Transfer of Phosphoryl Group between Two Regulatory Proteins Involved in Osmoregulatory Expression of the ompF and ompC Genes in *Escherichia coli*

(Received for publication, November 14, 1988)

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EnvZ is a cytoplasmic membrane protein which is involved in osmoregulatory expression of the *ompF* and *ompC* genes in *Escherichia coli* possibly by sensing the environmental osmotic signal. A truncated form of the EnvZ protein (EnvZ*), comprising 82% of EnvZ starting from the C terminus, was purified to homogeneity. The purified EnvZ* was autophosphorylated with ATP. The phosphoryl group on EnvZ* could then be rapidly transferred to OmpR, which is a positive regulator of the *ompF* and *ompC* genes and which was proposed to interact with EnvZ in the process of osmoregulation. In the presence of ATP, the phosphorylated OmpR was rapidly dephosphorylated. These results suggest that the transfer of the phosphoryl group between EnvZ and OmpR plays an important role in the signaling pathway in osmoregulation.

The outer membrane of *Escherichia coli* K-12 contains two major proteins, OmpF and OmpC, which serve for the passive diffusion of small hydrophilic molecules across the membrane. Expression of the genes coding for the two proteins, *ompF* and *ompC*, is affected in a reciprocal manner by the medium osmolarity. As the osmolarity increases the *ompC* gene is preferentially activated, whereas as the osmolarity decreases the *ompF* gene is preferentially activated (1, 2). This osmoregulation is controlled at the transcriptional level by the common regulator of the osmotic signal. A truncated form of EnvZ is phosphorylated in vitro.

Here we present experimental evidence that the EnvZ-OmpR system involves a phosphotransfer cascade mechanism. We demonstrated that the phosphoryl group was transferred from ATP to EnvZ and then to OmpR, and finally released as inorganic phosphate in the presence of ATP.

**EXPERIMENTAL PROCEDURES**

**Materials**

All restriction endonucleases, S1 nuclease, and T4 DNA polymerase were purchased from Takara Shuzo Co., Ltd. [γ-32P]ATP (30 Ci/mmol) and [α-32P]ATP (410 Ci/mmol) were obtained from Amersham International. Sephacryl S-200 (superfine) and Sephadex G-75 (superfine) were from Pharmacia LKB Biotechnology Inc. All other reagents were of reagent grade.

**Construction of Plasmids**

Plasmid pHAI-085 carrying a truncated *envZ* gene under the control of the tac promoter-operator, and a synthetic ribosome-binding site was constructed from plasmids pAT224 and pUS12. Plasmid pAT224 carries the intact *envZ* gene (31). Plasmid pUS12 is an expression vector harboring the tac promoter-operator and synthetic ribosome-binding site followed by an ATG initiation codon, under and with which any coding sequences can be connected (32). Plasmid pAT224 was digested with BstII and then the cleaved sites were blunt-ended by means of S1 treatment, followed by Sall digestion to isolate a 2.3-kilobase BstII-SalI fragment. This fragment encompasses a truncated *envZ* coding sequence corresponding to the amino acid sequence of EnvZ from Tyr-81 to C-terminal Gly-450. Plasmid pUS12 was digested with KpnI and then the cleaved sites

large C-terminal portion was suggested to be exposed to the cytoplasm. The results of genetic studies suggested that OmpR and EnvZ functionally interact with each other (20, 21). While EnvZ has been assumed to sense an environmental osmotic signal and then modulate the OmpR function (3), little is known about the biochemical events that take place in the transmembrane signaling.

As reported by Ronson et al. (22), a number of systems have been identified in prokaryotes which can be classified as two-component regulatory systems. They each comprise a “modulator” protein that detects environmental stimuli and a “regulator” protein that controls expression of particular genes (23). The EnvZ-OmpR system seems to be a paradigm of such systems. EnvZ and OmpR share highly homologous amino acid sequences with other modulator and regulator proteins, respectively, that belong to this family. Based on the results of extensive biochemical studies on the Ntr regulon (24, 25) and the chemotaxis system (26–28), it was suggested that a common cascade mechanism, i.e., the transfer of a phosphoryl group (phosphotransfer) between the two components, is involved in these regulatory systems (22, 23, 29). In fact, recently Igo and Silhavy (30) demonstrated that a truncated form of EnvZ is phosphorylated in *Escherichia coli*.
were blunted-ended with T4 DNA polymerase, followed by Sall digestion to isolate a large KpnI-Sall fragment. These BgII-Sall and KpnI-Sall fragments were ligated together to construct plasmid pHAI-085. The resultant plasmid was expected to produce a truncated form of EnvZ (see Fig. 1).

