Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 6052–6056, August 1989
Biochemistry

Phosphorylation of OmpR by the osmosensor EnvZ modulates expression of the *ompF* and *ompC* genes in *Escherichia coli*

(osmoregulation/histidine phosphorylation/phosphate transfer/transcriptional regulation)

STEVEN FORST*, JORGE DELGADO*, AND MASAYORI INOUYE

Department of Biochemistry, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, Piscataway, NJ 08854-5635

Communicated by Boris Magasanik, May 5, 1989

ABSTRACT **EnvZ and OmpR, the regulatory proteins for *ompF* and *ompC* expression in *Escherichia coli*, belong to a modulator–effector family of regulatory proteins which are essential for the response to environmental signals. We show that the soluble cytoplasmic domain of the transmembrane modulator protein EnvZ is phosphorylated *in vitro* by [γ-^{32}P]-ATP. We also demonstrate that the phosphate group can, in turn, be transferred to the transcription activator protein OmpR. The pH stability properties of the phosphate groups linked to EnvZ indicate that this molecule contains histidyl phosphate. The invariant His-243 of EnvZ corresponds to the phosphorylated His-48 of the chemotactic modulator protein CheA. Substitution of His-243 with valine produces an EnvZ that is refractory to phosphorylation and can no longer catalyze the transfer of phosphate to OmpR. Furthermore, in a Δ*envZ* strain of *E. coli*, containing the *envZ* Val-243 plasmid, *ompC* expression is elevated 7-fold relative to that found in cells carrying the wild-type *envZ* plasmid. Based on these results we propose a model in which the phosphorylated state of OmpR modulates the expression of the *ompF* and *ompC* genes.**

The pores in the outer membrane of *Escherichia coli* K-12 through which small hydrophilic molecules diffuse passively are formed by the porin proteins OmpF and OmpC (1). In most *E. coli* strains, both OmpF and OmpC are expressed when the bacterium is grown at 37°C in nutrient broth or minimal medium, while OmpC is preferentially expressed and OmpF is repressed when the cells are grown in high osmolarity medium (2–4). The *ompB* operon, which consists of *ompR* and *envZ*, regulates porin expression (5–7). The nucleotide sequence of this locus has been determined (8–10). OmpR is a DNA-binding protein consisting of 239 amino acid residues that activates transcription of *ompF* and *ompC* by binding to sequences ≈90 base pairs upstream of the transcription initiation site of these genes (10–15). EnvZ, a molecule consisting of 450 amino acid residues, is an inner membrane protein possessing a periplasmic domain of 115 amino acid residues and the cytoplasmic domain contains ≈270 amino acid residues (16).

EnvZ is thought to modulate the functional activity of OmpR (11). However, the precise biochemical events by which EnvZ affects OmpR activity have not been determined. The recent recognition that a class of modulator proteins, to which EnvZ belongs, share considerable amino acid sequence similarities suggests that a common mechanism may exist for the signaling to a cognate effector molecule (17, 18). Indeed, two members [CheA, NR_{II} (NtrB)] of this class of modulator proteins have been shown to possess autophosphorylating activity and, in turn, transfer their phosphate group to the cognate effector molecules [CheY and NR_{I} (NtrC), respectively] (19–25). That protein phosphorylation plays a role in osmoregulation now seems very likely since Igo *et al.* (26, 27) have recently shown that EnvZ is phosphorylated *in vitro* and phosphorylation of OmpR enhances the *in vitro* transcription of *ompF*.

In this report, we show that in the presence of ATP the soluble cytoplasmic fragment of EnvZ becomes phosphorylated *in vitro* and can transfer its phosphate group to OmpR. In addition, using site-directed mutagenesis of EnvZ we provide evidence to support a model in which phosphorylation of EnvZ and OmpR plays a central role in the osmoregulatory response in *E. coli*.

MATERIALS AND METHODS

Chemicals and Reagents. Nutrient broth was obtained from Difco. Nitrocellulose membranes were from Schleicher & Schuell. Alkaline phosphatase-conjugated goat anti-rabbit antibody was from Bio-Rad. [γ-^{32}P]ATP and the reagents for M-13 oligonucleotide site-directed mutagenesis were obtained from Amersham.

