependent binding to liposomes (Table 4). Furthermore, competition between synthetic liposomes and quinone-less inverted membrane vesicles (AN384) demonstrated that PutA does not have a significant preference for either form of phospholipid vesicle (Table 5). These results suggest that PutA associates with the cytoplasmic membrane strictly via protein–lipid interactions and the membrane association does not require additional membrane proteins.

To confirm this conclusion, we compared the reconstitution of proline dehydrogenase activity in liposomes containing a minimal electron transport chain versus inverted membrane vesicles (Table 6).

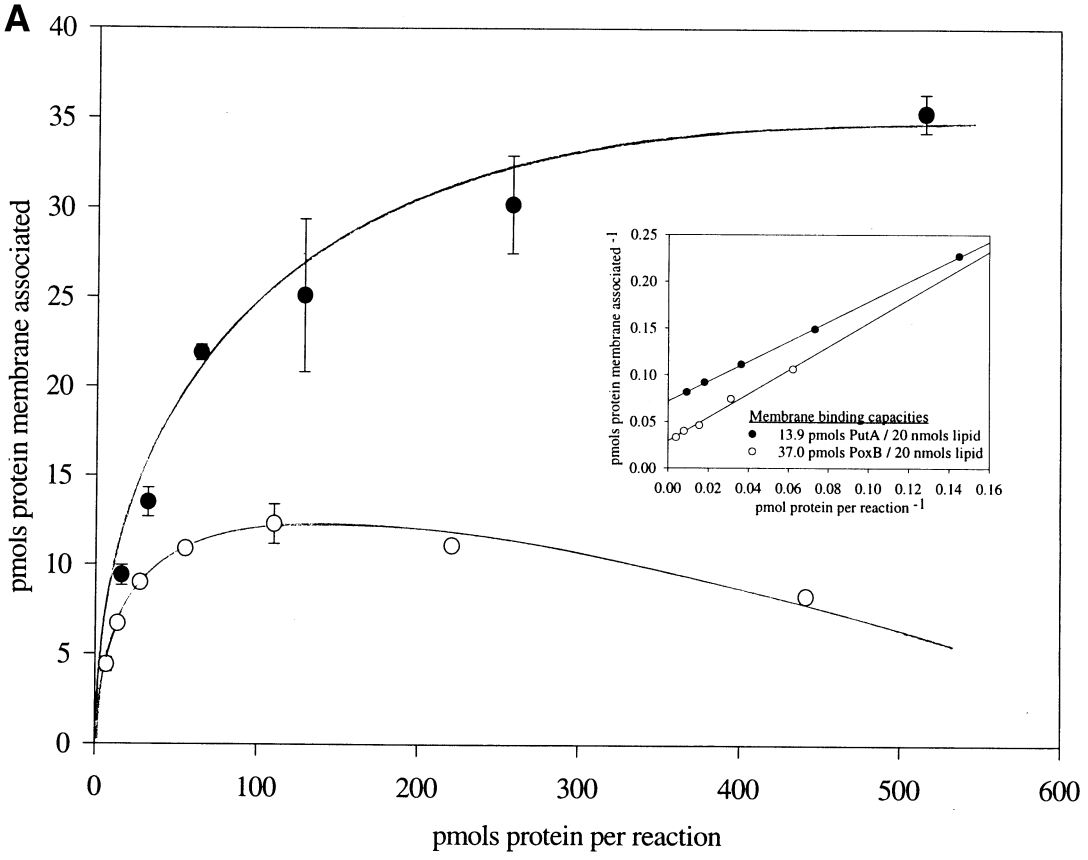
Table 4
PutA and PoxB liposome association

