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Function of Conserved Histidine-243 in Phosphatase Activity of EnvZ, the Sensor for Porin Osmoregulation in *Escherichia coli*

WEIHONG HSING AND THOMAS J. SILHAVY*

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544

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EnvZ and OmpR are the sensor and response regulator proteins of a two-component system that controls the porin regulon of *Escherichia coli* in response to osmolarity. Three enzymatic activities are associated with EnvZ: autokinase, OmpR kinase, and OmpR-phosphate (OmpR-P) phosphatase. Conserved histidine-243 is critical for both autokinase and OmpR kinase activities. To investigate its involvement in OmpR-P phosphatase activity, histidine-243 was mutated to several other amino acids and the phosphatase activity of mutated EnvZ was measured both in vivo and in vitro. In agreement with previous reports, we found that certain substitutions abolished the phosphatase activity of EnvZ. However, a significant level of phosphatase activity remained when histidine-243 was replaced with certain amino acids, such as tyrosine. In addition, the phosphatase activity of a previously identified kinase$^-$ phosphatase$^+$ mutant was not abolished by the replacement of histidine-243 with asparagine. These data indicated that although conserved histidine-243 is important for the phosphatase activity, a histidine-243–P intermediate is not required. Our data are consistent with a previous model that proposes a common transition state with histidine-243 (EnvZ) in close contact with aspartate-55 (OmpR) for both OmpR phosphorylation and dephosphorylation. Phosphotransfer occurs from histidine-243–P to aspartate-55 during phosphorylation, but water replaces the phosphorylated histidine side chain leading to hydrolysis during dephosphorylation.

Escherichia coli has two major porin proteins, OmpF and OmpC, which serve as channels for the passive diffusion of small, hydrophilic molecules through the outer membrane. The expression of *ompF* and *ompC* is regulated by a variety of environmental factors, including osmolarity, temperature, redox potential, and nutrient availability (for a recent review, see reference 32). Numerous studies demonstrate that a two-component system, including the sensor kinase EnvZ and the response regulator OmpR, mediates porin gene transcription in response to changes in osmolarity (for reviews, see references 4, 28, and 31).

EnvZ belongs to a large family of two-component sensor kinases. It is an integral membrane protein that has all of the conserved motifs common to this family: H, N, G1, D/F, and G2 boxes (30). Three enzymatic activities have been shown to be associated with the carboxy-terminal cytoplasmic domain of EnvZ: autokinase, OmpR kinase, and OmpR-phosphate (OmpR-P) phosphatase. At high osmolarity, EnvZ functions as an OmpR kinase, and the resulting high level of OmpR-P activates *ompC* transcription and represses *ompF* transcription. In contrast, at low osmolarity, EnvZ functions as an OmpR-P phosphatase, and the resulting low level of OmpR-P activates *ompF* only. Thus, OmpC is abundant at high osmolarity, and OmpF predominates at low osmolarity (31).

Most (if not all) of the two-component regulatory systems exhibit the same three biochemical activities outlined above for EnvZ (30). The mechanism for protein phosphorylation involves autophosphorylation of the sensor kinase by ATP at a conserved histidine residue in the H box (histidine-243 of EnvZ) and a subsequent phosphotransfer from the phosphohistidine to a conserved aspartic acid residue on the response regulator (aspartate-55 of OmpR). The mechanism for dephosphorylating the response regulator, however, remains unclear.

In some two-component systems, such as chemotaxis (Che), the phosphatase reaction is catalyzed by a protein that is distinct from the kinase: CheY-P dephosphorylation is stimulated by CheZ (9), not the kinase CheA. In others, the conserved histidine residue is not involved in the phosphatase reaction: replacing the conserved histidine with asparagine in NtrB (18), the sensor for nitrogen regulation (Ntr), or with glutamine in NarX (33), one of the dual sensors for nitrate and nitrite regulation, does not affect the phosphatase activity. However, recent studies suggest that the dephosphorylation of OmpR-P involves reversal of the OmpR kinase reaction with histidine-243–P serving as an obligate intermediate for the phosphatase reaction (3). This conclusion is based mainly on the observation that the phosphoryl group on OmpR-P can be transferred back to the kinase$^-$ phosphatase$^+$ (K^- P^+) mutant EnvZ.N347D at histidine-243 in vitro (3). However, this reaction has not been observed with wild-type EnvZ.

To investigate the role of histidine-243 of EnvZ for OmpR-P dephosphorylation more carefully, we developed a sensitive in vivo assay for phosphatase activity and used site-directed mutagenesis to change histidine-243 to a variety of other amino acids. Our results show that although this histidine is clearly important for phosphatase activity, a histidine-243–P intermediate is not required for the phosphatase reaction.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. The *E. coli* strains and plasmids used in this study are described in Table 1. Phage P1vir was used for transduction. Standard microbiological techniques were used for strain construction and bacterial growth (35). Cells were grown at 37 or 30°C with shaking in appropriate media. pEnvZ was constructed from pFR29 (34) by digestion with *Sma*I and *Ssp*I and religation. In effect, this removes the first 832 nucleotides of *ompB* on pFR29.

