Protein phosphorylation on serine, threonine, and tyrosine residues modulates membrane–protein interactions and transcriptional regulation in *Salmonella typhimurium*

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There exists a plethora of tyrosine kinases that play essential roles in regulation of eukaryotic proteins. Several dual specificity kinases that phosphorylate proteins on threonine, serine, and tyrosine residues also play critical roles in eukaryotic phosphorylation cascades. In contrast, very few prokaryotic proteins have been shown to be phosphorylated on tyrosine residues, and the functions of the rare examples remain obscure. Furthermore, no dual specificity kinases have been described in prokaryotes. Our results indicate that PutA protein from the bacterium *Salmonella typhimurium* autophosphorylates on several threonine, serine, and tyrosine residues. PutA protein both represses the proline utilization operon and degrades proline to glutamate. These two opposing functions are regulated by the availability of proline and the membrane sites needed for the proline dehydrogenase activity of PutA protein. In addition, these functions are modulated by phosphorylation of PutA protein. The rate of dephosphorylation of PutA protein is determined by the availability of proline and membranes. Dephosphorylated PutA protein has a higher DNA binding affinity than the phosphorylated protein and thus may prevent toxic overexpression of PutA protein in the absence of available membrane sites.

[Key Words: Dual specificity kinase/phophatase; transcriptional regulation; protein phosphorylation; membrane association; proline metabolism]

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Proline oxidation is widespread in prokaryotes (Costilow and Cooper 1978; Chen and Maloy 1991) and in the mitochondria of eukaryotic cells (Sacktor and Childress 1967; Stewart and Lai 1974, Sylvester et al. 1974; Downing et al. 1976; Brandrius and Magasanik 1979), allowing proline to be used as a source of carbon, nitrogen, and energy. In enteric bacteria, proline utilization requires two genes: The *putP* gene encodes the major proline permease, and the *putA* gene encodes a multifunctional protein (Maloy 1987). In the presence of proline, the PutA protein catalyzes the two enzymatic steps required to oxidize proline to glutamate, and in the absence of proline it acts as the transcriptional repressor of both *put* genes [Menzel and Roth 1981a; Maloy and Roth 1983; Ostrovsky de Spicer et al. 1991]. These functions of PutA protein occur in different cellular compartments: Oxidation of proline occurs at the cytoplasmic membrane where the tightly bound FAD cofactor donates electrons directly to the electron transport chain; repression of *put* transcription occurs in the cytoplasm [Abrahamson et al. 1983; Wood 1987; Wood et al. 1987; A.M. Muro-Pastor, P.C. Ostrovsky, and S. Maloy, in prep.].

The PutA protein senses the intracellular proline concentration and the physiological state of the membranes to mediate the decision between these two opposing roles. Reduction of the FAD cofactor causes an increase in the relative hydrophobicity of PutA protein, favoring its interaction with the cytoplasmic membrane [Ostrovsky de Spicer and Maloy 1993]. Thus, when both proline and membranes are available, PutA protein interacts with the membrane and is released from its specific binding sites in the *put* control region DNA [Muro-Pastor and Maloy 1995; Ostrovsky de Spicer and Maloy 1993; A.M. Muro-Pastor, P.C. Ostrovsky, and S. Maloy, in prep.]. These results suggested that the presence of proline causes PutA protein to be sequestered in the membrane where it can function as a dehydrogenase but where it lacks access to DNA.

The results presented here indicate that the mechanism for deciding between these two opposing roles also involves autophosphorylation. Protein phosphorylation
is widespread in eukaryotes and prokaryotes and often it is involved in the regulation of membrane receptors or enzymes (Cozzone 1993; Edelman et al. 1987; Hunter and Cooper 1985). As with many well-characterized examples of protein phosphorylation, phosphorylation of PutA protein seems to affect its regulatory function. The phosphorylation pattern observed with PutA protein, however, has previously only been observed in eukaryotes.