**Purification of the Truncated EnvZ Protein**

The truncated EnvZ protein (EnvZ*) was purified to homogeneity as described below. Throughout the purification, EnvZ* was identified on SDS-polyacrylamide gels.

Cultivation—E. coli AT142 (Δ enuZ) (8) transformed with plasmid pHAI-085 was grown in L-broth supplemented with 50 µg/ml ampicillin on a rotary shaker. To induce the synthesis of EnvZ*, IPTG (final concentration, 2 mM) was added to the logarithmic growth phase. After incubation for 6 h, the cells were harvested and then washed with a buffer containing 50 mM Tris-HCl (pH 7.8), 250 mM sucrose, and 1 mM dithiothreitol. About 14 g of cells was obtained from 5 liters of the culture.

**Purification of EnvZ**—To 14 g of cells was added 80 ml of the Tris-HCl buffer containing 2 mg of deoxyribonuclease I. The cell suspension was passed through a French pressure cell three times at 11,000 p.s.i. to obtain a total lysate. The lysate was then directly applied onto an stepwise sucrose gradient (30/40/55%, w/v). After centrifugation (10,000 g × 4 h), the precipitate was collected and dissolved in 140 ml of a buffer containing 50 mM Tris-HCl (pH 7.8), 6 mM guanidine hydrochloride, and 0.005% Tween 80. The solution was then diluted to 1,400 ml with adjustment of the buffer composition to 1 mM guanidine hydrochloride, 50 mM Tris-HCl (pH 7.8), 0.005% Tween 80, 2 mM reduced form of glutathione, and 0.02 mM oxidized form of glutathione. After the solution was incubated for 24 h at 4°C, the solution was dialyzed against a buffer containing 50 mM Tris-HCl (pH 7.8), 0.005% Tween 80, and 2 mM 2-mercaptoethanol. The dialyzed sample was applied to a DEAE-cellulose DE52 column (2 × 15 cm) previously equilibrated with the dialysis buffer and washed with the buffer containing 100 mM NaCl. Proteins were then eluted with 500 ml of a linear NaCl gradient, from 100 to 400 mM, at a flow rate of 30 ml/h. The EnvZ*-containing fractions eluted at around 220 mM NaCl, were pooled (150 ml). The pooled fractions were concentrated to a volume of 10 ml and then applied onto a Sephacryl S-200 column (3 × 95 cm) previously equilibrated with the same buffer as that used for the DE52 column chromatography. Elution was performed with the same buffer at a flow rate of 15 ml/h. The EnvZ*-containing fractions which were essentially free from other proteins were combined and used as the purified EnvZ* (see Fig. 2).

**Purification of the OprR Protein**

The OprR protein was purified as described by Jo et al. (10).

**Polyacrylamide Gel Electrophoresis**

An SDS-polyacrylamide gel (12.5% acrylamide) was used for most experiments (33).

**Amino Acid Sequencing**

Amino acid sequencing was performed with an automated amino acid sequencer (Applied Biosystems model 477A).

**Protein Labeling Experiments**

Protein labeling experiments were carried out as described by Hess et al. (26, 27), with slight modifications. In general, proteins labeled were analyzed by SDS-polyacrylamide gel electrophoresis as follows. Proteins were incubated in 15 µl of TEDG buffer (50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 2 mM dithiothreitol, 10% glycerol (v/v) containing 0.1 mM [γ-32P]ATP (10,000 cpm/pmol), 5 mM MgCl2, and 50 mM KCl). After incubation for 20 min at room temperature, the reaction was stopped by the addition of 4 µl of an SDS buffer (250 mM Tris-HCl (pH 6.8), 4% SDS, 6% 2-mercaptoethanol, 60% glycerol, 0.02% bromphenol blue). The sample was subjected to SDS-polyacrylamide gel electrophoresis, followed by autoradiography with Fuji x-ray film (RX-50). For quantitative studies, proteins were labeled as described under "Experimental Procedures" (lanes 3–6). The protein was purified to near homogeneity as a soluble form in a milligram quantity above. The reaction mixture was spotted onto a Whatman GF/A glass fiber filter. The filter was then immediately put into ice-cold 10% CCl4:COOH containing 1% sodium pyrophosphate. After 30 min in the solution, the filter was washed three times for 30 min each in ice-cold 5% CCl4:COOH containing 1% sodium pyrophosphate. The filter was then washed briefly in ethanol and subjected to radioactivity assaying in Bray's scintillator.