Media and reagants. Media were prepared as previously described (35). *o*-Nitrophenyl-β-D-galactopyranoside for β-galactoside assay was purchased from Sigma. Restriction enzymes, β-agarase, and T4 DNA ligase were from New England BioLabs, Inc. *Taq* polymerase and reagents used for PCR amplification,

* Corresponding author. Phone: (609) 258-5899. Fax: (609) 258-2769 or 6175. E-mail: tsilhavy@molecular.princeton.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
E. coli strains		
BW16463	*lacX74* Δ(*pta ackA hisQ hisP*) *zej-223*::Tn*10*	B. L. Wanner, 41
MC4100	*F*$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA flb-5301 ptsF25 deoC1 thiA1*	2
MH225	MC4100 Φ(*ompC′-lacZ*$^+$) *10-25*	6
MH513	MC4100 Φ(*ompF′-lacZ*$^+$)*16-23*	7
SG480Δ900	MC4100 Δ(*envZ-malP*)*900 malP*::*neo*	Laboratory stock
WH20	MC4100 *araB* Δ*ompB malPQ*::*neo* Φ(*ompF′-lacZ*$^+$)*16-23*	This study
WH56	MH225 *envZ*::*kan*	This study
WH57	MH513 *envZ*::*kan*	This study
WH58	MH513 *envZ H243Y*	This study
WH59	MH513 *envZ H243N*	This study
WH60	MH513 *envZ H243Q*	This study
WH62	MH513 *envZ N343K*	This study
WH66	WH57 Δ(*pta ackA hisQ hisP*) *zej-223*::Tn*10*	This study
WH67	WH57 *recA*::*cam*	This study
WH71	MH513 *envZ250+H243Y*	This study
WH72	MH513 *envZ250+H243N*	This study
WH73	MH513 *envZ250*	This study
WH149	MH513 *envZ H243A*	This study
WH254	MH513 *envZ H243C*	This study
WH255	MH513 *envZ H243S*	This study
Plasmids		
pFR29	*ompR*$^+$ *envZ*$^+$ Ap^r	34
pFR32	*ompR*$^+$ *envZ*$^+$ Cam^r(Ts)	33a
pEnvZ	*envZ*$^+$ Ap^r	This study
pMBP-OmpR	Φ(*malE′-′ompR*) Ap^r	12
pSG115	*envZ115* Ap^r	14

T4 DNA polymerase, and Sequenase were from United States Biochemical Corp. [γ-^{33}P]ATP (1,000 to 3,000 Ci/mmol, 10 mCi/ml) were from NEN Life Science Products. The oligonucleotide primers used for site-directed mutagenesis and DNA sequencing were provided by the Princeton University Department of Molecular Biology Synthesis/Sequencing Facility. The Sculptor in vitro site-directed mutagenesis kit was purchased from Amersham. NuSieve low-melting-point agarose was from FMC BioProducts. Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from National Diagnostics.

β-Galactosidase assay. β-Galactosidase activities were determined by using a microtiter plate assay that has been previously described (36). Cells were grown overnight in appropriate medium at 37°C, subcultured (1:40) into 2 ml of the same medium, and then grown to mid-log phase at 37°C. Activities are expressed as $(U/A_{600}) \times 10^3$, where U is micromoles of *o*-nitrophenol formed per minute. A minimum of four independent assays was performed for each strain, and the results were averaged for display as bar graphs.

PCR amplification and DNA sequence analysis. PCR amplification and DNA sequence analysis were performed essentially as previously described (34). DNA was amplified from either bacterial cells or plasmids. For sequencing analysis, the template DNA was either from PCR amplification or from restriction digestion. The template was purified by gel electrophoresis in 1% NuSieve low-melting-point agarose, and the agarose was digested with β-agarase. Sequence analysis was then performed as indicated in the supplier's instructions.

Site-directed mutagenesis. Double-stranded site-directed mutagenesis was performed on pEnvZ by following the manufacturer's instructions. Each mutation was first confirmed by DNA sequence analysis, subcloned onto pFR32, which has a temperature-sensitive origin of replication, and then recombined onto the chromosome as described by Hamilton et al. (8). MH225 was used as the recipient strain, and lactose-tetrazolium medium was used as an indicator to facilitate the screening of chromosomal recombinants. The presence of each mutation on the chromosomal location was confirmed by PCR amplification and sequence analysis. Each *envZ* allele was then moved into a clean strain background by P1 transduction by using WH20 as the recipient.

***In vitro* phosphorylation and dephosphorylation analysis.** The cytoplasmic membrane fractions were isolated from an *ompB* deletion strain (SG480Δ900) which harbors different *envZ* alleles on the multicopy plasmid pEnvZ by using a previously described procedure (44). The membranes were resuspended in the assay buffer described below. Amounts of EnvZ protein per unit of the isolated membranes were roughly the same as determined by immunoblot analysis with anti-EnvZ antibody. The MBP-OmpR fusion protein was overexpressed and purified by following the procedure suggested by the manufacturer (New England Biolabs). The truncated EnvZ protein (EnvZ115) was partially purified as previously described (14).

