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EnvZ, a Transmembrane Environmental Sensor of *Escherichia coli* K-12, Is Phosphorylated In Vitro

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By fusing the transcriptional and translational start signals of *lacZ* to *envZ*, we have obtained high-level synthesis of a truncated EnvZ protein (EnvZ115) in which the first 38 amino acids of EnvZ are replaced with the first 8 amino acids of LacZ. Using this construct, we have partially purified the EnvZ115 protein and demonstrated that this protein can be phosphorylated in vitro. We suggest that phosphorylation may be an important feature of EnvZ function.

Synthesis of the *Escherichia coli* K-12 major outer membrane porin proteins, OmpF and OmpC, is controlled by the products of the regulatory genes *ompR* and *envZ* in response to a variety of environmental conditions including changes in medium osmolarity (4, 19). EnvZ is an integral cytoplasmic membrane protein (3) that is thought to act as an environmental sensor. Its function is to signal OmpR, a DNA-binding protein (6), which then activates transcription of the appropriate porin gene (16). An issue that is central to understanding the regulation of the porin genes is the mechanism by which EnvZ signals OmpR.

DNA sequence analysis of *ompR* and *envZ* has revealed similarities between OmpR and EnvZ and numerous two-component sensory processing systems in bacteria (14, 18). Recent studies on the analogous regulatory pairs involved in response to nitrogen limitation (NtrB-NtrC) (12) and in chemotaxis (CheA-CheY) (5) suggest that protein phosphorylation is involved in signal transduction. To determine whether the EnvZ-OmpR regulatory pair also uses this mechanism, we tested whether EnvZ could be phosphorylated in vitro.

The examination of EnvZ phosphorylation in vitro is complicated by the fact that although *ompR* and *envZ* are transcribed as a single mRNA, the efficiency of *envZ* expression is much lower than that of *ompR*, suggesting that there is a strong translational barrier between these two genes (11). Accordingly, EnvZ is present at less than 10 copies per cell, making it difficult to obtain sufficient quantities of EnvZ for biochemical analysis. Previous results (10; S. Garrett, Ph.D. dissertation, The Johns Hopkins University, Baltimore, Md., 1986) have shown that the removal of this translational barrier can result in high-level synthesis of a truncated protein that exhibits partial EnvZ activity. We reasoned that cells containing such a construct might provide an enriched source of EnvZ.

Plasmid pSG115 (Garrett, Ph.D. dissertation) carries a large 3′ fragment of *envZ* fused to the transcriptional and translational start signals of *lacZ*. In the presence of the inducer isopropyl-thio-β-D-galactoside (IPTG), this plasmid directs high-level synthesis of a truncated EnvZ protein (EnvZ115) in which the first 38 amino acids in EnvZ are replaced with the first 8 amino acids of LacZ. Complementation analysis of an *envZ* null mutation by *envZ115* is shown in Fig. 1. In this analysis, envelope fractions were prepared from a wild-type strain (MC4100) (1) and an *envZ* insertion mutant (MC4100 *envZ60*::Tn*10*) (16) containing the plasmid pSG115 and from the same strains containing the plasmid pBR322. These samples were then compared on a urea–sodium dodecyl sulfate–11% polyacrylamide gel (9). Examination of this gel indicates that the 47,000-dalton EnvZ115 protein from this plasmid results in a significant increase in the amounts of OmpF and OmpC found in the outer membrane over the amounts seen in the *envZ* insertion mutant. A similar pattern of expression was observed when the strains were grown in the presence of 1 mM IPTG (data not shown). This result suggests that there is sufficient production of EnvZ115 in the absence of IPTG to complement the *envZ* insertion mutation.

We note that EnvZ115 does not restore normal fluctuation of OmpF and OmpC in response to changes in medium osmolarity. EnvZ (448 amino acids) is thought to be embedded in the membrane by two membrane-spanning segments, amino acids 16 to 46 and 163 to 179 (3). We suspect that the *envZ115* mutation, which removes part of the first membrane-spanning segment, may prevent correct assembly of EnvZ sequences in the membrane. This might interfere with the ability of the protein to sense osmotic changes. Nevertheless, *envZ115* complements the *envZ* null mutation, and thus EnvZ115 must retain the ability to communicate with OmpR.