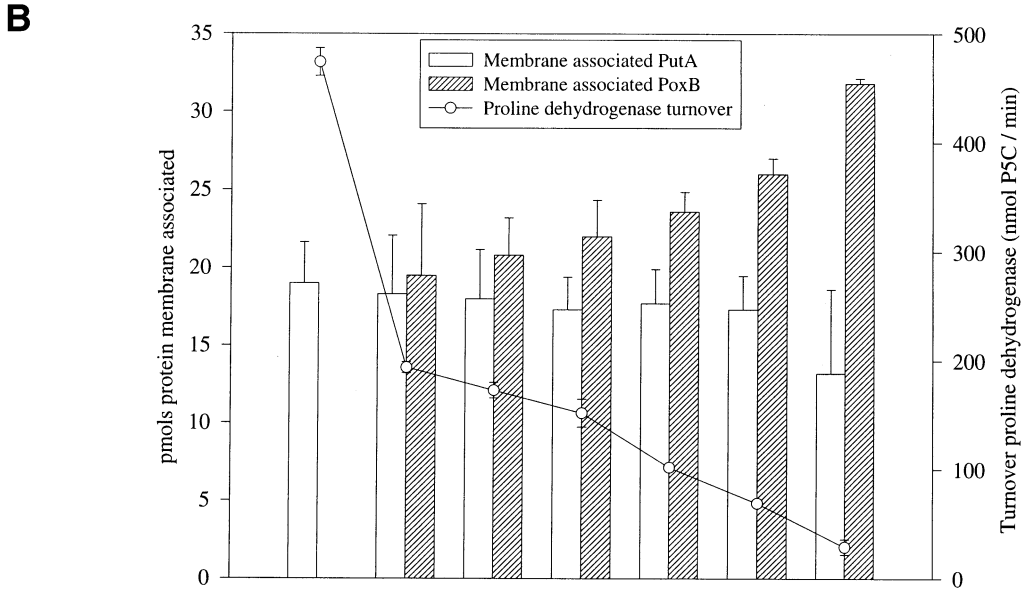
Reaction mix[a]	Fraction[b]	Protein[c]	% of Total	% Substrate-induced membrane association
PutA	L	0.25	41	–
	P	0.365	59	
PutA, proline	L	0.452	71	30
	P	0.188	29	
PoxB, TPP	L	0.179	32	–
	P	0.374	68	
PoxB, TPP, pyruvate	L	0.337	55	23
	P	0.272	45	

[a]All reactions were performed in Buffer 1 and contained 1 μg PutA or PoxB, 10 mM $MgCl_2$ and 134 nmol lipid per reaction. Where appropriate, 100 μM thiamine pyrophosphate (TPP), 100 mM L-proline, and 100 mM sodium pyruvate were added to the reactions. Reactions were incubated for 5 min at 37°C prior to loading a sucrose step-gradient.

[b]Fractions were collected throughout the gradient. Fractions containing liposome (L) and free protein (P) were reported.

[c]OD_{450} from ELISA using either polyclonal mouse anti-PutA antibody or rabbit anti-PoxB antibody with goat anti-mouse horseradish peroxidase or goat anti-rabbit horseradish peroxidase secondary antibody, respectively. Data based on an average of two independent trials with errors less than OD 0.06.





Protein/reaction							
pmols PoxB	0	16	32	64	128	256	512
pmols PutA	110	110	110	110	110	110	110

