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Biochemistry

PutA protein, a membrane-associated flavin dehydrogenase, acts as a redox-dependent transcriptional regulator

PAULA OSTROVSKY DE SPICER AND STANLEY MALOY

Department of Microbiology, University of Illinois, 131 Burrill Hall, 407 South Goodwin Avenue, Urbana, IL 61801

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ABSTRACT **The proline utilization (*put*) operon of *Salmonella typhimurium* is transcriptionally repressed by PutA protein in the absence of proline. PutA protein also carries out the enzymatic steps in proline catabolism. These two roles require different cellular localizations of PutA. Catabolism of proline requires PutA to associate with the membrane because reoxidation of the FAD cofactor in PutA needs the presence of an electron acceptor. Repression of the *put* operon requires PutA to bind to the *put* control-region DNA in the cytoplasm. The presence of proline, the inducer, is necessary but not sufficient for PutA to discriminate between its roles as an enzyme or as a repressor. Two conditions that prevent PutA protein binding to the *put* control region are (*i*) when proline and an electron acceptor or the cytoplasmic membrane are present or (*ii*) when PutA is reduced by dithionite. These two conditions increase the relative hydrophobicity of PutA protein, favoring membrane association and therefore enzymatic activity.**

The proline utilization (*put*) operon in *Salmonella typhimurium* and *Escherichia coli* consists of the two genes that allow cells to use proline as a sole source of carbon, nitrogen, and energy. The *putP* gene encodes the major proline permease, and the *putA* gene encodes a bifunctional dehydrogenase that catalyzes the oxidation of proline to glutamate (Fig. 1).

The *putA* gene product has been purified to homogeneity and its properties have been studied *in vitro*. The *putA* gene encodes a single polypeptide with both proline dehydrogenase (EC 1.5.99.8) and pyrroline-5-carboxylate (P5C) dehydrogenase (EC 1.5.1.12) activities (1). Proline dehydrogenase couples proline oxidation to reduction of a FAD cofactor which is tightly associated with the PutA protein (2). The electrons from the reduced FAD are directly transferred to the membrane-associated electron transport chain *in vivo* (3, 4). P5C dehydrogenase couples oxidation of P5C to glutamate with reduction of NAD. All *putA* null mutants and most *putA* missense mutants lack both enzymatic activities (5).

The *put* operon is induced 10- to 20-fold by proline (6). In addition, full induction of the *put* operon requires oxygen or another suitable terminal electron acceptor (6). However, the effect of oxygen seems to be indirect because the regulation by oxygen requires the PutA protein: *putA* mutants express the *put* operon at high constitutive levels regardless of the amount of oxygenation (6).

The phenotypes of *putA* mutants indicate that the PutA protein mediates the regulation of the *put* operon by proline. (*i*) Null mutations in the *putA* gene (including transposon insertions, deletions, nonsense mutations, and frameshift mutations) cause loss of PutA enzymatic activity and constitutive expression of *putP*: such mutants are designated *putA*(A^-C^-), where A^- indicates the loss of enzymatic activity and C^- indicates the loss of regulatory activity. (*ii*) There are three classes of *putA* missense mutants: *putA*(A^-C^-) mutants have the same phenotype as the null mutants; *putA*(A^-C^+) mutants lose the enzymatic activity but retain the regulatory activity; and *putA*(A^+C^-) mutants lose the regulatory activity but retain the enzymatic activity. These three types of mutations map throughout the *putA* gene, indicating that both regulatory and enzymatic properties are located in a single polypeptide (7). (*iii*) Haploid *putA::lac* operon fusions express β-galactosidase constitutively. However, when the *putA*$^+$ gene is provided in trans, expression of β-galactosidase from the *putA::lac* operon fusions is regulated by proline (6). Taken together, these genetic results strongly suggest that the PutA protein negatively regulates transcription of the *put* operon. *In vitro* studies support the genetic results. Both gel retardation studies (8) and *in vitro* DNase protection studies (unpublished work) demonstrate that purified PutA protein binds specifically to operator sites on DNA fragments from the *put* regulatory region.