The same assay buffer, which contains 0.1 M Tris-HCl buffer (pH 8.0), 50 mM KCl, 5 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol, was used for both the phosphorylation and dephosphorylation assays. For the autokinase assay, the assay mixture included 4 μl of isolated cell membranes (approximately 0.5 mg/ml), 1 μl of ATP (250 μM), 0.5 μl of [γ-^{33}P]ATP (1,000 to 3,000 Ci/mmol, 10 mCi/ml), and 4.5 μl of assay buffer. The reaction continued for 10 min at room temperature and was stopped by 5 μl of 3× SDS-sample buffer. Proteins were separated by SDS-PAGE and visualized through autoradiography. The kinase assay was essentially the same as the autokinase assay, except that 0.5 μl of MBP-OmpR (2.5 mg/ml) was also included in the assay mixture.

For the phosphatase assay, partially purified EnvZ115 was first phosphorylated by [γ-^{33}P]ATP through the autokinase reaction in a final volume of 250 μl. After being incubated for 10 min at room temperature, the reaction mixture was centrifuged and the EnvZ115-^{33}P present in the pellet was washed three times with 1 ml of assay buffer through resuspension and centrifugation. This EnvZ115-^{33}P was then incubated with MBP-OmpR (0.25 mg/ml, final concentration) in assay buffer for 10 min at room temperature. The MBP-OmpR-^{33}P was separated from EnvZ115 through centrifugation and used as the substrate for the dephosphorylation assay. The assay mixture contained 4 μl of membrane (0.5 mg/ml), 1 μl of ATP (10 mM), 0.4 μl of $MgCl_2$ (200 mM), and 5 μl of MBP-OmpR-^{33}P from the previous step. Note that the presence of 1 mM (final concentration) ATP or ADP is required for the assay. The reaction was carried out at room temperature and stopped after various time points with SDS-sample buffer. Proteins were separated by SDS-PAGE. The amount of MBP-OmpR-^{33}P was visualized through autoradiography and quantified by a densitometer.

RESULTS

Measurement of mutant EnvZ phosphatase activity in vivo. We and others have noticed that in the absence of EnvZ, OmpR can still be phosphorylated by some other mechanism(s), and this low level of OmpR-P activates *ompF* transcription to a significant degree (for example, see Fig. 1). Previous results showed that small-molecule phosphodonors such as acetyl phosphate can phosphorylate OmpR in vitro (21, 25). To determine if this happens in vivo, we constructed an *envZ* null strain (WH57 [Table 1]) carrying a deletion encompassing the genes required for the synthesis of acetyl phosphate, *ackA* (acetyl kinase) and *pta* (phosphotransacetylase). As shown in Fig. 1, this deletion reduced the level of *ompF* transcription in the *envZ* null background to a level similar to that of an *ompR envZ* deletion strain (Fig. 1). Although we do not understand the mechanism (22, 27, 38), this result demonstrates that acetyl phosphate is the major phosphate donor for OmpR in the absence of EnvZ in vivo.

Results presented in Fig. 1 suggest a sensitive assay to measure mutant EnvZ phosphatase activity in vivo. The low level of OmpR-P formed from acetyl phosphate in an *envZ* null strain causes an OmpF$^{+/-}$ phenotype. In contrast, in a K$^-$ P$^+$ *envZ* mutant background, the low level of OmpR-P is destroyed by the phosphatase activity of the mutant EnvZ, rendering the cell OmpF$^-$ (34). Thus, the phosphatase activity of an *envZ* K$^-$ P$^+$ mutant protein can be quantitatively measured as the decrease in *ompF* transcription compared to that of an isogenic *envZ* null strain. The lower the *ompF* transcription, the stronger the phosphatase activity. The level of *ompF* transcription can be simply quantified by β-galactosidase assays using an *ompF′-lacZ*$^+$ fusion. To test our system, we measured *ompF* transcription in several known K$^-$ P$^+$ mutant *envZ* backgrounds. As illustrated in Fig. 1, the level of *ompF* transcription specified by the β-galactosidase activity from an *ompF′-lacZ*$^+$ fusion decreased about 50% in an *envZ* null strain compared to that of the wild type in Luria broth medium. A K$^-$ P$^+$ mutation in the conserved N region (N343K) (11), which behaves very similarly