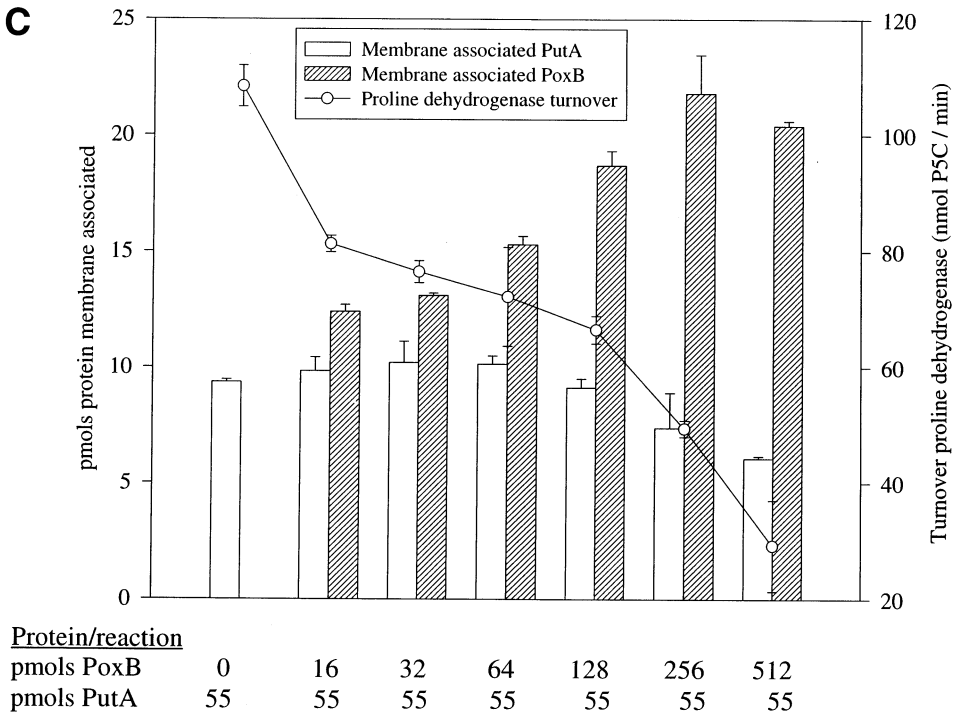


Fig. 3. Membrane binding sites for PutA and PoxB. (A) Membrane association of PutA and PoxB. All reactions were performed in Buffer 1 and contained the indicated quantities of either PutA or PoxB, 10 mM $MgCl_2$, and AN384 (*ubiA420 menA401 rpsL gal*) inverted membrane vesicle (20 nmol total lipid). In addition, PutA assays contained 200 mM L-proline, and PoxB assays contained 100 μM thiamin pyrophosphate and 200 mM sodium pyruvate. Reactions were incubated for 5 min at 37°C prior to loading the sucrose step-gradient. Double-reciprocal plots were used to determine the membrane binding capacities for both PutA and PoxB (see inset). (B) Using the protocol and data in A, increasing quantities of PoxB were pre-associated with AN384 inverted membrane vesicles for 5 min at 37°C. Following incubation, 110 pmol PutA and proline were added to each reaction and incubated for an additional 5 min at 37°C prior to loading the sucrose step-gradient. Location of PutA and PoxB were determined via ELISA using mouse anti-PutA and rabbit anti-PoxB antibodies, respectively. (C) Same as B, except 55 pmol PutA were included in each reaction. The quantity of membrane-bound protein was determined by comparison to an ELISA standard curve.

The results indicate that proline dehydrogenase activity can be efficiently reconstituted into both inverted membrane vesicles and liposomes containing ubiquinone and a terminal oxidase, demonstrating that PutA does not require an additional membrane protein to mediate transfer to the electron transport chain.

3.5. *PutA–membrane association is limited by the availability of membrane phospholipid*

Studies measuring the effect of increasing the concentration of PutA on proline dehydrogenase activity and autogenous repression suggest that PutA binds to a limited number of membrane binding sites in vivo [10]. However, membrane vesicles isolated from a *put*$^+$ strain grown in the presence of proline can be reconstituted with soluble PutA to yield increased proline dehydrogenase activity, suggesting that the membrane sites are not limiting in vitro [17]. To estimate the number of potential membrane binding sites, we calculated the surface area of available phospholipid and compared this value to the predicted surface area occupied by PutA and PoxB in vitro. Inverted membrane vesicles from strain AN384 contained 20 nmol total lipid per reaction. Based upon physical parameters of small unilamellar vesicles [33], the calculated vesicle surface area in each reaction was 6.28×10^8 μm^2. Using the simplifying assumption that the PutA dimer is a sphere with a Stoke's radius of 7.1 nm [12], and that PutA binds to the membrane as a monomer (unpublished studies), the surface area occupied by a single PutA molecule is roughly 3.96×10^{-5} μm^2. Therefore,