Purification of EnvZ Protein Fragments and OmpR. The *E. coli* strain MH1160 (*ompR1*; ref. 8), which does not contain detectable levels of the OmpR protein (28), was transformed with different *envZ*-containing plasmids. The *ompR1* strain containing the *ompC:envZ*^{c} plasmid [the *ompC* promoter (K. Tsung and M.I., unpublished data) and the cytoplasmic fragment of EnvZ (EnvZ^c; Arg-180 to Gly-450)] was grown in 100 ml of nutrient broth plus 20% (wt/vol) sucrose and was harvested during early exponential phase. The pellets were resuspended in 1.4 ml of 20 mM sodium phosphate (pH 7) and sonicated, and the soluble fractions were obtained after centrifugation (390,000 × *g* for 14 min). The soluble fraction was applied to a DE-52 column equilibrated in 100 mM NaCl/20 mM Tris·HCl, pH 7.8/10 mM 2-mercaptoethanol/5% (vol/vol) glycerol, and protein was eluted with a linear gradient of NaCl (100–500 mM). The EnvZ^c fragment (Arg-180 to Gly-450) was further purified by ammonium sulfate precipitation (40% saturation at 4°C for 40 min) and the resulting protein pellet was resuspended in 200 μl of distilled water and applied to a Sephacryl S-200 column equilibrated in 20 mM Tris·HCl, pH 7.8/10 mM 2-mercaptoethanol/5% glycerol/250 mM NaCl. Two other plasmids were created by cloning *envZ* fragments into pINIII expression vectors (16). The plasmid pSF102 contains the *Eco*RI/*Hin*dIII *envZ* fragment (Glu-106 to Gly-450) of pYK12 and pSF106 contains the same *envZ* fragment except His-243 was replaced by valine (see below). The EnvZ fragments from pSF102 and pSF106 were partially purified by DE-52 column chromatography.

OmpR was purified as described (28) with the slight modification that a 60% ammonium sulfate precipitation step was added between the DEAE-cellulose and Sephacryl S-200 columns.

Protein Phosphorylation and pH Stability Assays. The reaction mixture (14 μl) contained a standard phosphorylation buffer (50 mM Tris·HCl, pH 8.0/0.1 mM EDTA/4% glycerol/0.8 mM dithiothreitol) to which was added [γ-^{32}P]ATP (5000

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Ci/mmol; 1 Ci = 37 GBq), EnvZ fragments, and OmpR as indicated. The reaction was carried out for 30 min at 22°C at which time either 2 μl of stop buffer (10% $NaDodSO_4$/1.5 M dithiothreitol) or OmpR was added. After the reaction was terminated with OmpR, the reaction mixtures were applied to a 15% $NaDodSO_4$/polyacrylamide gel and proteins were subsequently blotted onto nitrocellulose as described (2) and subjected to autoradiography.

To study the stability of the phosphate linked to either EnvZ or OmpR, the reaction was stopped by adding $NaDodSO_4$/dithiothreitol and 2 μl of the following buffers were added: 1 M HCl (pH 1.0), 0.16 M HCl (pH 3.0), 0.2 M Na_2HPO_4 (pH 7.1), 1.8 M NaOH (pH 11.5), and 3.3 M NaOH (pH 13.5). The reaction mixture was incubated for 60 min at 43°C and the proteins were applied to a $NaDodSO_4$/polyacrylamide gel followed by transfer to nitrocellulose and autoradiography.

Plasmid Construction and *envZ* Mutagenesis. The plasmid pYK12 is a derivative of pBR322, which contains the *ompB* locus of *E. coli* (9). A unique *Eco*RI site was introduced into the Arg-180 codon of *envZ* of pYK12 by site-directed mutagenesis, thus creating pDR203 (29). Arg-180 defines the N terminus of the 271-amino acid residue cytoplasmic fragment of EnvZ. The 1.7-kilobase *Eco*RI/*Hin*dIII fragment derived from pDR203 containing *envZ*^{c} (Arg-180 to Gly-450) was cloned into an *ompC* promoter vector developed in our laboratory to create the *ompC*:*envZ*^{c} plasmid.

The mutagenesis of *envZ* was performed by cloning the 1.9-kilobase *Eco*RI/*Hin*dIII fragment of pYK12 (Glu-106 to Gly-450) into the same sites of M13mp19. The use of the *Eco*RI site in pYK12 facilitated cloning into M13. Site-directed mutagenesis was performed by using the Amersham *in vitro* mutagenesis kit (version 2). His-243 of *envZ* was thus changed to valine, which created a unique *Sal* I site. The Val-243 mutation was confirmed by both restriction enzyme analysis and DNA sequencing. The Val-243 mutation in *envZ* was finally created by recloning the *Eco*RI/*Hin*dIII *envZ* fragment contained in M13mp19 back into pYK12, creating the Val-243 plasmid (pSF112, $ompR^+$ $envZ^{Val\text{-}243}$). The *Eco*RI/*Hin*dIII fragments of either wild-type or Val-243 *envZ* were also cloned into the pINIIIA2 expression vector (30), thus creating pSF102 (wild type, Glu-106 to Gly-450) and pSF106 (Val-243, Glu-106 to Gly-450).

β-Galactosidase Assay. The β-galactosidase assay was performed as described (8).