**Purification of Phosphorylated EnvZ**

Purified EnvZ* (130 µg) was incubated with 0.1 mM [γ-32P]ATP (5,000 cpm/pmol), 5 mM MgCl2, and 50 mM KCl in TEDG buffer for 20 min at 37°C. The reaction mixture was immediately applied onto a Sephadox G-75 column previously equilibrated with TEDG buffer. The fractions containing the radiolabeled EnvZ*, which were essentially free from ATP, were collected.

**RESULTS**

**Purification of the Truncated EnvZ**—Since EnvZ is a cytoplasmic membrane protein that contains two long stretches of hydrophobic amino acid residues (Fig. 1), it is difficult to isolate it as a soluble form without using detergents. Therefore, we attempted to purify a truncated form of EnvZ, which lacks the N-terminal 80 amino acid residues including the N-terminal hydrophobic stretch. This was achieved by constructing a recombinant plasmid (pHAI-085) which carries a truncated enuZ-coding sequence connected downstream to the tac promoter-operator and a synthetic ribosome-binding site as shown schematically in Fig. 1. This plasmid was expected to produce a truncated EnvZ, corresponding to the portion of the intact EnvZ extending from Tyr-81 to C-terminal Gly-450 (Fig. 1). It should be emphasized that when this plasmid was transferred into an envZ mutant strain (MH1461; envZ11) (34), the characteristic phenotype of the EnvZ* mutant (OmpF* OmpC*) reverted to nearly the wild-type (OmpF* OmpC*) (data not shown).

A total cell lysate was prepared from strain AT142 (Δ enuZ) carrying plasmid pHAI-085 and then analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). A thick protein band appeared on the gel upon induction of the cloned gene for the truncated envZ* gene with IPTG (lanes 1–3). The apparent molecular weight of the protein was essentially the same as that calculated from the amino acid sequence deduced from the nucleotide sequence of this gene. The protein was fractionated from the total cell lysate as described under "Experimental Procedures" (lanes 3–6). The protein was purified to near homogeneity as a soluble form in a milligram quantity

**Fig. 1. Schematic representation of the structures of EnvZ* and the recombinant gene coding for EnvZ* a, the structure of the intact EnvZ is schematically represented by an open rectangle. The filled regions represent long stretches of hydrophobic amino acids in EnvZ, b, the structure of EnvZ* purified in this study is schematically presented. Relevant amino acid residues with their position numbers are indicated. c, the structure of the recombinant gene (envZ*), which codes for EnvZ*, is schematically presented. The hatched rectangle represents the tac promoter-operator (tac) followed by a synthetic ribosome-binding site (RB). The open rectangle represents the coding sequence for EnvZ*. This gene was constructed on a high copy number plasmid (pUS12) and the resultant plasmid was designated as pHAI-085."
The question then arose as to whether the observed modification of EnvZ* by ATP was due to phosphorylation or adenylylation. When we used [α-32P]ATP in place of the γ-labeled nucleotide, there was no significant radiolabeling of EnvZ* (Fig. 4). When an aliquot of the reaction mixture with [α-32P]ATP was analyzed by thin-layer chromatography, 32P-labeled ADP was found to be released (data not shown). From these results, we conclude that EnvZ* is phosphorylated in the presence of ATP.

The phosphorylation reaction took place only with the purified EnvZ* and ATP, suggesting the autophosphorylation nature of this protein. To confirm this, the initial rate of phosphorylation of EnvZ* was determined over a 10-fold EnvZ* concentration range in the presence of 0.1 mM [γ-32P]ATP, which was in a large molar excess with respect to EnvZ*. The initial rate of the phosphorylation appeared to be independent of the EnvZ* concentration, suggesting that EnvZ* can be autophosphorylated (Fig. 5).

Coupling of OmpR Phosphorylation with EnvZ Dephospho-
**Dependence of EnvZ* phosphorylation on the EnvZ* concentration.** Various amounts of EnvZ* (0.8–8 μM) were incubated under the same conditions as in the legend to Fig. 4. Aliquots of the reaction mixtures were removed at 15, 30, 45, and 60 s and the amount of trichloroacetic acid-precipitable phosphate at each time point was determined. The initial rates were calculated on the basis of the slopes of the time courses and expressed in picomoles/min/mg EnvZ* and plotted against the concentration of EnvZ*.

**Autoradiograms showing the time course of dephosphorylation of EnvZ* mediated by a substoichiometric amount of OmpR.** Purified OmpR (0.12 μM) was incubated with purified phosphorylated EnvZ* (1.2 μM), the amount of which was in a 10-fold molar excess with respect to OmpR, in a reaction mixture containing 5 mM MgCl₂ and 50 mM KCl at room temperature. Autoradiograms showing the time courses of OmpR-mediated dephosphorylation of EnvZ* in the absence (A) and the presence (B) of ATP are shown. Cold ATP was added at the concentration of 0.1 mM at the times indicated. Other details were the same as those in the legend to Fig. 6.