The following model was proposed to explain how a peripheral membrane protein that functions as a bifunctional enzyme autogenously regulates expression of the *put* operon (Fig. 2). In the absence of proline, PutA protein remains in the cytoplasm, where it binds to the *put* operators, preventing *put* gene expression. However, when PutA protein binds proline, it associates with the membrane, where it catalyzes the degradation of proline; the decrease in cytoplasmic PutA protein frees up the operator sites, allowing *put* gene expression. This model predicts that induction of the *put* operon by proline is not simply due to an allosteric effect that prevents DNA binding but due to a change in the cellular localization of PutA protein from the cytoplasm to the membrane. To test this prediction we studied the effect of proline on PutA binding *in vivo* and *in vitro*. The results indicate that PutA protein responds to a change in its redox state, and this determines whether PutA protein should remain bound to its operator sites in the cytoplasm or associate with the membrane.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions. All strains used in this study were derivatives of *S. typhimurium* LT2. The relevant genotypes of the three strains used for the *in vivo* footprinting studies were MST837, *putA*$^+$/pPC6 (*putP*$^+$ *putA*$^+$, *amp*^{r}) (9); MST2614, *putA1020*::MudJ/pPC6 (*putA*^{s}) (S. W. Allen and S.M., unpublished work); and MST284, *putA912*(A^+C^-)/pPC13. Plasmid pPC6 is a pBR322 derivative that contains the entire *S. typhimurium* wild-type *put* operon (9). Plasmid pPC13 is a deletion derivative of pPC6 that contains only the control region of the *put* operon and the 5′ ends of each gene; therefore it is *putP*$^-$ and *putA*$^-$ (9).

The strains were grown either with or without proline in NCE medium (10) with 0.6% succinate or 0.2% glucose as a carbon source and ampicillin at 60 μg/ml to maintain the plasmids.

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Abbreviations: INT, *p*-iodonitrotetrazolium; P5C, pyrroline-5-carboxylate.

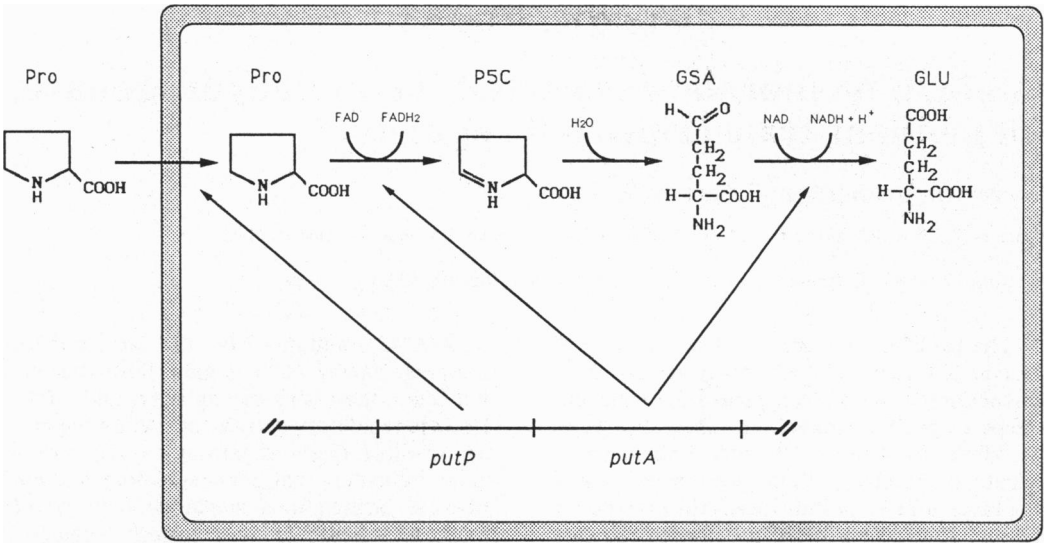

FIG. 1. Genes of the *put* operon and the roles of their gene products in proline utilization. The pathway for the degradation of proline to glutamate is shown at the top. GSA, glutamic semialdehyde.