Results

Purified PutA protein is phosphorylated in vitro

Several lines of evidence indicate that purified PutA protein is autophosphorylated in vitro (Fig. 1). (1) PutA protein becomes radioactively labeled when incubated with [γ-32P]ATP. (2) PutA protein is not radioactively labeled when incubated with [α-32P]ATP, indicating that labeling observed with [γ-32P]ATP is not simply due to noncovalent binding of ATP or covalent adenylation (Foster et al. 1989). (3) PutA protein retains the 32P label when subjected to denaturing polyacrylamide gel electrophoresis, indicating that PutA is covalently phosphorylated. (4) Most of the 32P label on PutA protein can be removed with nonspecific alkaline phosphatases (Figs. 1A and 2), confirming that PutA is phosphorylated not simply adenylylated, and suggesting that the phosphorylated residues are located on the surface of the protein.

PutA protein is phosphorylated on serine, threonine, and tyrosine residues

The 32P label is acid resistant and somewhat base labile (data not shown), indicating that PutA may be phosphorylated on multiple amino acid residues. However, interpretation of these results is complicated because the stability of phosphoamino acids can vary greatly depending on the nearby residues in the protein (Buss and Stull 1983). Therefore, to determine which types of amino acid residues were phosphorylated, we analyzed PutA protein by two-dimensional phosphoamino acid analysis and 31P-NMR. The two-dimensional phosphoamino acid analysis revealed phosphoserine, phosphothreonine, and phosphotyrosine residues (Fig. 2). 31P-NMR analysis also demonstrates that PutA protein is phosphorylated. The 31P-NMR results clearly showed peaks with chemical shifts corresponding to phosphoserine and phosphothreonine at pH 8.0 but did not reveal phosphorytrosine (Fig. 3A). The apparent discrepancy between the results obtained from these two different methods could be due to several factors: (1) The expected concentration of phosphorytrosine is at the threshold of the range of sensitivity of 31P-NMR measurements; (2) two-dimensional phosphoamino acid analysis of PutA protein treated with phosphatase or proline (Fig. 2) indicated that the phosphotyrosine may not be surface-exposed and therefore not detectable by the 31P-NMR measurements; and (3) the phosphate moiety of phosphorytrosine often has a rapid turnover (Hunter and Cooper 1985), so the phosphotyrosine in PutA protein might be spontaneously dephosphorylated during the extended time course of the NMR experiment.

PutA protein is also phosphorylated in vivo

The experiments described above were done with purified PutA protein in vitro. To determine if the phosphorylation of PutA protein is simply an in vitro artifact, we directly examined the phosphorylation of PutA protein expressed in vivo. PutA protein from crude cell extracts was recognized by a polyclonal anti-phosphotyrosine antibody in ELISA assays (Fig. 4A) and by monoclonal anti-phosphotyrosine, anti-phosphothreonine (Fig. 5), and anti-phosphoserine (data not shown) antibodies in Western blot immunoassays. These results indicate that PutA protein is also phosphorylated on serine, threonine, and tyrosine residues in vivo.

PutA protein contains protein kinase-like subdomain motifs

The deduced sequence of the PutA protein (EMBL accession code X70843) carboxyl terminus displays moderate similarity with several of the subdomains conserved among protein kinases (Hanks et al. 1988). A sequence similar to subdomain I, containing the ATP-binding motif, is present in PutA protein. Preliminary data suggest that mutation of the conserved glycines in this motif affects phosphorylation of PutA protein (S. Allen and S. Maloy, unpubl.). A motif similar to subdomain VI is also present, and it contains the sequence GXgLX6HRDLAXR usually indicative of a kinase with specificity for tyrosine (Hanks et al. 1988). In general, these domains in PutA protein show a higher similarity to protein–tyrosine kinase family members and in par-
Figure 2. Phosphoamino acid analysis of PutA protein reveals phosphoserine, phosphothreonine, and phosphotyrosine, but only phosphoserine, and phosphothreonine are sensitive to phosphatase and proline. (Top) The phosphoamino acids are phosphoserine (a), phosphothreonine (b), and phosphotyrosine (c) in the following conditions: (I) Untreated PutA protein, (II) PutA protein in 100 mM proline, (III) PutA protein treated with calf thymus alkaline phosphatase. (Bottom) Quantitation of each phosphoamino acid (a, b, and c) label in conditions I, II, and III, as described above.