RESULTS

Purification of the EnvZ Cytoplasmic Fragment: (EnvZ^c). The 271-amino acid residue (Arg-180 to Gly-450) cytoplasmic domain of EnvZ (EnvZ^c) is thought to interact with OmpR, thereby modulating the functional activity of this transcription activator protein. To study the putative interaction between EnvZ and OmpR, we cloned the DNA fragment encoding this cytoplasmic domain of EnvZ into an *ompC* promoter expression vector developed in our laboratory (K. Tsung and M.I., unpublished data). Fig. 1 shows that EnvZ^c (Arg-180 to Gly-450) was present in the soluble fraction of sonicated cells (lane 2). Subjecting the soluble EnvZ^c-containing fractions to ion-exchange chromatography and ammonium sulfate precipitation (lane 3) resulted in a nearly pure fraction of this EnvZ fragment. The purification steps were monitored by Western blotting analysis (data not shown). The nearly homogeneous EnvZ^c (lane 3) was further purified by gel filtration. The apparent molecular weight of EnvZ^c as judged by electrophoresis on $NaDodSO_4$/polyacrylamide gels was 37,000, which is consistent with the predicted molecular weight of 35,350.

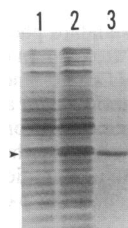

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of fractions from the purification of EnvZ^c. Coomassie blue-stained gel. Lanes: 1, soluble extract (20 μg) from *ompR1*/pBR322; 2, soluble extract (20 μg) from *ompR1*/*ompC*:*envZ*^{c}; 3, EnvZ^c after 40% ammonium sulfate precipitation. Arrowhead, position of EnvZ^c.

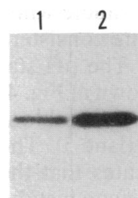

FIG. 2. Phosphorylation of the cytoplasmic fragment of EnvZ. Purified EnvZ^c (6 nM) was incubated with [γ-^{32}P]ATP (5000 Ci/mmol; 66 nM) in 15 μl for 30 min at 22°C. Lanes: 1, standard phosphorylation buffer (50 mM Tris·HCl, pH 8.0/0.8 mM dithiothreitol/0.1 mM EDTA/4% glycerol); 2, standard buffer plus 10 mM $MgCl_2$.

Phosphorylation of EnvZ^c and OmpR. Based on the knowledge that phosphorylation of the regulatory modulator proteins CheA and NR_{II} (NtrB) plays an important role in the function of these molecules and that they can be autophosphorylated *in vitro* (23–25), we examined whether EnvZ^c (Arg-180 to Gly-450) became phosphorylated in the presence of [γ-^{32}P]ATP. To determine whether the purified cytoplasmic domain of EnvZ (EnvZ^c, Arg-180 to Gly-450) was sufficient for the putative autophosphorylation activity of this fragment, EnvZ^c was incubated with [γ-^{32}P]ATP for 30 min at 22°C. The reaction was stopped by adding $NaDodSO_4$ and the protein was applied to a $NaDodSO_4$/polyacrylamide gel followed by blotting onto nitrocellulose paper. Fig. 2 shows that radiolabeled phosphate was transferred to EnvZ^c when incubated with [γ-^{32}P]ATP in the presence of 0.1 mM EDTA (lane 1) and that the phosphorylation activity was greatly stimulated by 10 mM Mg^{2+} (lane 2).

We next examined whether OmpR became phosphorylated in the presence of EnvZ^c. As with the effector molecules that share sequence similarities with OmpR, such as CheY and NR_I (NtrC), incubation of purified OmpR with [γ-^{32}P]ATP (Fig. 3, lane 2) did not result in the phosphorylation of OmpR. However, when EnvZ^c was first incubated with [γ-^{32}P]ATP for 30 min, and purified OmpR was subsequently added, the radiolabeled phosphate appeared rapidly on OmpR and was concomitantly removed from EnvZ^c. Fig. 3 shows that within 2 min (lane 3) after the addition to the phosphorylation assay OmpR had become phosphorylated and the incorporation of the phosphate group continued at a much slower rate thereafter (lanes 4 and 5; 5 min and 10 min, respectively). In the absence of added Mg^{2+} we could not detect the transfer of radiolabeled phosphate from EnvZ^c to purified OmpR (data not shown). These results demonstrate that the soluble cytoplasmic fragment of EnvZ is sufficient for the putative autophosphorylation activity of EnvZ and the transfer of its phosphate group to OmpR.