For protein purification, PutA was overexpressed from plasmid pPC34. Plasmid pPC34 is a derivative of pCKR101 (11) that contains the *putA* gene downstream of the *tac* promoter and the $lacI^q$ gene, allowing induction of PutA expression by isopropyl β-D-thiogalactopyranoside.

***In Vivo* Footprinting.** Methylation with dimethyl sulfate (12) and primer extension (13) were done as described to examine PutA protein binding to the *put* control region on multicopy plasmids *in vivo*.

Protein Purification. To purify PutA, 200 ml of a strain carrying plasmid pPC34 was grown to early logarithmic phase in plasmid broth (14) with ampicillin at 100 μg/ml and induced for 1 hr with a final concentration of 0.1 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested and ruptured in a French pressure cell as described (1). The membranes were removed from the crude extract by centrifugation at 110,000 × *g*. The supernatant was precipitated with saturated $(NH_4)_2SO_4$, dissolved in 500 μl of G buffer [20% (vol/vol) glycerol/70 mM Tris, pH 8.2], dialyzed against G buffer, brought to 60 mM KCl, and then applied to a Mono Q HR 5/5 FPLC column (Pharmacia). Elution was carried out with a linear gradient of 60–160 mM KCl in G buffer by an FPLC system (Pharmacia). Fractions were assayed for proline oxidase activity (7). Fractions with the highest proline oxidase activity were pooled, $(NH_4)_2SO_4$-precipitated, dissolved in 200 μl of G buffer, and applied to a Superose 12 FPLC column (Pharmacia). Elution was carried out with G-buffer. Fractions with proline oxidase activity were pooled, yielding 1 ml of PutA protein at 2 mg/ml with a specific activity of 2000 nmol of *p*-iodonitrotetrazolium (INT) reduced per minute per milligram of protein. These pooled fractions were used directly in gel retardation assays. Purified PutA protein was stored in G buffer at −70°C.

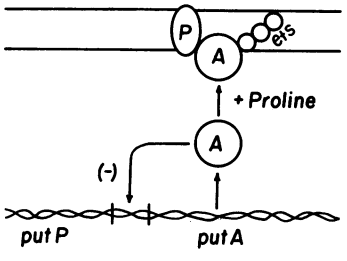

FIG. 2. Model for *put* regulation by PutA protein. In the absence of proline, PutA protein (A) remains in the cytoplasm and binds the DNA in the *put* regulatory region to repress transcription. In the presence of proline, PutA associates with the membrane to carry out proline degradation and allow induction of the *put* genes. ets, Electron transport system. P, *putP* gene product.

Gel Retardation Assays. Each assay mixture contained 150 nM PutA protein in G buffer (final concentration, 0.2×) and 0.1 nM ^{32}P-labeled *put* control-region DNA in 1× binding buffer. DNA was 5′ labeled with [γ-^{32}P]ATP as described (8). The 1× binding buffer contained calf thymus DNA (0.185 μg/ml), 12 mM Tris·HCl (pH 8.0), 5 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM dithiothreitol, and bovine serum albumin (100 μg/ml). Where indicated, the following additional reagents were used at the indicated concentrations: 680 mM proline, 640 mM INT, or 350 mM dithionite. PutA protein, DNA, and additional reagents were incubated at room temperature for 15 min before loading on the gel or before addition of the last reagent when the order of addition was varied; when the order of addition was varied, the mixture was incubated for an additional 10 min after addition of the last reagent. The DNA fragments were then separated by electrophoresis in an 8% polyacrylamide gel with a 30:0.8 acrylamide/*N*,*N*′-methylenebisacrylamide weight ratio. Electrophoresis was carried out in 89 mM Tris/89 mM boric acid/2.5 mM EDTA at constant voltage (15 V/cm) for 2 hr (Fig. 4) or 4 hr (data not shown). The gels were stained for proline dehydrogenase activity by submerging them in a proline oxidase assay mix (1) for 15 min at 37°C. After staining for proline oxidase, the gels were dried and exposed to a phosphor screen for 18 hr and analyzed with a PhosphorImager (Molecular Dynamics) to assay for radiolabeled DNA.

