Regulation of Gene Expression by Repressor Localization: Biochemical Evidence that Membrane and DNA Binding by the PutA Protein Are Mutually Exclusive

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The PutA protein from *Salmonella typhimurium* is a bifunctional enzyme that catalyzes the oxidation of proline to glutamate, a reaction that is coupled to the transfer of electrons to the electron transport chain in the cytoplasmic membrane. The PutA protein is also a transcriptional repressor that regulates the expression of the *put* operon in response to the availability of proline. Despite extensive genetic and biochemical studies of the PutA protein, it was not known if the PutA protein carries out both of these two opposing functions while membrane associated or if instead it carries them out in different cellular compartments. To distinguish between these alternatives, we directly assayed the binding of purified PutA protein to DNA and membranes *in vitro*. The results indicate that wild-type PutA does not simultaneously associate with DNA and membranes. In addition, PutA superrepressor mutants that exhibit increased repression of the *put* genes show a direct correlation between decreased membrane binding and increased DNA binding. These results support a model in which the PutA protein shuttles between the membrane (where it acts as an enzyme but lacks access to DNA-binding sites) and the cytoplasm (where it binds DNA and acts as a transcriptional repressor), depending on the availability of proline.

Escherichia coli and *Salmonella typhimurium* can use proline as a sole carbon and nitrogen source. Proline utilization requires expression of two gene products. The *putP* gene encodes the major proline permease. The *putA* gene encodes a bifunctional enzyme with both the proline dehydrogenase (EC 1.5.99.8) and pyrroline-5-carboxylic acid dehydrogenase (EC 1.5.1.12) activities required for the oxidation of proline to glutamate (2, 11). The proline dehydrogenase reaction couples proline oxidation with reduction of a flavin adenine dinucleotide (FAD) cofactor tightly associated with the PutA protein (2, 12). The electrons are then transferred to the electron transport chain in the cytoplasmic membrane *in vivo* (1). In addition to its catalytic functions, in the absence of proline PutA acts as a transcriptional repressor that regulates the expression of both *put* genes by binding to specific operator sites in the *put* regulatory region (2, 15).

Several lines of evidence suggest that the functional coupling of the proline dehydrogenase activity of PutA with the membrane-associated electron transport chain is required for derepression of the *put* genes. First, full induction of the *put* genes *in vivo* requires both proline and a terminal electron acceptor (9). Second, the stable association of wild-type PutA with membranes *in vitro* requires both proline and the transfer of electrons to the electron transport chain (3, 19). Third, *putA* mutants that have diminished proline dehydrogenase activity show increased repression of the *put* genes *in vivo* (14).

On the basis of these studies, the following model has been proposed to explain how PutA functions as both a membrane-

associated enzyme and a transcriptional repressor (8, 10). In the absence of proline PutA remains in the cytoplasm where it binds to the *put* operators and represses *put* gene expression. When a sufficient concentration of proline is available, PutA binds proline and functionally associates with the electron transport chain in the cytoplasmic membrane where it is enzymatically active. As it becomes associated with the membrane, the cytoplasmic concentration of PutA protein drops below that required for repression, allowing expression of the *put* genes. Thus, this model predicts that the derepression of *put* gene expression is due to the functional compartmentalization of the PutA protein in the membrane where it lacks access to its operator sites.

This repressor localization model fits the available data, but there was no direct evidence that the choice between the regulatory and enzymatic activities of PutA protein is determined by its cellular localization. By analogy with the ToxR protein (13), it seemed possible that both functions of the PutA protein may occur while it is associated with the membrane and that derepression may simply be due to an allosteric change in the protein conformation which decreases its affinity for DNA. To answer this question, we developed *in vitro* assays to determine whether the binding of PutA protein to membranes and DNA occurred simultaneously or was mutually exclusive.

PutA protein does not simultaneously associate with DNA and cytoplasmic membranes *in vitro*. PutA protein binds to DNA containing the *put* operator sites *in vitro* (2, 14, 15) and *in vivo* (16). Proline decreases the affinity of the PutA protein for DNA *in vivo*, resulting in derepression of the *put* genes (9, 16). In contrast, proline only prevents DNA binding *in vitro* if an artificial electron acceptor is also available (16). These results suggest that the interaction of PutA protein with the membrane-associated electron transport chain may prevent it from binding DNA *in vivo*. To determine whether the association of the PutA protein with cytoplasmic membranes prevents it from binding to DNA, we assayed the binding of purified PutA protein to DNA by gel mobility shift assays (4)