Proline stimulates PutA dephosphorylation

The phosphorylation state of PutA protein changes in response to the physiological signals required for proline utilization. Addition of proline resulted in the rapid loss of ~60% of the total 32P-radioactive label from PutA protein in vitro (Fig. 1A,C; Table 1). This proline-stimulated dephosphorylation coincided with an increase in the inorganic phosphate peak in 31P-NMR experiments, but PutA protein was rapidly rephosphorylated in the presence of excess ATP (Fig. 3B). Two-dimensional phosphoamino acid analysis of PutA protein treated with proline or phosphatase revealed that both treatments resulted in dephosphorylation of the phosphoserine and phosphothreonine residues but not the phosphotyrosine residues (Fig. 2). The proline-induced dephosphorylation was prevented by addition of membranes isolated from an E. coli strain deleted for the put genes (Fig. 1C; Table 1).

Dephosphorylation increases the affinity of PutA protein for DNA

To determine whether the phosphorylation of PutA protein has a physiological role, we tested the effects of phosphorylation on each of its known functions. Phosphorylation did not significantly affect the enzymatic activities of PutA protein (data not shown). However, phosphorylation did affect the apparent $K_d$ for DNA binding as measured by gel mobility retardation experiments (Fried and Crothers 1981; Carey 1991). Preincubation of PutA protein with ATP, which increases PutA phosphorylation in vitro, decreased the apparent affinity of PutA for DNA about eightfold (Fig. 4C). In addition, preincubation of PutA protein with proline, which induces put gene expression in vivo, decreased the apparent affinity of PutA for DNA about twofold (Fig. 4C). Addition of both proline and ATP further decreased the apparent affinity of PutA for DNA (Fig. 4C). Although proline stimulates dephosphorylation of PutA protein, in the presence of excess ATP, it is rapidly rephosphory-
Figure 3. $^{31}$P-NMR spectra of PutA protein confirms the presence of phosphoserine and phosphothreonine. (A) Spectra in the absence of proline. The assigned peaks correspond to phosphoserine (+4.66 ppm), phosphothreonine (+4.18 ppm), inorganic phosphate (+2.9 ppm), the γ-phosphate of ATP (~5.35 ppm) and the α-phosphate of ATP (~9.43 ppm) [Burt 1987]. (B) Same as A but with 100 mM proline. The chemical shifts are the same as in A but the integrated area of the inorganic phosphate peak has increased by 68%.
Discussion

These results indicate that the *S. typhimurium* PutA protein is covalently phosphorylated both in vitro and in vivo. PutA protein is phosphorylated on serine, threonine, and tyrosine residues, a phosphorylation pattern common to dual-function kinases in eukaryotes (Nishida and Gotoh 1993) but not previously observed in bacteria.

Does phosphorylation of PutA protein play an important physiological role? Expression of PutA protein is necessary for cells to use proline as a carbon, nitrogen, or energy source. However, like many other many membrane proteins, overexpression of PutA protein is toxic. Thus, maintaining the optimal expression of the *put* genes requires a way of responding to both the availability of proline and the availability of functional membrane sites. Previous studies have demonstrated that reduction of the PutA protein during proline catabolism increases its hydrophobicity, favoring the dissociation of PutA protein from the DNA and its partitioning into the membrane (Ostrovsky de Spicer and Maloy 1993; Muro-Pastor and Maloy 1995; A.M. Muro-Pastor, P.C. Ostrovsky, and S. Maloy, in prep.). These results explain how proline induces the expression of the *put* genes, but not how toxic overexpression of PutA protein is avoided when proline is present and the functional membrane sites are saturated.

Phosphorylation may serve as a mechanism to avoid the toxic overexpression of PutA protein in the absence of functional membrane sites. In the absence of membrane-binding sites, proline stimulates dephosphorylation of PutA protein, increasing the affinity of PutA protein for DNA and repressing the *put* operon. In contrast, when both membrane-binding sites and proline are available, PutA protein remains phosphorylated, decreasing the affinity of PutA protein for DNA and derepressing the *put* operon.