The protocol used to partially purify EnvZ115 from the overproducing strain was as follows. Strain MC4100 *envZ60*::Tn*10* containing plasmid pSG115 was grown in 1 liter of LB medium (15) to the mid-exponential phase of growth and then split into two equal portions. To one of the cultures, 1 mM IPTG was added to induce synthesis of EnvZ115; the other culture remained untreated and served as a control for the subsequent experiments. After 1 h, the cells were collected by centrifugation, and the pellets were stored overnight at −70°C. The cells were thawed, suspended in 2.5 ml of phosphate-buffered saline (2), passed through a French press three times (15,000 lb/in^2), and gently sonicated for 20 s. Unbroken cells were removed by centrifugation at 3,000 rpm (Sorvall rotor SS-34) for 10 min; the crude extracts (Fig. 2A, lanes 1 and 2) were then spun at 10,000 rpm for 10 min (Sorvall rotor SS-34). Examination of the pellet from the induced culture revealed that it was enriched for EnvZ115. (The reason for the partitioning of this protein to the pellet is unclear, but may be the result of aggregation.) The pellets (Fig. 2A, lanes 3 and 4) were washed extensively with phosphate-buffered saline to re-

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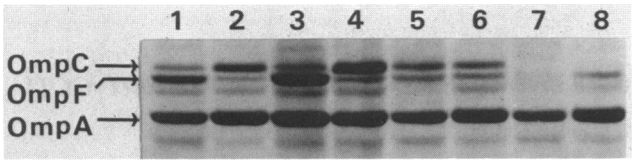

FIG. 1. Complementation analysis of an *envZ* insertion mutation by the plasmid pSG115. Cellular envelopes were prepared from the plasmid-containing strains grown in the absence of IPTG in either A medium (7) (lanes 1, 3, 5, and 7) or A medium supplemented with 15% sucrose (lanes 2, 4, 6, and 8). The samples were analyzed on an 11% SDS-polyacrylamide gel containing 4 M urea. The positions of the outer membrane proteins OmpC, OmpF, and OmpA are indicated on the left. Lanes: 1 and 2, MC4100 (pBR322); 3 and 4, MC4100 (pSG115); 5 and 6, MC4100 *envZ60*::Tn*10* (pSG115); 7 and 8, MC4100 *envZ60*::Tn*10* (pBR322).

move contaminating nucleotides and were then suspended in 0.5 ml of phosphate-buffered saline and stored at −70°C.

To test for phosphorylation of the partially purified EnvZ115 protein in vitro, samples of the crude extract and the pellet fraction from both the uninduced and the induced cells were incubated with 5 mM $MgCl_2$ and 0.02 μM [γ-^{32}P]ATP (4,500 Ci/mmol) for 5 min at room temperature. The reactions were terminated by addition of sodium dodecyl sulfate sample buffer (8), heated to 55°C, and analyzed on a 10% polyacrylamide gel. After electrophoresis, the gel was dried under vacuum and subjected to autoradiography. As can be seen in Fig. 2B (lane 4), a band that appears to correspond to EnvZ115 is labeled.

To confirm that the phosphorylated protein observed was indeed EnvZ115, immunoprecipitation studies with anti-EnvZ antiserum were performed. For these experiments, the phosphorylation reaction was terminated by adding an equal volume of 2% sodium dodecyl sulfate–2 mM EDTA and then heating to 55°C for 5 min. The sample was then spun for 10 min in a Microfuge, and the soluble fraction was used for immunoprecipitation (17). The immunoprecipitates were analyzed by gel electrophoresis followed by autoradiography (Fig. 3A). To demonstrate the specificity of the rabbit anti-EnvZ antiserum, we show that an EnvZ-LacZ hybrid protein is also recognized (Fig. 3B, lane 4). Because of the

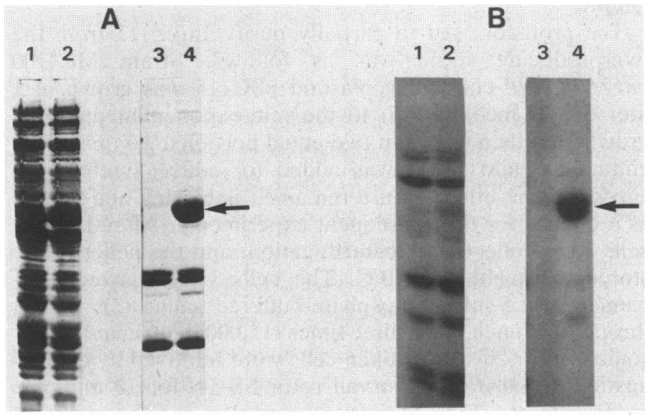

FIG. 2. Phosphorylation of EnvZ115. Crude cell extracts from the uninduced (lanes 1) and induced (lanes 2) cultures and pellets from the uninduced (lanes 3) and induced (lanes 4) cultures are shown. (A) Coomassie blue stain of the samples analyzed on a 10% SDS-polyacrylamide gel. (B) Autoradiogram of the same samples incubated in the presence of [γ-^{32}P]ATP as described in text. Arrows indicate the position of the 47,000-dalton EnvZ115 protein.