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FIG. 1. Effect of membranes on DNA binding by PutA. Gel retardation assays were performed in the presence (+) or absence (-) of 230 mM proline as described previously (16). The presence or absence of 0.8 μ g of wild-type PutA and 250 ng of *E. coli* EM41 membranes in the samples is indicated. Where indicated, membranes were added after 15 min of preincubation of PutA with DNA (14). The positions of *put* control region free and bound DNAs are indicated. Membranes were prepared as follows. Cells grown at 37°C overnight in 400 ml of minimal E medium (18) without citrate and supplemented with 0.6% succinate were collected by centrifugation at 6,000 rpm in a Sorvall GSA rotor for 10 min. The cells were washed with one volume of 0.85% NaCl, centrifuged, and resuspended in 20 ml of 0.1 M cacodylic buffer, pH 6.8. The suspension was passed twice through a French pressure cell at 10,000 to 12,000 psi, and the crude extract was centrifuged for 10 min at 10,000 rpm in a Sorvall SS34 rotor. The supernatant was removed and centrifuged at 42,000 rpm on a Beckman 50 Ti rotor. The supernatant was discarded, and the pellet containing the membrane vesicles was resuspended in 3 ml of the same buffer containing 5% glycerol. The protein concentration in membrane samples was determined as described previously (17).

in the presence or absence of membranes. Membranes from *S. typhimurium* could not be used for these studies because they contain nucleases that rapidly degrade the DNA. However, membranes from *E. coli* lack nuclease activity, and purified PutA protein from *S. typhimurium* functionally associates with membranes from *E. coli*. Therefore, we used membranes isolated from an *E. coli put* deletion mutant. The results are shown in Fig. 1. In the absence of proline the addition of membranes did not affect DNA binding by the PutA protein. However, when PutA was bound to DNA in the presence of proline, the addition of membranes caused the dissociation of the PutA protein from DNA. These results indicate that, at least in vitro, the PutA protein does not bind to both DNA and membranes at the same time.

We also assayed membrane association in the presence or absence of DNA. PutA was incubated with membranes and DNA in the presence or absence of proline. The soluble and membrane fractions were then separated, and the distributions of PutA protein and DNA between the two fractions were determined. The results are shown in Fig. 2. Although proline promoted the association of PutA with cytoplasmic membranes, the distribution of the *put* control region DNA was not altered: both the *put* control region DNA and the nonspecific control DNA (ϕ X174) remained in the soluble fraction. Furthermore, the results obtained for the reaction without DNA indicated that the presence of DNA in the samples did not alter the association of PutA with membranes in the presence of proline. Thus, these results, obtained from a different biochemical assay, confirm that PutA protein does not bind to both DNA and membranes at the same time.

PutA superrepressor mutants are defective in membrane association and reduction of the FAD cofactor. The functional association of the PutA protein with cytoplasmic membranes can be monitored by assaying the formation of a colored complex between pyrroline-5-carboxylate (the product of the proline dehydrogenase reaction) and *o*-aminobenzaldehyde (5). This reaction uses oxygen as the final electron acceptor and depends on the transfer of electrons via the membrane-associated respiratory chain (6, 19).

We previously isolated mutants of the PutA protein that show a superrepressor phenotype in vivo (14). An analysis of purified PutA proteins from the superrepressor mutants indicated that they all had diminished proline dehydrogenase activity (14), suggesting that the mutant proteins may not associate with membranes as well as the wild-type protein. To determine whether the increased level of repression by the superrepressor mutants was due to decreased ability to functionally associate with membranes, we assayed the binding of the purified wild-type and mutant (PutA1222, PutA1223, and PutA1224) proteins to membranes in vitro. The results are shown in Fig. 3. In the presence of proline, wild-type PutA protein appeared exclusively in the membrane fraction. In the absence of proline, a fraction of the wild-type PutA protein remained associated with membranes. This "nonspecific" membrane association in the absence of proline was also observed for the *E. coli* PutA protein (3). In contrast, the PutA proteins from the superrepressor mutants showed different extents of membrane association, and the defect in membrane association of each protein was proportional to the severity of its superrepressor phenotype in vivo (14). PutA1222, a weak superrepressor, associated with membranes about as well as the wild-type PutA protein. PutA1223, a moderate superrepressor, had an intermediate defect in membrane association: although membrane association of PutA1223 increased slightly in the presence of proline, much of the protein remained in the soluble fraction. PutA1224, the strongest superrepressor, was very defective in membrane association: PutA1224 remained