Table 1. Effect of proline and membranes on PutA autophosphorylation

| Membranes a | Proline b | Label intensity b | Percent label |
|-------------|-----------|------------------|---------------|
| +           | −         | 790 × 10^3       | 100.0         |
| +           | +         | 510 × 10^3       | 64.6          |
| −           | +         | 317 × 10^3       | 40.1          |

aThe conditions indicated here correspond to lanes 1, 2, and 3 of Fig. 1C.
bThe label intensity was quantitated by use of a PhosphoImager.

Figure 4. PutA protein from crude cell extracts is recognized by a polyclonal anti-phosphotyrosine antibody. (A) Immunoassays of crude extracts of *S. typhimurium* cells overexpressing PutA protein (+ PutA) grown with and without proline (+ Pro and −Pro, respectively), crude extracts of *S. typhimurium* cells that do not express PutA protein (−PutA), or pure phosphoamino acids (P-AA). (B) Immunoassays of purified PutA protein, bovine serum albumin with ATP (BSA + ATP), or pure phosphoamino acids (P-AA). (C) *K_{{DNA}}* of purified PutA protein under different conditions as indicated.

Figure 5. PutA protein from crude cell extracts is recognized by monoclonal phosphothreonine and phosphotyrosine antibodies in Western immunoblots. Lanes 1 and 4 contain extracts of *S. typhimurium* cells that do not express PutA protein [MST2489]. Lanes 2, 3, 5, and 6 contain extracts of *S. typhimurium* cells that express PutA protein under the control of the P_{vac} promoter [MST2830]; in lanes 2 and 5, the cells were not induced, but in lanes 3 and 6, the cells were induced with 0.1 mM IPTG. Lane 7 contains 4 txg of purified PutA protein. The samples in lanes 1–3 were sonicated after induction and ATP was added to 10 mM; the samples in lanes 4–6 were directly solubilized in SDS sample buffer, and no ATP was added. (A) Blot probed with anti-phosphothreonine monoclonal antibody. (B) Blot probed with anti-phosphotyrosine monoclonal antibody.
How does the availability of proline and membrane sites control PutA phosphorylation? Proline increases the relative hydrophobicity of PutA protein [Ostrovsky de Spicer and Maloy 1993], thus promoting either membrane binding or dimerization of PutA protein [possibly via interactions between hydrophobic surfaces on the monomers [Menzel and Roth 1981b]]. Dimerization plays a critical role in controlling the phosphorylation of many other proteins [Ninfa et al. 1993; Resh 1993]. By analogy, proline-induced dephosphorylation may occur between monomeric subunits of PutA dimers. An experimental observation that supports this idea is that although both dimers and monomers of PutA protein can be observed on native polyacrylamide gels, only the monomeric form of PutA protein seems to be labeled by $^{32}$P]ATP (data not shown).

A model that accounts for these results is shown in Figure 6. The proline-dependent increase in hydrophobicity would direct PutA protein toward two alternative routes: dimerization or association with the membrane. When no membrane sites are available, PutA protein would dimerize in the cytoplasm, allowing intermolecular dephosphorylation, and thus resulting in an increased DNA affinity and repression of the put operon. When membrane sites are available, PutA protein would associate with the membrane allowing the functional interaction with the electron transport chain required for enzyme activity and preventing dephosphorylation, thus resulting in a lower DNA affinity and derepression of the put operon. Hence, phosphorylation would allow PutA protein to rapidly respond to the presence or absence of both membrane sites and proline.