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**Effect of ATP on the dephosphorylation of EnvZ*.** After incubating the purified phosphorylated EnvZ* with OmpR for 1.5 min under exactly the same conditions as in Fig. 6A, 0.1 mM nonlabeled ATP was added to the reaction mixture. The addition of ATP resulted in dramatic acceleration of the OmpR dephosphorylation (t₁/₂ < 30 s). Interestingly, AMP-PNP, a nonhydrolyzable ATP analogue, could replace ATP as to enhancement of the
OmpR dephosphorylation, suggesting that ATP acts as an allosteric effector for the OmpR dephosphorylation reaction (data not shown).

To further characterize the mode of action of OmpR, a similar experiment as in Fig. 6 was carried out in a reaction mixture containing the phosphorylated EnvZ* in a 10-fold molar excess over OmpR. As shown in Fig. 7, all of the radioactivity present in the phosphorylated EnvZ* was eventually released by the stoichiometric amount of OmpR upon the addition of ATP. These results support the view that the phosphorylated EnvZ* is dephosphorylated through the rapid transfer of its phosphoryl group to OmpR and the phosphoryl transfers of EnvZ described in this paper demonstrated that EnvZ is presumably a protein kinase of EnvZ* by cycling between its phosphorylated and non-phosphorylated forms.

**DISCUSSION**

We have been able to demonstrate biochemical interactions between the products of the two genes that are known to be involved in the osmoregulatory expression of the ompF and ompC genes coding for the respective outer membrane proteins. The results of biochemical analyses of a truncated form of EnvZ described in this paper demonstrated that EnvZ is autophosphorylated, its phosphoryl group being rapidly transferred to OmpR. Thus, EnvZ is presumably a protein kinase and OmpR is a specific substrate for the kinase. While the phosphorylated OmpR was dephosphorylated slowly in the absence of ATP, rapid dephosphorylation was induced by ATP. It was suggested that the hydrolysis of ATP appears not to be required for the OmpR dephosphorylation. We do not know at present whether EnvZ is required for the OmpR dephosphorylation reaction. Thus, it is uncertain at present whether OmpR is an ATP-dependent autophosphatase or, alternatively, whether EnvZ is a phosphoprotein phosphatase. Furthermore, nothing is known about the chemical nature of the phosphorylated amino acid residues in EnvZ and OmpR.

A number of systems have been identified in prokaryotes which can be classified as two-component regulatory systems that respond to environmental stimuli (22, 23). There are extensive similarities in the amino acid sequences among the two sets of components: one is thought to sense environmental stimuli and to transmit the information to the other, which then regulates gene expression or cellular behavior. In the light of recent findings as to the Ntr system (24, 25) and the chemotaxis system (CheA-CheY system) (26-28), the scenario described here as to the EnvZ-OmpR system implies that a common cascade mechanism, i.e., phosphotransfer, is involved in signal transmission in the two-component regulatory systems in prokaryotes. It should be emphasized that EnvZ was postulated to be located in the cytoplasmic membrane (17-19), whereas NtrB and CheA, which are related to EnvZ, are cytoplasmic soluble proteins. Thus, the EnvZ-OmpR system will provide us with a unique opportunity to elucidate mechanisms underlying signal transmission across the cytoplasmic membrane. EnvZ* characterized in this paper is missing the transmembrane segment that presumably anchors the protein in the cytoplasmic membrane. In order to elucidate mechanisms underlying the signal transmission, the intact EnvZ remains to be characterized.

It is not known at present how the phosphotransfer process, observed for EnvZ and OmpR in vitro, might be involved in osmoregulatory expression of the ompF and ompC genes in vivo. Hall and Silhavy (3) previously proposed the idea that EnvZ directs OmpR to take on one of a few alternative structures, which respectively activate the ompF and ompC genes. The phosphorylated and nonphosphorylated forms of OmpR might represent the two presumed alternative structures. A number of ompR and enz mutants, which exhibit different phenotypes as to osmoregulation (5-7, 20), remain to be characterized with respect to the phosphotransfer process. This will certainly facilitate the understanding of the molecular mechanism involved in vivo. An in vitro system for EnvZ/OmpR-dependent expression of the ompF and ompC genes should also be established. Experiments along relevant lines are currently underway in our laboratory.

**Acknowledgments**—We are deeply indebted to Dr. T. Shibusawa (Mitsubishi-Kasei Co.) for the kind gift of plasmid pUS12 and to Dr. T. Sasaki for the help to determine the amino acid sequence. We also wish to thank F. Takeuchi for secretarial assistance.

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