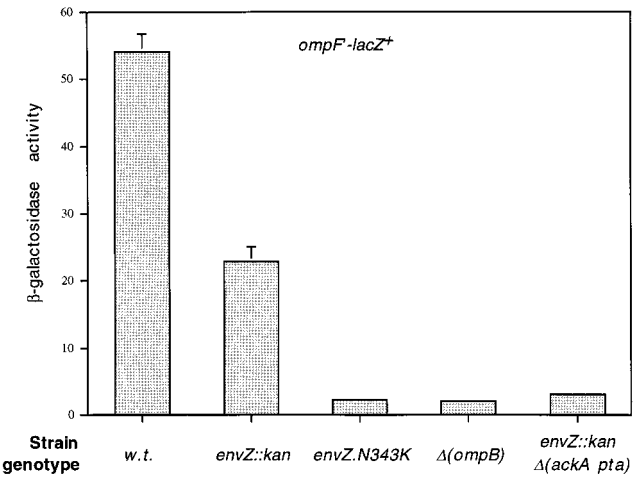

FIG. 1. Levels of *ompF* transcription in different strain backgrounds. Transcription of *ompF* was monitored as β-galactosidase activity through an *ompF'-lacZ*$^+$ fusion. β-Galactosidase activity was measured as described in Materials and Methods. Wild type (*w.t.*) cells and *envZ*::*kan*, *envZ.N343K*(K^- P^+), Δ*ompB* (*ompB* is the *envZ*/*ompR* locus), and *envZ*::*kan* Δ(*ackA pta*) mutant cells were tested.

to the N347D mutant used in the reverse phosphotransfer study described previously (3), decreased *ompF* transcription even further. Results similar to that observed with the N343K mutation were obtained with several other K^- P^+ mutants tested, such as *envZ247* and *envZ250* (34 and data not shown). These data verified our assay system for measuring EnvZ phosphatase activity in *envZ* K^- P^+ backgrounds.

Replacement of histidine-243 with several amino acids did not abolish the phosphatase activity of EnvZ. As noted in the introduction, the conserved histidine of two-component sensors is absolutely required for kinase activity. Thus, the substitution of any amino acid for histidine-243 of EnvZ will render the mutant protein K^-. Under these conditions, the assay described in the previous section can then be used to measure the phosphatase activity of the mutant proteins. Site-directed mutagenesis was performed to replace His-243 with several other amino acids in an *envZ* gene carried on multicopy plasmid pEnvZ. After confirming the mutation by DNA sequence analysis, the mutant pEnvZ plasmids were transformed into a *recA envZ*::*kan* strain carrying an *ompF'-lacZ*$^+$ fusion, and the β-galactosidase activity was measured. When His-243 was replaced by glutamine or aspartic acid, the level of *ompF* transcription was indistinguishable from that of the vector control, indicating that the two replacements abolished both the kinase and phosphatase activities of EnvZ. These results demonstrate the importance of His-243 for phosphatase activity and are in agreement with previous results obtained by valine or arginine substitution (5, 19). However, when the conserved histidine was replaced with tyrosine, the mutant EnvZ proteins caused a significant (~50%) decrease in *ompF* transcription compared to the *envZ* null, suggesting that this mutant EnvZ protein possesses a significant level of phosphatase activity. Other amino acid substitutions for histidine-243, such as cysteine, alanine, serine, or glutamic acid, decreased *ompF* transcription as well, although to a lesser extent (20 to 30%), implying that these substitutions also maintain some level of phosphatase activity.

A trivial explanation for the variable phenotypes conferred by histidine-243 substitution mutations is that these mutations destabilize the EnvZ protein to different degrees. To investigate this possibility, immunoblot analysis was performed with an anti-EnvZ antibody. We found that the level of EnvZ in these mutant strains was comparable to that in the wild type (data not shown). Thus, alterations in protein stability are not the cause of the observed phenotypes.

To further analyze these histidine-243 substitution mutations, the *envZ* mutations were recombined onto the chromosome as described in Materials and Methods. The level of *ompF* transcription in mutant cells which contained a single copy of mutated *envZ* was again measured by assaying the β-galactosidase activity produced by the *ompF'-lacZ*$^+$ fusion. As shown in Fig. 2, the asparagine and glutamine substitutions inhibited phosphatase activity, the tyrosine substitution mutant maintained a significant level of phosphatase activity, and the alanine, serine, and cysteine replacements exhibited phosphatase activity as well, although to a lesser extent. The β-galactosidase activities of these mutant strains were comparable to results obtained with the corresponding pEnvZ plasmids. However, we noted that on the basis of mutant colony color on lactose-MacConkey agar, the mutant genes in single copy were less effective than the plasmid-encoded genes in reducing β-galactosidase activity.

Histidine-243–P is not involved in the phosphatase reaction. Results presented above indicate that histidine-243 is not absolutely required for EnvZ phosphatase activity. One interpretation of these results is that a phosphoenzyme intermediate is not involved in the phosphatase reaction. However, a feature common to the amino acids serine, cysteine, and tyrosine is that, like histidine, they can all be phosphorylated. Thus, it could be argued that histidine-243 is required for EnvZ phosphatase activity, and the observed phosphatase activity in serine, cysteine, or tyrosine replacements is due to the ability of these amino acids to mimic the function of histidine. Although the result obtained with the alanine substitution argues against this hypothesis, the phosphatase activity of this mutant protein is weak. Accordingly, the following experiment was performed to clarify the issue.

envZP159S is a previously isolated K^- P^+ mutant allele that causes a P159S amino acid change in the periplasmic domain near transmembrane segment II (*envZ250* in reference 34). Thus, it must alter enzymatic activity indirectly, presumably by altering protein conformation. Indeed, the kinase activity is