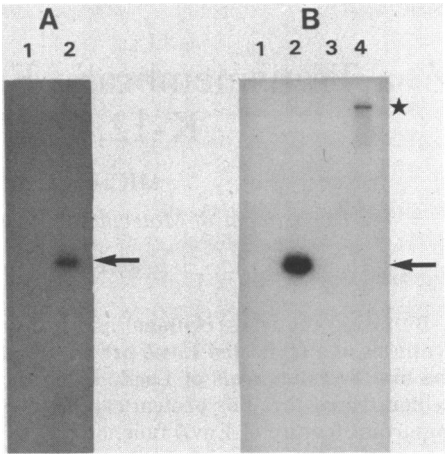

FIG. 3. Autoradiogram of EnvZ115 after immunoprecipitation. (A) EnvZ115 was incubated in the presence of [γ-^{32}P]ATP and then immunoprecipitated with preimmune serum (lane 1) or rabbit anti-EnvZ antiserum (lane 2). (B) MC4100 *envZ60*::Tn*10* containing the plasmid pSG115 (lanes 1 and 2) or containing a derivative of this plasmid (pSG115Δ10; Garrett, Ph.D. dissertation) in which a functional LacZ was fused to the carboxy terminus of EnvZ (lanes 3 and 4) was grown in minimal media (15) containing 1 mM IPTG, labeled for 10 min with [^{35}S]methionine, and subsequently treated as described by Stader et al. (17). The samples were then immunoprecipitated with preimmune serum (lanes 1 and 3) or rabbit anti-EnvZ antiserum (lanes 2 and 4). Arrows indicate the position of EnvZ115. Symbol: ★, position of the 145,000-dalton EnvZ-LacZ hybrid protein.

difficulties in solubilizing EnvZ115 without removing the phosphate, most of the phosphorylated protein remained in the pellet fraction. Therefore, recovery of labeled protein in the immunoprecipitation was low. Nevertheless, we conclude that the labeled protein is indeed EnvZ.

A series of experiments was performed to probe the nature of the EnvZ-labeling reaction and the properties of the labeled protein (Fig. 4). These data demonstrate that EnvZ modification specifically requires the γ-phosphate of ATP (Fig. 4, lanes 1 to 3) and that the labeled protein is sensitive to trichloroacetic acid (Fig. 4, lanes 7 to 9) but is stable in alkali (Fig. 4, lane 4) at room temperature. These results are

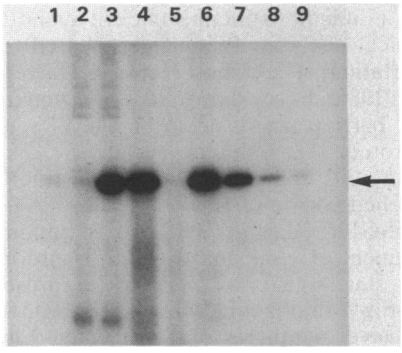

FIG. 4. Nucleotide specificity of the phosphorylation reaction and properties of labeled EnvZ115. EnvZ115 was incubated in the presence of [α-^{32}P]ATP (lane 1), [γ-^{32}P]GTP (lane 2), or [γ-^{32}P]ATP (lane 3) or was incubated in the presence of [γ-^{32}P]ATP and then treated for 20 min as follows: with 0.5 M NaOH at 23°C (lane 4) or 55°C (lane 5); with 7.5% CH_3COOH at 23°C (lane 6); and with 5% CCl_3COOH at 0°C (lane 7), 23°C (lane 8), or 90°C (lane 9). The arrow indicates the position of EnvZ115.

qualitatively similar to those obtained with phosphorylated CheA (5) and NtrB (12; V. Weiss and B. Magasanik, Proc. Natl. Acad. Sci. USA, in press).