Triton X-114 Phase Separation. This procedure was based on the methods of Bordier (15) and Pryde (16). First, 6 μl of purified PutA protein was added to 94 μl of 150 mM NaCl/10 mM Tris·HCl, pH 7.2, or to 94 μl 350 mM dithionite/150 mM NaCl/10 mM Tris·HCl, pH 7.2. The solution was mixed and then 9 μl of 10 mM K_2HPO_4, pH 6.0/150 mM NaCl-equilibrated, precondensed Triton X-114 was added. The solutions were mixed at 0°C, incubated in ice for 1 min, and then shifted to 30°C for 5 min. The aqueous and hydrophobic phases were separated by centrifugation at 2500 × *g* for 5 min. Both phases from the oxidized and reduced (dithionite-treated) PutA samples were analyzed in an SDS/polyacrylamide gel (14). Gels were stained with Fast Stain (Zoion Research, Allston, MA) and dried.

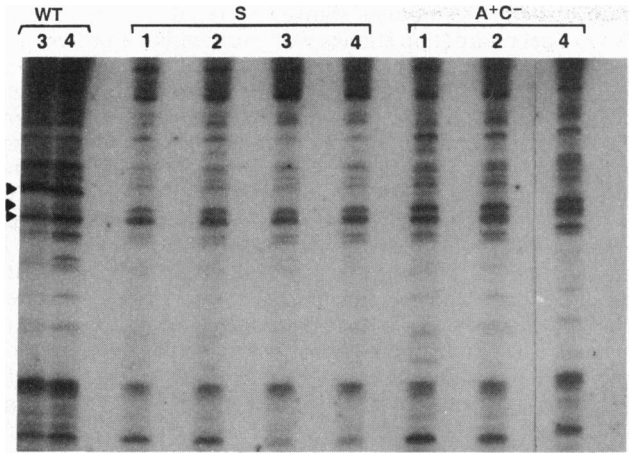

FIG. 3. *In vivo* methylation protection of a *put* operator site. The *putA* genotypes of the strains assayed were as follows: WT (wild type), *putA*$^+$; S, *putA* super-repressor mutant; A$^+$C$^-$, *putA* constitutive mutant. The growth conditions under which the strains were assayed were as follows: 1, NCE plus glucose; 2, NCE plus glucose plus proline; 3, NCE plus succinate; 4, NCE plus succinate plus proline. Arrowheads at left indicate guanine residues in the operator site.

RESULTS

Proline Disrupts PutA Protein–DNA Binding *in Vivo*. PutA protein binding to the *put* operator sites can be demonstrated *in vivo* (Fig. 3): (*i*) in a *putA*$^+$ strain the PutA binding sites are sensitive to methylation in the presence of proline but protected from methylation in the absence of proline; (*ii*) in a *putA* super-repressor mutant (S. W. Allen and S.M., unpublished work) the PutA binding sites are protected from methylation in the presence or absence of proline; (*iii*) in a *putA*(A$^+$C$^-$) constitutive mutant (7) the PutA operator sites are sensitive to methylation in the presence or absence of proline. These results indicate that proline prevents PutA protein from binding to the *put* operator sites *in vivo*. In addition, these *in vivo* footprinting experiments indicate that the PutA protein itself (and not some minor contaminant that copurifies with PutA) is the *put* repressor.