Table 5
Preference of PutA for liposomes or inverted bacterial membrane vesicles

Lipid vesicle (nmol lipid)[a]		Fraction[b]	PutA[c]	% of Total	% Associated	% Lipids[d]	Corrected % proline-induced lipid association[e]
Liposome	AN384						
		L	0.459	55	55	–	–
67	–	P	0.278	33			
		M	0.100	12	–	–	–
		L	0.105	17	–	–	–
–	134	P	0.184	32			
		M	0.295	51	51	–	–
		L	0.134	23	23	33	38
67	134	P	0.194	32			
		M	0.273	45	45	77	45

[a]All reactions were performed in Buffer 1, containing 1 μg PutA, 10 mM $MgCl_2$, and 100 mM L-proline. Where appropriate, reactions contained 67 nmol lipid. We assumed that AN384 (*ubiA420 menA401 rpsL gal*) inverted membrane vesicles contained 50% inner membrane, to accommodate 134 nmol was used.
[b]Fractions were collected throughout the gradient. Fractions containing liposome (L), PutA (P), and membrane vesicle (M) were reported.
[c]OD_{450} from ELISA using polyclonal mouse-anti PutA antibody with goat anti-mouse horseradish peroxidase secondary antibody. Data were based on an average of two independent trials with errors less than OD 0.075.
[d]Lipid content was determined from pertaining fractions by ester quantitation. Content was described as percent lipid from in liposome and membrane vesicle containing fractions.
[e]Percent proline-induced lipid association was corrected for lipid content in pertaining fractions.

1.59×10^{13} molecules of PutA would be required to occupy the surface of 20 nmol of membrane vesicle lipid. The observed amount of PutA bound to this concentration of membrane vesicles was 13.9 pmol (8.37×10^{12} molecules) (Fig. 3A). Thus, PutA occupied 53% of the calculated available lipid in vitro. Using a similar calculation, PoxB (62 kDa), occupied 81% of the calculated available lipid (Fig. 3A). Thus, both PutA and PoxB seem to nearly saturate the available membrane lipid in vitro.

If PutA and PoxB compete for binding to membrane lipids, then pre-association of PoxB to the membrane would be expected to decrease PutA–membrane association. To test this possibility, we pre-associated increasing amounts of PoxB to 20 nmol AN387 membrane vesicle lipid and then measured the membrane association of saturating (Fig. 3B) or half saturating (Fig. 3C) concentrations of PutA. Preassociation of PoxB did not significantly affect PutA–membrane association. However, preassociation of PoxB decreased the reconstitution of proline dehydrogenase enzyme activity, indicating that although excess protein can associate with the membrane only a limited amount of the membrane-associated PutA is functional. Similar results were obtained for reconstitution of PoxB when PutA was preassociated with membrane vesicles and PoxB was added subsequently (data not shown).

Table 6
Minimal membrane requirements for PutA proline dehydrogenase activity[a]

Lipid vesicle[b]	$K_{m\ L\text{-proline}}$ (mM)	V_{max} (nmol min^{-1} mg^{-1})	K_{cat} (s^{-1})
EM1445	15	256	614
Liposome Ubi Cytbo	15	156	374

[a]Proline dehydrogenase activity was determined by measuring the proline-dependent formation of a yellow colored complex between P5C and *o*-aminobenzaldehyde (*o*-ab).
[b]Proline dehydrogenase activity was determined in the presence of equal molar lipid EM1445 (*thi rpsL ndh*::Cam) inverted membrane vesicles, and Liposome Ubi Cytbo (liposomes reconstituted with 10 nmol ubiquinone-10 and 0.41 nmol of heme from cytochrome *bo*).