Chemical Properties of Phosphorylated EnvZ^c and OmpR. Table 1 shows that the phosphate group of EnvZ^c is very

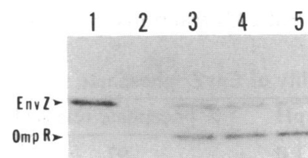

FIG. 3. Transfer of phosphate from EnvZ^c to purified OmpR. EnvZ^c (6 nM) was incubated with [γ-^{32}P]ATP for 30 min at 22°C in the standard phosphorylation buffer containing 10 mM $MgCl_2$ (lanes 1, 3, 4 and 5). Purified OmpR (300 nM) was subsequently added and the reaction was stopped by adding $NaDodSO_4$/2-mercaptoethanol (final concentration, 1.0% and 0.16 M, respectively) at 2 min (lane 3), 5 min (lane 4), and 10 min (lane 5). OmpR (300 nM) was incubated alone at 22°C in the standard buffer plus 10 mM $MgCl_2$ and [γ-^{32}P]ATP (lane 2).

unstable in acidic conditions (pH 1.0) and very stable when incubated in alkaline conditions. These results are consistent with the properties of histidyl phosphate (31). The pH stability characteristics of OmpR phosphate are shown in Fig. 4. The results indicate that the phosphate group was unstable at pH 1.0 (lane 1), pH 11.5 (lane 4), and pH 13.5 (lane 5). The instability at the extreme pH conditions indicates that the phosphate group of OmpR is neither a hydroxyl nor a histidyl phosphate, consistent with the properties of an acyl phosphate (31).

Substitution of His-243 of EnvZ. The results from the pH stability experiments indicated that EnvZ^c was phosphorylated at a histidine residue. An invariant histidine residue was found by Stock *et al.* (18) when a sequence comparison was made of eight different modulator proteins involved in regulatory pathways in various bacteria. In the chemotactic modulator protein CheA, a conserved sequence (Leu-Thr-**His**-Leu-Val-Arg) centering around the invariant histidine residue occurring at position 393 of this molecule was identified. The histidine residue (His-48) that was found to be phosphorylated in CheA resides within a similar sequence (Ala-Ala-**His**-Ser-Ile-Lys). In EnvZ, the invariant histidine residue is found at position 243 within the sequence Val-Ser-**His**-Asp-Leu-Arg. Since the histidine residue was found within the conserved sequence of the six other modulator proteins examined (18), it seemed likely that this histidine residue was involved in a common regulatory reaction, such as phosphorylation. These considerations prompted us to mutate the invariant His-243 of EnvZ (ref. 18; see *Materials and Methods*). His-243 was replaced with valine and both a wild-type *envZ* fragment and the Val-243-containing *envZ* fragment, both of which encoded the EnvZ sequence Glu-106 to Gly-450, were cloned into a pINIII expression vector, which contains an isopropyl β-D-thiogalactopyranoside-inducible promoter. This larger EnvZ fragment (344 amino acid residues) was used for the mutagenesis experiments, as compared with the 271-amino acid residue fragment used for the purification of EnvZ^c, due to the convenience of cloning the larger *envZ* fragment into M13. The resultant plasmids, pSF102 (wild type, Glu-106 to Gly-450) and pSF106 (Val-243, Glu-106 to Gly-450), were introduced into the *ompR1* strain of *E. coli* (32). The 344-amino acid residue EnvZ fragments produced from pSF102 (wild type) and pSF106 (Val-243) were found in both the pellet and soluble fractions derived from cells grown in nutrient broth plus 1 mM isopropyl β-D-thiogalactopyranoside. The EnvZ fragment in the soluble fractions was partially purified by ion-exchange chromatography. Fig. 5*A* shows the Western blot analysis of the EnvZ-containing fractions prepared from *ompR1*/pSF102 (wild type, lane 2) and *ompR1*/pSF106 (Val-243, lane 3). The total protein applied to the $NaDodSO_4$/polyacrylamide gel was 250 ng for *ompR1*/pBR322 and *ompR1*/pSF102 (lanes 1 and 2, respectively) and 750 ng for *ompR1*/pSF106 (lane 3). We found that the *ompR1*/pSF102 (wild type) fractions contained ≈6 times more EnvZ fragment than did the *ompR1*/pSF106 (Val-243) fractions as measured by Western

Table 1. pH stability of EnvZ^c phosphate

pH	% phosphate released
1.0	97
3.0	85
7.5	0
11.5	0
13.5	0

Phosphorylated EnvZ^c was aliquoted into buffers and incubated at 43°C for 60 min. Values represent data obtained from laser scans of autoradiographs and are expressed as the amount of phosphate remaining as a percentage of the phosphorylated EnvZ^c kept at 4°C at pH 8.0 during the incubation period.