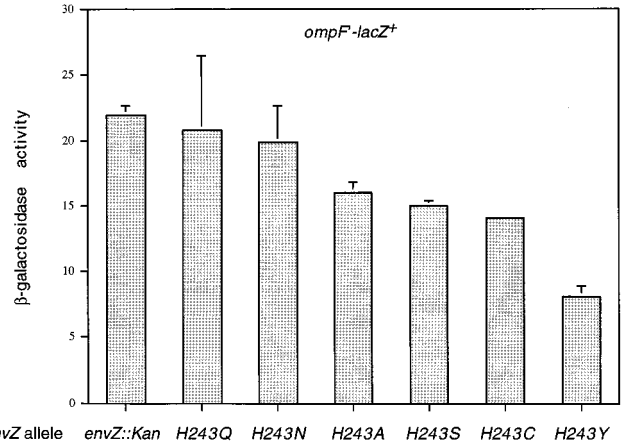

FIG. 2. Replacement of histidine-243 with some amino acids decreased *ompF* transcription when the *envZ* gene was present in single copy inside the cell. The histidine-243 substitution mutations were recombined onto the chromosome as described in Materials and Methods. The levels of *ompF* transcription were measured as β-galactosidase activity from an *ompF'-lacZ*$^+$ fusion.

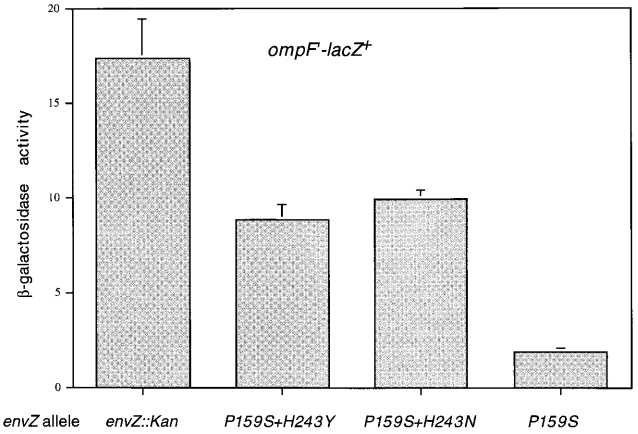

FIG. 3. Phosphatase activity of EnvZP159S was not abolished by the histidine-243 substitution. Histidine-243 on *envZP159S* was replaced with tyrosine or asparagine through in vitro DNA manipulation. The *envZ* alleles with double mutation P159S+H243Y or P159S+H243N were then placed in single copy at the chromosomal position. The phosphatase activities of the mutant EnvZ proteins were measured as decreases in *ompF* transcription compared to the *envZ* null. The level of *ompF* transcription was measured as β-galactosidase activity from an *ompF'-lacZ*$^+$ fusion.

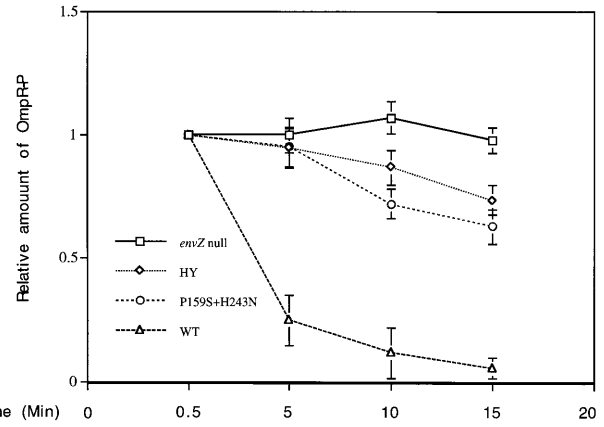

FIG. 4. In vitro dephosphorylation assay. Purified MBP-OmpR was phosphorylated by EnvZ115-^{33}P, and the phosphorylated MBP-OmpR (MBP-OmpR-^{33}P) was then used as the substrate for the dephosphorylation assay. Membranes enriched for different EnvZ mutant proteins were assayed for phosphatase activity. Reactions were stopped at different time points after incubating MBP-OmpR-^{33}P, membranes, and 1 mM cold ATP in the assay buffer. Proteins were separated by SDS-PAGE and subjected to autoradiography. The density of MBP-OmpR-^{33}P on the autoradiograph was measured with a densitometer and normalized to that of the first time point to yield the relative amount of MBP-OmpR-^{33}P, which was then plotted against time. Error bars indicate variation observed with different membrane preparations in five different experiments. HY, *envZ H243Y* mutant cells; WT, wild-type cells.

restored in vitro by solubilization from the membrane (34). We reasoned that residual phosphatase activity may be more easily detected in this conformationally altered EnvZ. If histidine-243 is required for phosphatase activity, then replacement of this residue with asparagine, which cannot be phosphorylated, should abolish the phosphatase activity of EnvZP159S. Two double mutants, one possessing P159S and H243N and one possessing P159S and H243Y, were constructed on pEnvZ. Each double mutant was then placed in single copy at the normal chromosomal location (see Materials and Methods). The phosphatase activity of each of the double mutants was measured by using the in vivo assay system described above. As shown in Fig. 3, the level of *ompF* transcription is decreased by the presence of either double mutant *envZ* allele compared with that of an *envZ* null. Thus, the phosphatase activity of *envZP159S* is not abolished by replacing histidine-243 with either tyrosine or asparagine. These results argue strongly that a histidine-243–P intermediate is not required for the phosphatase reaction.