Disruption of the PutA Protein–DNA Complex *in Vitro* Requires Proline and an Electron Acceptor. Binding of PutA protein to the *put* control-region DNA was studied *in vitro* by gel retardation assays (17, 18). The position of the DNA on the gel was visualized with a PhosphorImager, and the position of the PutA protein was visualized by staining for proline dehydrogenase activity. Purified PutA retarded the mobility of *put* control-region DNA (Fig. 4*A*). The migration of the retarded DNA complex coincided with the proline dehydrogenase activity (Fig. 4*B*), strongly suggesting that the retardation was mediated by PutA binding to *put* control-region DNA. Preincubation of PutA with proline did not prevent the retardation of *put* control-region DNA (Fig. 4*A*). However, when PutA was preincubated with proline and the electron acceptor INT, the retardation of the *put* control region disappeared (Fig. 4*A*), indicating that the PutA–DNA complex did not form. In this case, the proline dehydrogenase activity did not comigrate with the DNA: proline dehydrogenase formed a low-mobility complex that did not enter the gel after 2 hr of electrophoresis (Fig. 4*B*) and barely entered the gel after 4 hr of electrophoresis (data not shown). Upon addition of *put* control-region DNA to PutA protein that had been preincubated with the electron acceptor INT in the absence of proline, both the proline dehydrogenase activity and the DNA formed a large aggregate at the well, but they remained together (data not shown). Also, when INT alone was added to *put* control region that had been preincubated with PutA, the *put* control region was retarded to the same extent as with PutA alone (data not shown). These results suggest that proline *and* INT disrupt the PutA–DNA complex but that neither compound alone has this effect.

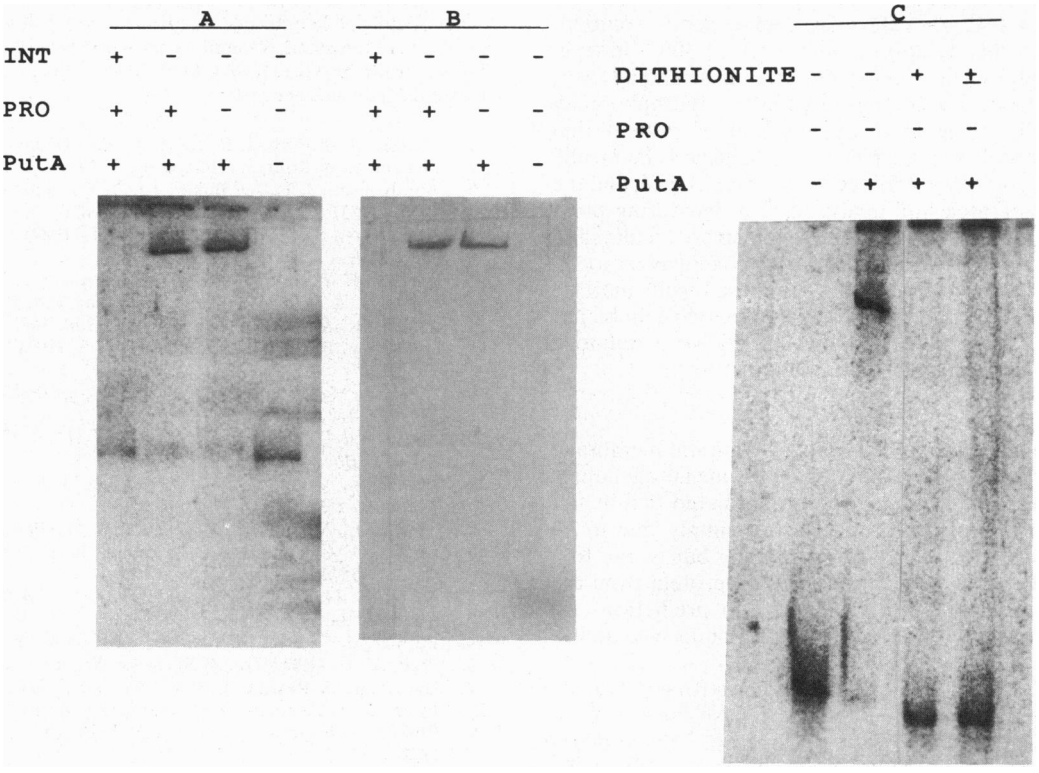

FIG. 4. Gel retardation assays of *put* control-region DNA with PutA protein and the indicated reagents. PRO, proline. (*A* and *C*) Position of radiolabeled DNA fragments on the gels. (*B*) Same gel as in *A* but stained for proline dehydrogenase activity. When dithionite was added last, its addition (+) is shown underlined (±).