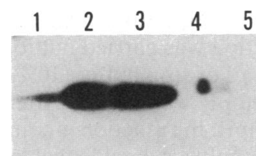

FIG. 4. The pH stability of the phosphate group of phosphorylated OmpR. OmpR (600 nM) was phosphorylated as described in Fig. 3 (lane 5). After stopping the phosphorylation reaction by adding $NaDodSO_4$, the pH was adjusted, as described in *Materials and Methods*, to pH 1 (lane 1), pH 3 (lane 2), pH 7.5 (lane 3), pH 11.5 (lane 4), and pH 13.5 (lane 5), and the reaction mixture was incubated for 60 min at 43°C. The phosphorylated OmpR was electrophoresed on a $NaDodSO_4$/polyacrylamide gel, transferred to nitrocellulose, and detected by autoradiography.

blot analysis. This difference may be due to several factors including the decreased stability and/or antigenicity of the Val-243 fragment. To assess the effect of replacing His-243 of EnvZ on the *in vitro* phosphorylation activity, equal amounts of the EnvZ fragments, based on the Western analysis, were added to each assay reaction mixture. The results of the phosphorylation assay are shown in Fig. 5*B*. When partially purified protein fractions derived from the *ompR1* strain lacking an *envZ* plasmid (prepared in exactly the same way as the EnvZ fragment-containing fractions) were assayed for phosphorylation activity an unidentified protein, "a," was found to incorporate radiolabeled phosphate (lane 1) but the phosphate was not transferred to purified OmpR (lane 2). We used this unidentified phosphorylated protein as an internal control for the phosphotransfer reaction. As expected, the wild-type EnvZ fragment (pSF102) was phosphorylated (lane 3) and in turn transferred its phosphate to purified OmpR (lane 4). In contrast, the Val-243 EnvZ fragment encoded by pSF106 was not phosphorylated (lane 5). Note that the total protein applied in lane 5 was ≈6 times that applied in lane 3. Furthermore, when purified OmpR was included in the phosphorylation assay with the Val-243 fragment (lane 6), the activator protein did not become phosphorylated. Thus, the phosphate bound to the considerable amount of phosphoprotein a was not transferred to OmpR. These results indicate that His-243 is involved in the phosphorylation of EnvZ and that the phosphotransfer reaction in the *in vitro* assay is specific for phosphorylated EnvZ fragment.

To assess whether the inability of EnvZ to become phosphorylated had a functional correlate, an *ompB* deletion

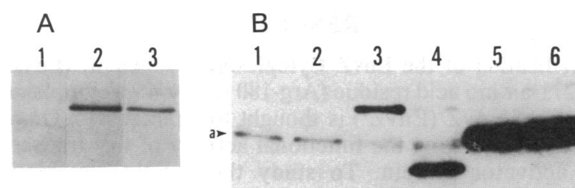

FIG. 5. Western blot and phosphorylation assay of wild-type and His-243 (valine) EnvZ fragments. (*A*) Partially purified fractions derived from *ompR1*/pBR322 (lane 1, 250 ng), *ompR1*/pSF102 (lane 2, 250 ng), and *ompR1*/pSF106 (lane 3, 750 ng) were electrophoresed on a $NaDodSO_4$/polyacrylamide gel and subsequently processed for Western blot analysis. (*B*) Equal amounts of the EnvZ fragment partially purified from cells containing either pSF102 (lanes 3 and 4) or pSF106 (lanes 5 and 6), as estimated from the Western blot analysis (*A*), were incubated with [γ-^{32}P]ATP in the standard phosphorylation buffer containing 10 mM Mg^{2+} at 22°C. After 30 min of incubation, either $NaDodSO_4$ (lanes 3 and 5) or purified OmpR (lanes 4 and 6) was added. The incubation with OmpR was continued for an additional 10 min before $NaDodSO_4$ was added. Identical fractions from *ompR1*/pBR322 were incubated in the same manner and either $NaDodSO_4$ (lane 1) or OmpR (lane 2) was added. a, An unidentified phosphorylated protein that appears in various amounts in all partially purified fractions.