In vitro phosphorylation and dephosphorylation assays of histidine-243 substitution mutants. To confirm results from our in vivo assays, in vitro protein phosphorylation and dephosphorylation assays were performed. Membranes enriched for the mutant or wild-type proteins were isolated from strains carrying the pEnvZ plasmids. Not surprisingly, none of the histidine-243 substitution mutants could be autophosphorylated by ATP and thus could not phosphorylate OmpR in vitro under our assay conditions (data not shown). As expected from the in vivo studies, the phosphatase activities of the histidine-243-substituted proteins were much lower than that of the wild type; however, they were clearly detectable. Assays showed that membranes enriched for either EnvZ H243Y or EnvZP159S+H243N dephosphorylated OmpR-P, while the control membranes from an *envZ* null strain did not (Fig. 4). We concluded that the His-243-substituted EnvZ mutant proteins have phosphatase activity.

The phosphatase activity of histidine-243 substitution mutant EnvZs did not appear to be osmoregulated. Because the histidine-243 replacement mutations completely abolish the autokinase and OmpR kinase activities of EnvZ, any effect of medium osmolarity on strains producing these mutant proteins should solely reflect osmoregulation of the phosphatase activity. Our in vivo phosphatase assay depends on the phosphorylation of OmpR by acetyl phosphate. As illustrated in Fig. 5, the *envZ* null *ompF'-lacZ*$^+$ fusion strain exhibits higher β-galactosidase activity when grown in high-osmolarity medium, indicating that high osmolarity increases OmpR-P even in the absence of EnvZ. Introduction of the *ackA pta* deletion into the *envZ* null fusion strain abolished this increase, suggesting that these changes in *ompF* transcription depend on acetyl-phosphate under our assay conditions. It is important to note that *ompF'-lacZ*$^+$ expression in wild-type strains is much higher in low osmolarity and that high osmolarity causes repression. In *envZ* null strains, OmpR-P never accumulates to

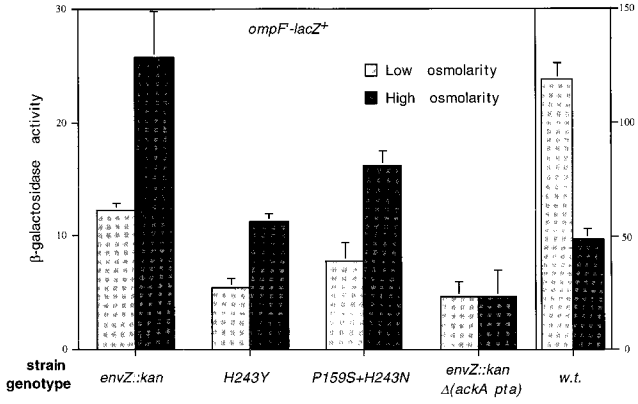

FIG. 5. Osmoregulation of *ompF* transcription with histidine-243 substitution mutants. Transcription of *ompF* was monitored through an *ompF'-lacZ*$^+$ fusion. β-Galactosidase assays were performed with cells grown under either high (A medium plus 15% sucrose) or low (A medium) osmolarity at log phase. *envZ*::*kan*, *envZ H243Y*, *envZP159S + H243N*, and *envZ*::*kan* Δ(*ackA pta*) mutant and wild type (*w.t.*) cells were tested. Note the difference in scale between the β-galactosidase activities of mutant and wild-type cells.

high enough levels to cause repression. Under these conditions, changes in OmpR-P are reflected by changes in *ompF* transcription activation only.

If the phosphatase activity of the histidine-243 substitution mutants is regulated by medium osmolarity, increased osmolarity should result in decreased phosphatase activity and, correspondingly, more OmpR-P. Thus, we would expect a smaller fold reduction of *ompF* transcription in the histidine-243 substitution mutants compared to the *envZ* null control at high osmolarity relative to low osmolarity. However, our results (Fig. 5) show that the relative ratios of *ompF* transcription at low and high osmolarities were unchanged by the histidine-243 substitution mutants compared with the *envZ* null strain. Expression of the *ompF′-lacZ*$^+$ fusion was reduced by the phosphatase activity of the mutant sensors equally under both conditions. These results suggest that the phosphatase activity of histidine-243 substitution mutant EnvZ protein is not regulated by osmolarity.