FIG. 2. Effect of DNA on membrane binding by PutA. PutA was incubated with DNA in the presence (+) or absence (-) of 200 mM proline for 15 min as previously described (14). Membranes from *E. coli* EM41 were then added, and the incubation was continued for 30 min. The soluble (S) and precipitable (M) fractions were separated by centrifugation at 12,000 rpm in an Eppendorf microcentrifuge. The distribution of PutA protein was determined by assaying its proline dehydrogenase activity in the presence of an artificial electron acceptor (12). The distribution of the *put* control region DNA, as well as the distribution of nonspecific DNA fragments also included in the samples (ϕ X174 *Hae*III fragments), was determined by spotting two dilutions of each sample on Gene-Screen Plus membranes and hybridizing with labeled *put* control DNA (from plasmid pPC6) (7) or labeled ϕ X174 *Hae*III fragments. Samples contained wild-type PutA (3 μ g), proline (200 mM), DNA (500 ng of a mixture of *put* control region DNA and ϕ X174 *Hae*III fragments), and *E. coli* EM41 membranes (150 μ g), as indicated. The distributions of PutA protein, *put* control region DNA, and control DNA (ϕ X174) between soluble and precipitable fractions are shown.

FIG. 3. Membrane association of wild-type and superrepressor PutA proteins. Western immunoblot detection of PutA protein in soluble (S) or membrane (M) fractions after incubation of samples containing PutA and membranes in the absence (–) or presence (+) of 200 mM proline is shown. Wild-type and mutant PutA proteins were purified as previously described (14). Membranes from *S. typhimurium* MST63 [*del(putA)559*] were prepared as described in the legend for Fig. 1. Strain MST63 carries a deletion of the *put* genes, so these membranes lack endogenous proline dehydrogenase activity. Seven and one-half micrograms of purified PutA was incubated with 270 μ g of the membranes in the presence or absence of proline in 0.1 M cacodylic buffer, pH 6.8, containing 10 mM $MgCl_2$. After 30 min at room temperature, the membrane and soluble fractions were separated through a step gradient containing a layer of 25% (wt/wt) sucrose and a layer of 65% (wt/wt) sucrose by centrifugation for 60 min at 50,000 rpm in a Beckman SW-55 Ti rotor. Material collected from the cushion on top of the 25% sucrose layer contained the soluble fraction, and the material collected between the two layers contained the membrane fraction. All samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Immobilon), and assayed by Western immunoblotting by using a polyclonal antibody against PutA protein.

almost entirely in the soluble fraction even in the presence of proline. These results indicate that the decreased association of PutA protein with cytoplasmic membranes is directly correlated with increased repression of the *put* genes.

Reduction of the FAD cofactor in PutA protein is not the signal for PutA membrane association. The functional association of the *E. coli* PutA protein with cytoplasmic membranes was shown to occur concomitant with the reduction of the FAD cofactor in PutA (3, 6). Furthermore, the FAD cofactor reaches its half-maximum reduction at a concentration of proline (0.11 mM) strikingly similar to the concentration of proline at the midpoint for membrane association equilibrium (0.1 mM) (3). This led to the suggestion that the reduction of the FAD cofactor promotes membrane association of PutA (3). Thus, we studied the reduction of the FAD cofactor in each of the three superrepressor mutant proteins described above. The results are shown in Fig. 4. The kinetics of FAD reduction in wild-type PutA protein from *S. typhimurium* is essentially identical

FIG. 4. Reduction of the FAD cofactor in wild-type and mutant PutA proteins. The graph shows the dependence of protein absorbance at 450 nm on proline concentration. Proline concentrations ranged from 0.001 to 100 mM. Scans were performed in samples containing 0.75 mg of protein/ml (wild-type PutA, PutA1222, or PutA1223) or 1.5 mg of protein/ml (PutA1224).

to the kinetics reported for the *E. coli* PutA protein (3). However, all three of the mutant proteins are severely altered in the reduction of FAD, requiring a much higher concentration of proline before they reach the midpoint of bleaching at 450 nm (the characteristic absorbance peak of flavins). Although protein from weak superrepressor mutant PutA1222 is severely defective in FAD reduction, it associates with membranes as well as does the wild-type PutA protein. Furthermore, although proteins from the three superrepressor mutants show similar kinetics of FAD reduction, their abilities to interact with cytoplasmic membranes are very different (see Fig. 3). Thus, the superrepressor phenotype correlates more closely with the defect in membrane association than with the kinetics of FAD reduction. These results suggest that the reduction of FAD by proline is not the inducing signal that determines the subcellular localization of PutA.