strain (33) lacking *ompR* and *envZ* was transformed with either a wild-type *ompB* clone (pYK12; $ompR^+$ $envZ^+$) or a Val-243-containing *ompB* clone (pSF112; $ompR^+$ $envZ^{Val\text{-}243}$) and grown in nutrient broth at 37°C. Fig. 6 shows that in the parent strain MC4100 (lane 1) and in Δ*ompB*/pYK12 ($ompR^+$ $envZ^+$) both OmpC and OmpF were produced to a similar extent (lane 3). In contrast, the OmpC production in Δ*ompB*/pSF1112 ($ompR^+$ $envZ^{Val\text{-}243}$) was markedly increased and there was a noticeable reduction in the amount of OmpF in the outer membrane (compare lanes 3 and 4). When the cells were grown in nutrient broth containing 20% sucrose, the outer membrane porin production of the Δ*ompB*/pSF112 cells (lane 8) was essentially the same as that in the parent strain (lane 5). To determine whether the substitution of His-243 in EnvZ directly affected the transcription of *ompC*, we used a Δ*envZ*/*ompC*:*lacZ* operon fusion strain, RU1012, that was constructed in our laboratory by transducing the Δ*envZ* locus of AT142 (31) into the *ompC*:*lacZ* fusion strain MH215 (6). It was previously shown that OmpC production in the Δ*envZ* strain AT142 and in the *envZ22* (amber) strain of *E. coli* was greatly reduced in cells grown in nutrient broth (refs. 2 and 34; see *Discussion*). As shown in Table 2 *ompC* expression in RU1012 transformed with the control plasmid pBR322 was very low. When the RU1012 strain was transformed with the wild-type *ompB* clone, pYK12, ($ompR^+$ $envZ^+$) *ompC* expression, as measured by β-galactosidase activity, was significantly increased. In contrast, introduction of pSF112 ($ompR^+$ $envZ^{Val\text{-}243}$) into the RU1012 strain produced very high levels of *ompC* expression. Thus, the level of β-galactosidase activity in RU1012/pSF112 was 7-fold greater than that found in RU1012/pYK12. These results indicate that blocking the phosphorylation of EnvZ and OmpR increased the expression of the *ompC* gene in cells grown in nutrient broth.

DISCUSSION

We determined that the purified soluble cytoplasmic fragment of EnvZ is phosphorylated in the presence of [γ-^{32}P]ATP and that phosphorylated EnvZ is capable of transferring its phosphate to OmpR. These results are strikingly similar to the autophosphorylation of the homologous modulator proteins CheA and NR_{II} (NtrB) and the subsequent transfer of their phosphate groups to the cognate effector proteins CheY and NR_I (NtrC), respectively. CheA has been shown to be phosphorylated at His-48 contained within the sequence Ala-Ala-**His**-Ser-Ile-Lys (21). Mutations of His-48 abolished the ability of CheA to autophosphorylate and transfer its phosphate moiety to CheY as well as eliminated the ability of CheA to complement a *cheA* deletion strain. NR_{II} is also autophosphorylated at a histidine residue (24). In EnvZ, His-243 is contained within the sequence Val-Ser-**His**-Asp-Leu-Arg, which occurs in a conserved region of the modulator set of regulatory proteins (18). We show that the substitution of valine for His-243 eliminated the ability of the EnvZ fragment to be phosphorylated and subsequently transfer its phosphate to OmpR. These results demonstrate that the cytoplasmic domain of EnvZ is sufficient for autophosphorylation activity and for the transfer of its phosphate to OmpR.

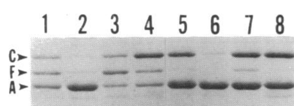

FIG. 6. Coomassie blue-stained outer membrane proteins. Cells were grown in either nutrient broth (lanes 1–4) or nutrient broth plus 20% sucrose (lanes 5–8). Lanes: 1 and 5, parent strain (MC4100); 2 and 6, Δ*ompB*/pBR322; 3 and 7, Δ*ompB*/pYK12 ($ompR^+$ $envZ^+$); 4 and 8, Δ*ompB*/pSF112 ($ompR^+$ $envZ^{Val\text{-}243}$). F, C, and A, OmpF, OmpC, and OmpA, respectively.

Table 2. ompC expression in RU1012 containing either pBR322, pYK12, or pSF112

Plasmid	β-Galactosidase activity, units
pBR322	60
pYK12	280
pSF112	1600

The β-galactosidase activity represents the average values derived from four independent experiments.