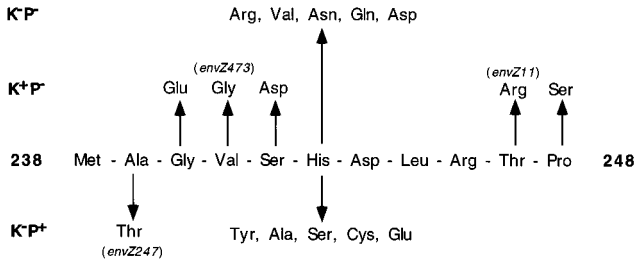

FIG. 6. Known mutations in the conserved histidine-243 region. Some of the histidine-243 substitutions abolish both kinase and phosphatase activities (5, 19; this study). Mutations that strongly affect phosphatase include G240E, V241G, S242D, T247R, and P248S (1, 40, and references therein). A239T, as well as some of the histidine-243 substitution mutations, strongly affect kinase activity (reference 34 and this study).

DISCUSSION

It has been suggested that the dephosphorylation of OmpR-P involves reversal of the OmpR kinase reaction using the identical conserved histidine residue of EnvZ, histidine-243 (3). The reverse phosphotransfer model, which is based on studies with EnvZ.N347D, is attractive for several reasons. First, it explains the null phenotype conferred by histidine-243 replacement with either valine or arginine (5, 19) or, as we show here, with asparagine, glutamine, or aspartic acid, as well. Second, it is conceptually similar to the well-characterized phosphorelay, which signals the initiation of sporulation in *Bacillus subtilis*, where phosphate is transferred sequentially from histidine to aspartate to histidine to aspartate residues on four different proteins (10). However, several observations in the original study are troubling. The reverse phosphotransfer reaction is inhibited by ADP, yet with wild-type EnvZ, ADP or ATP stimulates the phosphatase reaction (15). Indeed, the reverse transfer reaction cannot be demonstrated with wild-type EnvZ. Also, the molar ratio of OmpR to EnvZ.N347D was 1:5 in the reverse transfer experiments, whereas in vivo, OmpR is nearly 100-fold more abundant than EnvZ. We agree that reverse phosphotransfer can occur under certain conditions (11), but we do not believe that this explains the phosphatase activity of wild-type EnvZ.

To test the reverse phosphotransfer model directly, we changed conserved histidine-243 to a variety of other amino acids and assayed the phosphatase activities of the resulting mutant EnvZ proteins both in vivo and in vitro. We found that all of the amino acid substitutions tested reduced the phosphatase activity. Clearly, histidine-243 plays an important role in this reaction. However, in several cases, such as the tyrosine, alanine, cysteine, serine, and glutamine substitutions, significant phosphatase activity remains. Furthermore, an H243N change does not abolish the phosphatase activity of kinase-defective mutant protein EnvZP159S. The fact that we can replace the conserved histidine with several different residues, including alanine, but still maintain some degree of phosphatase activity indicates that histidine-243 is not required for the phosphatase activity. These results invalidate the reverse phosphotransfer model.

A second two-component phosphatase model proposes that the response regulator has intrinsic phosphatase activity and, in analogy to the co-protease function of RecA, the phosphatase is really a co-phosphatase that functions to stimulate the autodephosphorylation reaction (23, 30, 39). Supporting evidence for the co-phosphatase model comes from studies of the stability of certain phosphorylated response regulators. When assayed in vitro under nondenaturing conditions, the half-life of CheY-P is only a few seconds (9) and that of NtrC-P (42) or PhoB-P (26) is a few minutes. However, under denaturing conditions, the half-lives of phosphorylated regulators are generally several hours at neutral pH and ambient temperature (37, 42), corresponding to the intrinsic rate of hydrolysis of the acylphosphate group. This implies that these response regulators have autophosphatase activity. The co-phosphatase may modulate this activity by causing a conformational change in the phosphorylated response regulator rather than by participating directly in the hydrolysis reaction. Accordingly, the co-phosphatase should contribute no important residues to the active site. Replacement of the conserved histidine residue with certain amino acids should not affect the phosphatase activity, as has been observed for NtrB (18).

Several observations hinder the generalization of the co-phosphatase model to all two-component regulatory systems. First, not all phosphorylated response regulators have obvious autophosphatase activity. The half-lives of OmpR-P (15), VanR-P (43), and VirG-P (16) are more than 1 h under either nondenaturing or denaturing conditions. Second, in these more orthodox systems, the sensor kinase catalyzes dephosphorylation directly; there are no ancillary proteins, such as CheZ or PII (9, 20, 29). Third, while it may be true that EnvZ contributes no essential residues to the phosphatase active site, available mutant data indicate that the H-box region, especially histidine-243, is indeed important. A variety of amino acid substitutions at and around histidine-243 strongly affect the phosphatase activity of EnvZ (Fig. 6). Accordingly, it seems misleading to label EnvZ a co-phosphatase.

For EnvZ, the available data best fit a model that proposes that it is a true phosphatase but that the kinase and phosphatase active sites overlap substantially. This model predicts that changes in the H-box region could affect the kinase, the phosphatase, or both activities, and this is certainly true. As shown in Fig. 6, there are known mutations in the region that confer every possible phenotype. Moreover, many of these changes, such as *envZ473* (13) or any of the histidine-243 substitutions, clearly affect both activities simultaneously.