This model can account for the modulation of PutA expression under a spectrum of physiological conditions. When proline and membrane sites are available, PutA protein would remain phosphorylated so PutA-DNA binding would be weak, resulting in the maximal expression of the put genes. In the absence of one of these components, PutA protein would have intermediate DNA binding affinity resulting in intermediate levels of put gene expression. Finally, when neither proline or membrane sites are available, PutA protein would be fully dephosphorylated so PutA would have the highest DNA binding affinity, resulting in minimal levels of put gene expression. These changes in PutA protein, caused by proline, phosphorylation, and the interaction of PutA protein with the membrane, would result in a gradient of DNA binding affinities that would optimize the level of put gene expression depending on the physiological conditions. Because the availability of membrane sites may depend on the intracellular concentration of other dehydrogenases or other aspects of membrane physiology, the phosphorylation state of PutA protein could provide a means for the cell to monitor the availability of functional membrane sites and to modulate put gene expression in response to the availability of these sites.

This model accounts for most of the available data, but many critical questions about the phosphorylation of PutA protein still need to be answered. We do not yet know which particular serine, threonine, and tyrosine residues in PutA protein are phosphorylated. The analogous eukaryotic proteins are often phosphorylated on multiple serine, threonine, and tyrosine residues. Systematic site-directed mutagenesis to change each of these amino acids in the phosphorylated protein is a common approach to elucidate which residues are phosphorylated and the physiological relevance of their phosphorylation. However, this approach is not feasible for PutA protein because it is quite large (144.2 kD) and contains 61 serine residues, 67 threonine residues, and 35 tyrosine residues.

In summary, phosphorylation seems to modulate the
switch that determines whether PutA protein from *S. typhimurium* functions as a membrane-associated enzyme or as a DNA-binding protein. Phosphorylation of PutA protein on serine, threonine, and tyrosine residues changes the DNA binding affinity of PutA protein, and consequently fine tunes the expression of the *put* genes in response to changes in inducer concentration and availability of membrane sites in the cell. The pattern of phosphorylation of this bacterial protein is similar to certain eukaryotic protein kinases. These results hint that protein tyrosine kinases may play important regulatory roles in prokaryotes as they do in eukaryotes.

**Materials and methods**

**Strains and growth conditions**

The following strains were used: EM41, *E. coli* K-12 * thy thi Δ[putPA101], MST2830, *S. typhimurium* LT2 * leu-414(Am)* putA826::Tn10/pPC34 pCKR101 lacI* P* tac-putA*, MST2489 *S. typhimurium* LT2 * leu-414(Am)* putA826::Tn10/pPC34 pCKR101 lacI*, MST58 *S. typhimurium* LT2 Δ[putPA523].

**Purification of PutA protein**

PutA protein was purified from strain MST2830 [pCKR101 P* tac-putA*] following induction with 0.1 mm isopropyl-β-D-thiogalactoside (IPTG) as described previously [Menzel and Roth 1981b, Ostrovsky de Spicer and Maloy 1991]. The final purified samples contained between 1.6 and 4 mg of PutA protein per milliliter. Purified samples were stored frozen at -70°C until use. Electrophoresis in SDS–polyacrylamide gels was carried out as described with discontinuous gels composed of a 4% stacking gel and an 8% separating gel. Proteins in SDs or native gels were stained with FastStain [ZoionResearch, Allston, MA].

**Phosphorylation with [γ-32P] ATP**

In vitro phosphorylation of PutA protein was carried out as follows: 2.5 μg of purified PutA protein was incubated with 2.5 mM [γ-32P]ATP in 1× buffer A (12 mM Tris, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM NaCl, 0.1 mM dithiothreitol, 100 μg/ml bovine serum albumin, and 5% glycerol) at pH 8.0 at 21°C for 5 min. For the experiments shown in Figure 1, PutA protein was phosphorylated as described above, incubated at room temperature for 5 min under the indicated conditions, then the samples were analyzed by polyacrylamide gel electrophoresis and autoradiography.

Membrane preparations from *S. typhimurium* have a high background of nuclease activities, which are absent from membrane preparations from *E. coli* [A.M. Muro-Pastor and S. Maloy, unpubl.]. Therefore, the membranes used were purified from strain EM41 [E. coli Δput] grown in minimal medium with sucrose and proline [Muro-Pastor and Maloy 1993].