The Val-243 mutation stimulated *ompC* expression 7-fold in cells grown under conditions in which *ompC* is normally expressed at lower levels (Table 1). This mutation also produced a noticeable reduction in the amount of OmpF protein found in the outer membrane (Fig. 6). Based on the *in vitro* phosphorylation results and the β-galactosidase assays, nonphosphorylated OmpR is predicted to function as a transcription activator for *ompC*. On the other hand, phosphorylated OmpR is thus considered to be a transcription activator for *ompF* and to be unable to activate *ompC*. Thus the phosphorylation of EnvZ would occur in cells growing in, for example, nutrient broth (low osmolarity), and the phosphate group of EnvZ would subsequently be transferred to OmpR. The phosphorylated OmpR in turn activates the transcription of *ompF*. The recent results of Igo *et al.* (27), showing that phosphorylated OmpR enhances ompF transcription *in vitro*, supports this idea. Since growth of cells in nutrient broth results in the production of both OmpF and OmpC, it is predicted that the nonphosphorylated OmpR also exists in the cell. Nonphosphorylated OmpR could result from the dephosphorylation of preexisting phosphorylated OmpR, or the incomplete phosphorylation of *de novo* synthesized OmpR. In any case, it is predicted that both forms of OmpR are active as regulatory proteins for porin gene expression, which is an interesting difference from the regulation of *glnA* expression in which only the phosphorylated form of NR_I can promote gene expression (23–25). The phosphorylation cascade described above appears to represent a primary pathway for the modulation of *ompF* and *ompC* expression but does not explain the lack of *ompC* expression in the Δ*envZ* strain RU1012 (see Table 1) grown in nutrient broth. It is possible that in the total absence of *envZ* function [e.g., RU1012 or *envZ22* (amber)] another activity that is associated with *envZ* is lacking. This activity, which is perhaps a phosphatase-like activity that removes the phosphate group from phosphorylated OmpR, would not be present in RU1012 but may still be active in the $EnvZ^{Val\text{-}243}$ molecule encoded by pSF112. It should also be noted that in the Δ*ompB*/pSF112 ($ompR^+$ $envZ^{Val\text{-}243}$) cells grown in nutrient broth the OmpF production is reduced but not completely eliminated. Since it is speculated that the phosphorylated form of OmpR is required for *ompF* expression, we propose that homologous modulator proteins such as CheA or NR_{II} are capable of inefficiently phosphorylating OmpR. Such cross-talk has been demonstrated between CheA and NR_I and between NR_{II} and CheY (35). In addition, we have previously shown that *ompC* can be induced by high osmolarity in the absence of *envZ* function (2). In this context, it is possible that high osmolarity generally suppresses phosphorylation in *E. coli* such that newly synthesized OmpR would not be phosphorylated, thereby activating *ompC* expression or that an *envZ*-independent dephosphorylating pathway may exist in *E. coli* converting preexisting phosphorylated OmpR into the dephosphorylated form during a shift to high osmolarity growth medium.

The results of the pH stability profile of phosphorylated OmpR support the idea that OmpR contains an acyl phosphate. The acyl phosphate of CheY (22) and NR_I (24) is thought to be linked to invariant aspartic acid residues

located in two different regions at the N-terminal region of the effector class of molecules. The invariant aspartic acid residues were shown to be clustered in an acidic pocket of the recently resolved three-dimensional structure of CheY (36). The invariant aspartic acid residues in OmpR are located at positions 12 and 55. This is particularly intriguing since genetic information indicates that the modulation of the functional activity of OmpR occurs at the N-terminal region of OmpR. In the *envZ11* strain, Thr-247 is replaced by arginine, four residues away from His-243, resulting in high levels of *ompC* expression in cells grown in low osmolarity medium (34). The *envZ11* phenotype is suppressed in a strain that contains an *ompR* allele in which Leu-16 is replaced by glutamine (37), providing genetic evidence that this portion of OmpR interacts with EnvZ. These results suggest that His-243 constitutes an essential amino acid residue of the phosphorylated domain of EnvZ and that the N-terminal region of OmpR interacts with EnvZ in this region resulting in a transfer of the phosphate group to a carboxylate group of OmpR. The linking of the phosphate to OmpR would then presumably result in a conformational change in this activator protein.

We have previously found that the OmpR purified from wild-type cells grown in high osmolarity medium and from the *envZ11* mutant strain grown in nutrient broth exhibited a 200-fold higher DNA-binding activity for the upstream OmpR binding sequences of *ompF* than did the OmpR purified from cells grown in low osmolarity medium (28). This tight binding correlates with the repression of OmpF production in the *envZ11* strain and in cells grown in high osmolarity. Presently, we are not able to conclude whether the phosphorylation of OmpR plays a role in the dramatic differences in the DNA-binding properties of OmpR or whether other covalent modifications or other protein components may influence the DNA-binding properties of OmpR as well. It is also not yet known how the putative modification of the amino-terminal region of OmpR influences the binding affinity of the carboxyl-terminal DNA-binding domain of OmpR (37, 38).

We thank Ryutaro Utsumi for the use of the RU1012 strain, Kangla Tsung for the use of the *ompC* promoter vectors, T. Silhavy for providing the *E. coli* strains used in this study, and T. Mizuno for providing the Δ*envZ* strain AT142. We thank Bert Lampson and Arfaan Rampersaud for their critical reading of the manuscript and Karen Goudie for her excellent secretarial assistance. This work was supported by Public Health Service Grant GM19043 from the National Institute of General Medical Services, Grant N1387N from the American Cancer Society (to M.I.), and National Research Service Award GM1553 (to S.F.). S.F. and J.D. contributed equally to the work presented here.