Lukat et al. (24) have proposed closely related transition states for the kinase and phosphatase reactions that fit the overlapping-site model well. As redrawn in Fig. 7, the kinase reaction involves the transfer of phosphate from histidine-243–P of EnvZ to aspartate-55 of OmpR. In the phosphatase reaction, the active site is altered slightly so that water replaces the histidine leading to hydrolysis. This model could also explain the importance of EnvZ histidine-243 for the phospha-

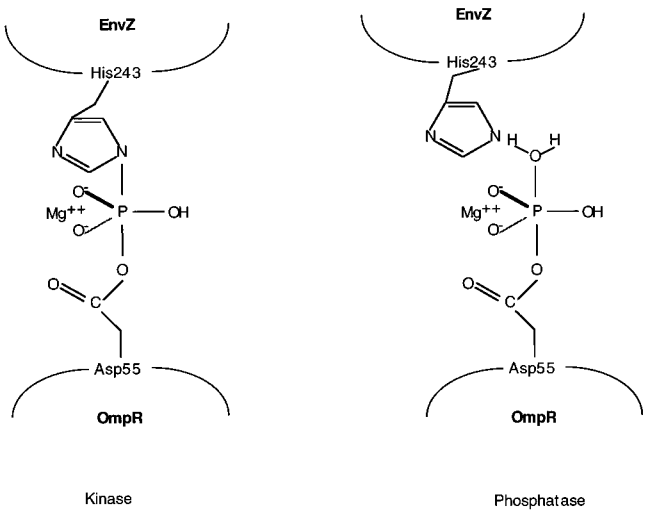

FIG. 7. Model for the mechanism of OmpR phosphorylation and dephosphorylation. A common transition state with histidine-243 of EnvZ in close contact with aspartate-55 of OmpR is involved in both OmpR phosphorylation and dephosphorylation reactions. Phosphotransfer occurs from histidine-243–P to aspartate-55 during phosphorylation, and water replaces the phosphorylated histidine side chain leading to hydrolysis during dephosphorylation.

tase reaction; it may function as a general base to polarize the water molecule or to facilitate opening of the OmpR active site, allowing water to enter.

It is generally agreed that EnvZ regulates porin gene expression simply by controlling the levels of OmpR-P and that it does so by varying the ratio of its kinase and phosphatase activities (3, 31, 34). Genetic analysis also suggests that EnvZ can exist in two opposed signalling states, kinase active and phosphatase active (31, 34). The overlapping-site model fits these views nicely. EnvZ can certainly exist in two forms, phosphorylated and unphosphorylated. For EnvZ to function as a kinase, it must exist in the EnvZ-P form. If the overlapping-site model is correct, it must exist in the unphosphorylated form to function as a phosphatase. Thus, osmoregulation could be accomplished by controlling the ratio of EnvZ-P to EnvZ.

It is not known mechanistically how the activities of EnvZ are regulated by medium osmolarity. By using a chimeric receptor (Taz) which contains the periplasmic domain and the transmembrane helices of the chemoreceptor Tar and the cytoplasmic domain of EnvZ, Jin and Inouye (17) have obtained data suggesting that it is the phosphatase activity of EnvZ that is regulated. They found that added aspartate stimulated the phosphatase activity of Taz. We do not dispute these data, but given the hybrid nature of the Taz receptor, we think these results should be viewed with caution. By using the K^- P^+ histidine-243 substitutions, we found no evidence that the remaining phosphatase activity is regulated by osmolarity (Fig. 5). In striking contrast, increased medium osmolarity does stimulate the kinase activity of the K^+ P^- mutant protein EnvZ473 (34). Although both of these are mutant proteins, they more closely resemble wild-type EnvZ than does Taz. Perhaps they provide a more useful model for what the wild-type protein actually does.

We propose that the simplest way to control the activities of EnvZ is to vary the ratio of EnvZ-P to EnvZ, the two forms of EnvZ predicted by the overlapping-site model, by regulating the autokinase activity of EnvZ. Indeed, this is the regulated activity with CheA (39). Accordingly, high osmolarity activates the autokinase activity, resulting in kinase-active EnvZ-P; low osmolarity inactivates the autokinase activity, resulting in phosphatase-active EnvZ. This model explains what happens with the various EnvZ mutant proteins. The histidine-243 substitutions can never be autophosphorylated; thus, they always exhibit phosphatase activity that is unaffected by medium osmolarity (Fig. 5). The K^+ P^- mutant protein EnvZ473 cannot function as a phosphatase, even though it can presumably exist in both the phosphorylated and unphosphorylated forms. Since increased medium osmolarity can still produce an increased level of EnvZ473-P, the model predicts that some level of osmoregulation will occur, and this is what has been observed (34).

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ADDENDUM IN PROOF

In a recent study, K. Skarphol, J. Waukau, and S. A. Forst (J. Bacteriol. **179:**1413–1416, 1997) reached similar conclusions regarding the role of His-243 in the phosphatase activity of EnvZ.

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