In conclusion, we have shown that EnvZ115 can be phosphorylated in vitro in the presence of [γ-^{32}P]ATP. Based on the similarities between EnvZ, CheA, and NtrB, we suggest that EnvZ is a protein kinase that catalyzes phosphorylation of itself and that phosphorylated EnvZ may serve as a phosphate donor to the transcriptional activator, OmpR. Although demonstration of the physiological relevance of the phosphorylation of EnvZ remains to be determined, these results provide an important clue to the mechanism of signal transduction in the porin regulon and greatly strengthen the view that the two-component regulatory systems share a common mechanism (13).

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LITERATURE CITED

1. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104**:541–555.
2. **Dulbecco, R., and M. Vogt.** 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. **99**:167–182.
3. **Forst, S., D. E. Comeau, S. Norioka, and M. Inouye.** 1987. Localization and membrane topology of EnvZ, a protein involved in osmoregulation of OmpF and OmpC in *Escherichia coli*. J. Biol. Chem. **262**:16433–16438.
4. **Hall, M. N., and T. J. Silhavy.** 1981. Genetic analysis of the *ompB* locus in *Escherichia coli* K-12. J. Mol. Biol. **151**:1–15.
5. **Hess, J. F., K. Oosawa, P. Matsumura, and M. I. Simon.** 1987. Protein phosphorylation is involved in bacterial chemotaxis. Proc. Natl. Acad. Sci. USA **84**:7609–7613.
6. **Jo, Y. L., F. Nara, S. Ichihara, T. Mizuno, and S. Mizushima.** 1986. Purification and characterization of the OmpR protein, a positive regulator involved in osmoregulatory expression of the *ompF* and *ompC* genes in *Escherichia coli*. J. Biol. Chem. **261**: 15252–15256.
7. **Kawaji, H., T. Mizuno, and S. Mizushima.** 1979. Influence of molecular size and osmolarity of sugars and dextrans on the synthesis of outer membrane proteins O-8 and O-9 of *Escherichia coli* K-12. J. Bacteriol. **140**:843–847.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
9. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* K-12 into four bands. FEBS Lett. **58**:254–258.
10. **Mizuno, T., and S. Mizushima.** 1987. Isolation and characterization of deletion mutants of *ompR* and *envZ*, regulatory genes for expression of the outer membrane proteins OmpC and OmpF in *Escherichia coli*. J. Biochem. **101**:387–396.
11. **Mizuno, T., E. T. Wurtzel, and M. Inouye.** 1982. Osmoregulation of gene expression. II. DNA sequence of the *envZ* gene of the *ompB* operon of *Escherichia coli* and characterization of its gene product. J. Biol. Chem. **257**:13692–13698.
12. **Ninfa, A. J., and B. Magasanik.** 1986. Covalent modification of the *glnG* product, NR*I*, by the *glnL* product, NR*II*, regulates the transcription of the *glnALG* operon in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83**:5909–5913.
13. **Ninfa, A. J., E. G. Ninfa, A. N. Lupas, A. Stock, B. Magasanik, and J. Stock.** 1988. Crosstalk between bacterial chemotaxis signal transduction proteins and regulators of transcription of the Ntr regulon: evidence that nitrogen assimilation and chemotaxis are controlled by a common phosphotransfer mechanism. Proc. Natl. Acad. Sci. USA **85**:5492–5496.
14. **Ronson, C. W., B. T. Nixon, and F. M. Ausubel.** 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell **49**:579–581.
15. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions, p. 217–222. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Slauch, J. M., S. Garrett, D. E. Jackson, and T. J. Silhavy.** 1988. EnvZ functions through OmpR to control porin gene expression in *Escherichia coli* K-12. J. Bacteriol. **170**:439–441.
17. **Stader, J., S. A. Benson, and T. J. Silhavy.** 1986. Kinetic analysis of *lamB* mutants suggests the signal sequence plays multiple roles in protein export. J. Biol. Chem. **261**:15075–15080.
18. **Stock, A., D. E. Koshland, Jr., and J. B. Stock.** 1985. Homologies between the *Salmonella typhimurium* CheY protein and proteins involved in the regulation of chemotaxis, membrane protein synthesis, and sporulation. Proc. Natl. Acad. Sci. USA **82**:7989–7993.
19. **van Alphen, W., and B. Lugtenberg.** 1977. Influence of osmolarity of the growth medium on the outer membrane protein pattern of *Escherichia coli*. J. Bacteriol. **131**:623–630.