1. Nikaido, H. (1979) in *Bacterial Outer Membrane: Biogenesis and Functions*, ed. Inouye, M. (Wiley, New York), pp. 361–408.
2. Forst, S., Delgado, J. & Inouye, M. (1988) *J. Bacteriol.* **170**, 5080–5085.
3. Kawaji, H., Mizuno, T. & Mizushima, S. (1979) *J. Bacteriol.* **140**, 843–847.
4. Verhoef, C., Lugtenberg, B., VanBoxtel, R., DeGraaff, P. & Verheij, H. (1979) *Mol. Gen. Genet.* **169**, 137–146.
5. Hall, M. N. & Silhavy, T. J. (1979) *J. Bacteriol.* **140**, 342–350.
6. Hall, M. N. & Silhavy, T. J. (1981) *J. Mol. Biol.* **146**, 23–43.
7. Sarma, V. & Reeves, P. (1977) *J. Bacteriol.* **132**, 23–27.
8. Comeau, D. E., Ikenaka, K., Tsung, K. & Inouye, M. (1985) *J. Bacteriol.* **164**, 578–584.
9. Mizuno, T., Wurtzel, E. T. & Inouye, M. (1982) *J. Biol. Chem.* **257**, 13692–13698.
10. Forst, S. & Inouye, M. (1988) *Annu. Rev. Cell Biol.* **4**, 21–42.
11. Hall, M. N. & Silhavy, T. J. (1981) *J. Mol. Biol.* **151**, 1–15.
12. Ikenaka, K., Ramakrishnan, G., Tsung, K. & Inouye, M. (1986) *J. Biol. Chem.* **261**, 9316–9320.
13. Jo, Y.-L., Nara, F., Ichihara, S., Mizuno, T. & Mizushima, S. (1986) *J. Biol. Chem.* **261**, 15252–15256.
14. Mizuno, T. (1987) *Gene* **57**, 57–64.
15. Norioka, S., Ramakrishnan, G., Ikenaka, K. & Inouye, M. (1986) *J. Biol. Chem.* **261**, 17113–17119.
16. Forst, S., Comeau, D., Norioka, S. & Inouye, M. (1987) *J. Biol. Chem.* **262**, 16433–16438.
17. Nixon, B. T., Ronson, C. W. & Ausubel, F. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7850–7854.
18. Stock, A., Chen, T., Welsh, D. & Stock, J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1403–1407.
19. Wylie, D., Stock, A., Wong, C.-Y. & Stock, J. (1988) *Biochem. Biophys. Res. Commun.* **151**, 891–896.
20. Hess, J. F., Oosawa, K., Kaplan, N. & Simon, M. I. (1988) *Cell* **53**, 79–87.
21. Hess, J. F., Bourret, R. B. & Simon, M. I. (1988) *Nature (London)* **336**, 139–143.
22. Stock, A. M., Wylie, D. C., Mottonen, J. M., Lupas, A. N., Ninfa, E. G., Ninfa, A. J., Schutt, C. E. & Stock, J. B. (1989) *Cold Spring Harbor Symp. Quant. Biol.* **53**, 49–57.
23. Ninfa, A. J. & Magasanik, B. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5909–5913.
24. Weiss, V. & Magasanik, B. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8919–8923.
25. Kerner, J. & Kustu, S. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4976–4980.
26. Igo, M. M. & Silhavy, T. J. (1988) *J. Bacteriol.* **170**, 5971–5973.
27. Igo, M. M., Ninfa, A. J. & Silhavy, T. J. (1989) *Genes Dev.* **3**, 598–605.
28. Forst, S. A., Delgado, J. & Inouye, M. (1989) *J. Bacteriol.*, in press.
29. Comeau, D. (1986) Ph.D. Thesis (SUNY at Stony Brook, Stony Brook, NY).
30. Inouye, S. & Inouye, M. (1985) *Nucleic Acids Res.* **13**, 3101–3110.
31. Mizuno, T. & Mizushima, S. (1987) *J. Biochem. (Tokyo)* **101**, 387–396.
32. Martensen, T. M. (1984) *Methods Enzymol.* **107**, 3–23.
33. Garrett, S., Taylor, R. K. & Silhavy, T. J. (1985) *J. Bacteriol.* **162**, 840–844.
34. Matsuyama, S.-I., Mizuno, T. & Mizushima, S. (1986) *J. Bacteriol.* **168**, 1309–1314.
35. Ninfa, A. J., Ninfa, E. G., Lupas, A. N., Stock, A., Magasanik, B. & Stock, J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5492–5496.
36. Stock, A. M., Mottonen, J. M., Stock, J. B. & Schutt, C. E. (1989) *Nature (London)* **337**, 745–749.
37. Tsung, K., Brissette, R. E. & Inouye, M. (1989) *J. Biol. Chem.* **264**, 10,104–10,109.
38. Tate, S., Kato, M., Nishimura, Y., Arata, Y. & Mizuno, T. (1988) *FEBS Lett.* **242**, 27–30.