**Phosphoamino acid analysis**

Purified, labeled PutA protein (100 μg) that was untreated, treated with 5 units of calf thymus alkaline phosphatase, or treated with 100 mM proline, precipitated in 20% TCA with 10 mg of bovine serum albumin by incubation on ice for 1 hr followed by centrifugation for 20 min, and air-dried. The dried pellet was resuspended in boiling 6% HCl then incubated at 110°C for 2 hr. The hydrolysate was resuspended in pH 1.9 buffer containing 1 mg/ml free phosphothreonine, phosphoserine, and phosphotyrosine (Sigma) then analyzed on a thin-layer cellulose plate by electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension [Boyle et al. 1991]. The position of the phosphoamino acids was determined by ninhydrin staining followed by autoradiography. The amount of label in each phosphoamino acid was quantitated with a PhosphorImager.

**31P-NMR analysis**

The sample prepared for 31P-NMR analysis contained 0.2 mM PutA protein, 2 mM ATP, 30% D₂O in 1× buffer A (see above). The acquisition parameters were set as follows: 39 ° pulse (15 msec), spectral width ± 3048.78 Hz, data size 32,768, DW 164 msec, pulse width 15 msec, delay time 500 msec, 10 Hz line broadening, 5000 scans for Figure 3A and 3388 scans for Figure 3B.

**Enzyme-linked immunoassays**

Enzyme-linked immunoassays were done essentially as described by Harlow and Lane (1988). Crude extracts of MST2830 [ΔputA[pCKR101 P* tac-putA*]] induced with 0.1 mM IPTG and grown in minimal medium with or without proline, and MST58 [ΔputA], or samples of purified PutA protein were used for the immunodetection assays shown in Figure 4. Positive controls contained free phosphoserine, phosphothreonine, or phosphotyrosine, and negative controls contained bovine serum albumin. Samples with crude cell extracts contained 500 μg of total protein per assay. Samples with purified PutA protein contained 10 μg of protein per assay. Samples with free phosphoserine, phosphothreonine, or phosphotyrosine (Sigma) contained 1 μg of each phosphoamino acid per assay. Control samples with bovine serum albumin (Sigma) contained 20 μg of protein per assay. Each assay was done with 5 μg/ml anti-phosphotyrosine polyclonal antibody [UBI, Lake Placid, NY].

**Western blot immunodetection**

Crude extracts of strain MST2830 [pCKR101 P* tac-putA*] induced with 0.1 mM IPTG or uninduced, strain MST2489 [pCKR101] treated in a similar manner, or purified PutA protein were separated by SDS–polyacrylamide gel electrophoresis. The proteins were then electrophoretically transferred to nitrocellulose membranes. The transfer was followed by two washes with phosphate-buffered saline [PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, at pH 7.3] plus 0.05% Tween 20. The washed membranes were blocked by soaking in PBS plus 5% nonfat dry milk for 1 hr, washed 3 times with PBS plus 0.05% Tween 20, then incubated for 3 hr in PBS plus 5% nonfat dry milk with anti-phosphoserine [clone PSR-49], anti-phosphothreonine [clone FT-66], or anti-phosphotyrosine [clone PTR-8] monoclonal antibodies (Sigma) at the dilution suggested by the manufacturer. After incubation with the primary antibody, the membranes were washed 4 times with PBS plus 0.05% Tween 20, and incubated in PBS plus 5% nonfat dry milk with 0.2 μg/ml of horseradish peroxidase [HRP]-conjugated goat anti-mouse IgG (GIBCO; BRL) for 1 hr. After incubation with the secondary antibody, the membranes were washed 4 times with PBS plus 0.05% Tween 20. The membranes were then incubated in the chemiluminescent ECL reagent mix [Amersham] for 1 min, covered with plastic, and exposed to X-ray film for 1 min.

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Gel mobility retardation assays

PutA–DNA binding was quantitated by use of a gel mobility retardation assay with 32P-labeled DNA from the put control region. The DNA binding reactions were carried out in 1 × buffer A (see above) as previously described [Ostrovsky de Spicer et al. 1991]. The free DNA was quantitated using a Phosphorlmager.

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