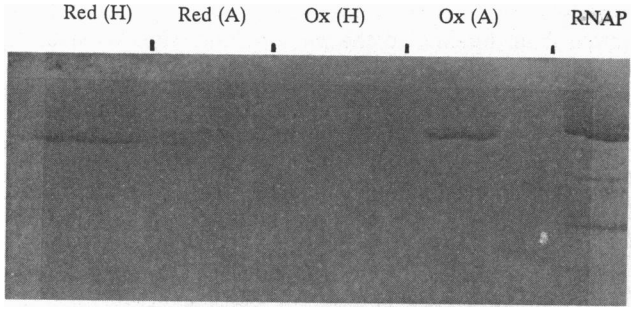

FIG. 5. Denaturing polyacrylamide gel of Triton X-114 phases. The oxidized samples (Ox) contained PutA protein that was not treated with dithionite. The reduced (Red) samples contain PutA protein that had been reduced with dithionite. Both the aqueous phase (A) and the hydrophobic phase (H) were analyzed. The RNAP lane contained 1 unit of *E. coli* RNA polymerase holoenzyme (Boehringer Mannheim) as a molecular weight marker.

Reduction of PutA Protein Prevents DNA Binding. To determine whether the effect of proline plus INT on PutA–DNA binding was due to reduction of PutA, we chemically reduced PutA with dithionite. Chemical reduction of PutA with dithionite disrupted the PutA–DNA complex, regardless of the order of addition of dithionite and PutA protein to DNA (Fig. 4*C*), or the presence or absence of proline (data not shown). In addition, reduction of PutA with dithionite caused the proline dehydrogenase activity to migrate as a low-mobility complex that remained near the well (data not shown), as had been observed when INT and proline were added. A potential explanation for these results could be that reduction alters the conformation of PutA protein, exposing hydrophobic residues and causing the protein to aggregate.

Reduction of PutA Protein Increases Its Relative Hydrophobicity. To test whether the relative hydrophobicity of purified PutA changes upon reduction, we assayed phase partitioning in the detergent Triton X-114. When proteins are mixed with Triton X-114 at 0°C, they form a homogeneous solution. However, when this solution is incubated at 30°C, it separates into two phases, an upper, aqueous phase and a lower, hydrophobic phase. The increase in relative hydrophobicity will cause an increase in the proportion of protein that partitions to the hydrophobic phase. Oxidized and chemically reduced PutA protein were mixed with Triton X-114, and the phases were separated and analyzed in a denaturing polyacrylamide gel. Oxidized PutA was exclusively localized in the aqueous phase and reduced PutA was exclusively localized in the hydrophobic phase (Fig. 5). These results indicate that reduction of PutA increases its relative hydrophobicity. In addition, mixing PutA with proline and INT or ubiquinone also increased PutA protein's hydrophobicity.

DISCUSSION

The model proposed to explain how a peripheral membrane protein that functions as a bifunctional enzyme autogenously regulates expression of the *put* operon predicted that induction of the *put* operon by proline is not simply due to an allosteric effect that prevents DNA binding but is due to a change in the cellular localization of PutA protein from the cytoplasm to the membrane. To test this prediction, we studied the effect of proline on PutA binding *in vivo* and *in vitro*.

In vivo and *in vitro* DNA-binding studies strongly indicate that the membrane-associated dehydrogenase PutA also directly mediates repression of the *put* operon. *In vivo* methylation protection studies showed specific protection of the *put* control region in *putA*$^+$ strains, and the protection was eliminated by proline; *putA* super-repressor mutants and *putA* constitutive mutants showed no regulation by proline. *In vitro* gel retardation studies with purified PutA protein and *put* control-region DNA demonstrated that proline dehydrogenase activity comigrates with the retarded DNA. Both proline and an electron acceptor were needed to prevent PutA protein from binding its operator sites *in vitro*. Chemical reduction of PutA also abolished its DNA-binding activity and increased its relative hydrophobicity. Reduction of other flavoproteins also causes conformational changes that increase their relative hydrophobicity (19).