Summary. These results indicate that the PutA protein does not interact simultaneously with membranes and DNA; that is, binding of the PutA protein to membranes or DNA is mutually exclusive. This conclusion supports the model that the PutA protein shuttles between the cytoplasm and the membrane. In the absence of proline PutA remains in the cytoplasm where it acts as a repressor, and in the presence of proline PutA binds to the cytoplasmic membrane where it acts as a catabolic enzyme. While membrane associated, the PutA protein is functionally sequestered in a cell compartment where it is unable to bind DNA. The signal for membrane binding requires the proline dehydrogenase activity of PutA. Thus, PutA represses the *put* genes unless the conditions are appropriate for the proline dehydrogenase reaction (availability of both proline and membrane sites), avoiding wasteful synthesis of PutA protein.

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REFERENCES

1. Abrahamson, J. L. A., L. G. Baker, J. T. Stephenson, and J. M. Wood. 1983. Proline dehydrogenase from *Escherichia coli* K12. Properties of the membrane-associated enzyme. *Eur. J. Biochem.* **134**:77–82.
2. Brown, E. D., and J. M. Wood. 1992. Redesign purification yields a fully functional PutA protein dimer from *Escherichia coli*. *J. Biol. Chem.* **267**:13086–13092.
3. Brown, E. D., and J. M. Wood. 1993. Conformational change and membrane association of the PutA protein are coincident with reduction of its FAD cofactor by proline. *J. Biol. Chem.* **268**:8972–8979.
4. Carey, J. 1991. Gel retardation. *Methods Enzymol.* **208**:103–117.
5. Dendinger, S., and W. Brill. 1970. Regulation of proline degradation in *Salmonella typhimurium*. *J. Bacteriol.* **103**:144–152.
6. Graham, S. B., J. T. Stephenson, and J. M. Wood. 1984. Proline dehydrogenase from *Escherichia coli* K12. Reconstitution of a functional membrane-association. *J. Biol. Chem.* **259**:2656–2661.
7. Hahn, D. R., R. S. Myers, C. R. Kent, and S. R. Maloy. 1988. Regulation of proline utilization in *Salmonella typhimurium*: molecular characterization of the *put* operon, and DNA sequence of the *put* control region. *Mol. Gen. Genet.* **213**:125–133.
8. Maloy, S. 1987. The proline utilization operon, p. 1513–1519. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.) *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
9. Maloy, S. R., and J. R. Roth. 1983. Regulation of proline utilization in *Salmonella typhimurium*: characterization of *put::Mu d(Ap, lac)* operon fusions. *J. Bacteriol.* **154**:561–568.
10. Maloy, S., and V. Stewart. 1993. Autogenous regulation of gene expression. *J. Bacteriol.* **175**:307–316.
11. Menzel, R., and J. Roth. 1981. Purification of the *putA* gene product. A

- bifunctional membrane-bound protein from *Salmonella typhimurium* responsible for the two-step oxidation of proline to glutamate. J. Biol. Chem. **256**:9755–9761.
12. **Menzel, R., and J. Roth.** 1981. Enzymatic properties of the purified *putA* protein from *Salmonella typhimurium*. J. Biol. Chem. **256**:9762–9766.
13. **Miller, V. L., R. K. Taylor, and J. J. Mekalanos.** 1987. Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. Cell **48**:271–279.
14. **Muro-Pastor, A. M., and S. Maloy.** 1995. Proline dehydrogenase of the transcriptional repressor PutA is required for induction of the *put* operon by proline. J. Biol. Chem. **270**:9819–9827.
15. **Ostrovsky de Spicer, P., K. O'Brien, and S. Maloy.** 1991. Regulation of proline utilization in *Salmonella typhimurium*: a membrane-associated dehydrogenase binds DNA in vitro. J. Bacteriol. **173**:211–219.
16. **Ostrovsky de Spicer, P., and S. Maloy.** 1993. PutA, a membrane-associated flavin dehydrogenase, acts as a redox-dependent transcriptional regulator. Proc. Natl. Acad. Sci. USA **90**:4295–4298.
17. **Peterson, G. L.** 1977. A simplification of the protein assay of Lowry *et al.* which is more generally applicable. Anal. Biochem. **83**:346–356.
18. **Vogel, H., and D. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–106.
19. **Wood, J. M.** 1987. Membrane association of proline dehydrogenase in *Escherichia coli* is redox dependent. Proc. Natl. Acad. Sci. USA **84**:373–377.