4. Discussion

PutA must shuttle between the cytoplasmic membrane where it is enzymatically active and the cytoplasm where it functions as an autogenous transcriptional repressor. To accomplish these events PutA must respond to the concentration of proline and the availability of membrane binding sites. To determine what causes PutA to release *put* operator DNA and partition into the membrane, we quantitated the effect of FAD redox on PutA–membrane association. In addition, to determine what membrane properties are required we defined the minimal membrane components necessary for PutA–membrane association and for functional reconstitution of enzymatic activity.

When the internal concentration of proline reaches the K_m for proline binding, PutA partitions from the cytoplasm and associates with the cytoplasmic membrane [34]. The initial driving force for PutA–membrane association is the reduction of FAD due to the oxidation of proline (Table 1). The partitioning into the membrane is correlated with an increase in the hydrophobicity of PutA upon reduction of FAD. This FAD-induced membrane association involves an alteration in the conformation of PutA [35–37], possibly due to a redox induced twist in FAD [38].

Reoxidation of $FADH_2$ by the membrane-bound quinone pool is critical for PutA enzymatic activity [16]. To determine if membrane association requires interactions with the quinone pool, we assayed the effect of quinones in the PutA–membrane interaction. Although quinones are essential for proline dehydrogenase activity (Table 3), they are not required for PutA to associate with the cytoplasmic membrane (Table 2).

To determine if PutA–membrane association is strictly due to hydrophobic protein–lipid interactions, we assayed the association of PutA with synthetic liposomes. The results demonstrate that PutA associates efficiently with both liposomes that lack protein and inverted membrane vesicles (Table 5). Furthermore, liposomes containing both ubiquinone and cytochrome *bo* allow efficient reconstitution of proline dehydrogenase activity (Table 6). Together, these results demonstrate that PutA–membrane association does not require an additional membrane protein component; rather, PutA interacts directly with the quinone pool in the absence of a protein mediator. Furthermore, these results demonstrate that the PutA–membrane interaction is not dependent on the transfer of electrons to the membrane-associated electron transport chain as previously suggested [14].

Comparison of in vivo and in vitro membrane binding capacities indicate that the maximum amount of PutA associated with membranes in vivo is 10-fold less than in vitro. These results support previous studies which suggest that there are a limited number of PutA–membrane binding sites in vivo [10,14]. This difference could simply be due to competition by other peripheral membrane proteins for the membrane surface. The greater amount of PutA binding in vitro may be due to the dissociation of competing peripheral membrane proteins during the membrane extraction procedure. Competition of PutA and PoxB for membrane association in vitro provides support for this hypothesis. Saturation of the membrane with PoxB prevents PutA from functionally interacting with membrane vesicles, indicating that PoxB and PutA share common membrane sites (Fig. 3C). Thus, this class of FAD-dependent dehydrogenases may compete for functional interactions with the electron transport chain or proper membrane lipid residues in vivo. Saturation of membrane sites in vivo might prevent essential protein–membrane interactions, possibly explaining why overexpression of *putA* in vivo is lethal. Autogenous regulation of *putA* expression normally limits the amount of PutA to 10-fold less than the concentration that can functionally bind to the membrane in vitro, preventing such toxic overproduction of PutA.

In summary, proline utilization requires PutA to adopt a variety of conformations that direct PutA to function as an autogenous transcriptional repressor or membrane associated dehydrogenase. Proline oxidation and concurrent FAD reduction increases the relative hydrophobicity of PutA and, together with available membrane lipid, stimulates PutA to release *put* operator DNA and associate with the cytoplasmic membrane. Therefore, in addition to the cytoplasmic proline concentration, PutA senses the availability of membrane lipid, and in the absence of either of these two conditions PutA remains as a transcriptional repressor. Finally, PutA interacts directly with the membrane associated quinone pool to

permit proline dehydrogenase activity. Thus, the PutA–membrane interaction is dependent on the availability of membrane lipid and is a strict protein–lipid interaction.

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