Induction of the *put* operon cannot be achieved with proline alone: both proline and the membrane-associated electron transport chain or an artificial electron acceptor are needed. The results indicate that induction of the *put* operon may be due to a change in the conformation of PutA protein following interaction with proline and an electron acceptor, which reduces the protein, increases its relative hydrophobicity, and thus causes it to preferentially associate with the membrane.

This regulatory scheme would allow cells to turn off a catabolic gene when it is not needed (i.e., in the absence of inducer), but it may also provide a clever way to avoid making a membrane-associated protein when the necessary membrane sites are saturated (e.g., PutA protein or other flavin dehydrogenases are already bound to all available sites at the membrane). This strategy is analogous to other autoregulatory systems in which titration of functional sites results in repression of the gene products that require those sites for function (20).

Noted Added in Proof. Brown and Wood (21) have shown that, like *S. typhimurium* PutA, PutA protein from *E. coli* binds DNA *in vitro* and DNA binding is not prevented by proline. In addition, Brown and Wood have found that reduction of the FAD cofactor changes the conformation of PutA protein (E. D. Brown and J. M. Wood, personal communication), which may promote membrane association.

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1. Menzel, R. & Roth, J. R. (1981) *J. Biol. Chem.* **256,** 9755–9761.
2. Menzel, R. & Roth, J. (1981) *J. Biol. Chem.* **256,** 9762–9766.
3. Abrahamson, J. L. A., Baker, L. G., Stephenson, J. T. & Wood, J. M. (1983) *Eur. J. Biochem.* **134,** 77–82.
4. Graham, S., Stephenson, J. & Wood, J. (1984) *J. Biol. Chem.* **259,** 2656–2661.
5. Ratzkin, B. & Roth, J. (1978) *J. Bacteriol.* **133,** 733–744.
6. Maloy, S. & Roth, J. (1983) *J. Bacteriol.* **154,** 561–568.
7. Menzel, R. & Roth, J. (1981) *J. Mol. Biol.* **148,** 21–44.
8. Ostrovsky, P., O'Brien, K. & Maloy, S. (1991) *J. Bacteriol.* **173,** 211–219.
9. Hahn, D., Myers, R., Kent, C. & Maloy, S. (1988) *Mol. Gen. Genet.* **213,** 125–133.
10. Berkowitz, D., Huston, J., Whitfield, H., Roth, J. & Ames, B. (1968) *J. Bacteriol.* **96,** 215–220.
11. Lee, C. E., Gumport, R. I. & Gardner, J. F. (1990) *J. Bacteriol.* **172,** 1529–1538.
12. Giniger, E., Varnum, S. M. & Ptashne, M. (1985) *Cell* **40,** 767–774.
13. Brewer, A. C., Marsh, P. J. & Patient, R. K. (1990) *Nucleic Acids Res.* **18,** 5574.
14. Maloy, S. (1989) *Experimental Techniques in Bacterial Genetics* (Jones & Bartlett, Boston).
15. Bordier, C. (1981) *J. Biol. Chem.* **256,** 1604–1697.
16. Pryde, J. G. (1986) *Trends Biochem. Sci.* **11,** 160–163.
17. Garner, M. & Revzin, A. (1981) *Nucleic Acids Res.* **9,** 3047–3060.
18. Fried, M. & Crothers, D. (1981) *Nucleic Acids Res.* **9,** 6505–6525.
19. Russell, P., Hager, L. P. & Gennis, R. B. (1977) *J. Biol. Chem.* **252,** 7877–7882.
20. Maloy, S. & Stewart, V. (1993) *J. Bacteriol.* **175,** 307–316.
21. Brown, E. D. & Wood, J. M. (1992) *J. Biol. Chem.* **267,** 13086–13092.