The Critical Role of the Conserved Thr\textsuperscript{247} Residue in the Functioning of the Osmosensor EnvZ, a Histidine Kinase/Phosphatase, in Escherichia coli*

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The histidine kinase/phosphatase EnvZ helps Escherichia coli adapt to osmotic shock by controlling the phosphorylation state of the transcription factor OmpR, which regulates the levels of the outer membrane porin proteins OmpF and OmpC. We examined the effects of mutating the highly conserved Thr\textsuperscript{247} residue in EnvZ. Using purified C-terminal domains of wild-type and mutant EnvZ proteins, we demonstrate that Thr\textsuperscript{247} plays a vital role in EnvZ function, variously affecting its autokinase and phosphotransferase activities, but mostly its function as a phosphatase. The cytoplasmic domain of EnvZ (EnvZc) is composed of three segments: the linker domain (residues 180–222), domain A (residues 223–289), and domain B (residues 290–450). It has been shown that the isolated domain A itself can dephosphorylate phosphorylated OmpR. Here we show that mutating Thr\textsuperscript{247} to Arg in domain A abolishes its phosphatase activity. Furthermore, using an \textit{in vivo} \(\beta\)-galactosidase activity assay of Taz1-1 (hybrid of the aspartate receptor Tar and EnvZ) constructs of the Thr\textsuperscript{247} mutants in RU1012 cells expressing \textit{ompC-lacZ}, we demonstrate that the external signal primarily down-regulates the phosphatase activity of EnvZ. Of the nine EnvZc(Thr\textsuperscript{247} mutant) constructs expressing \textit{ompC-lacZ} activity assay of Taz1-1 (hybrid of the aspartate receptor Tar and EnvZ) constructs of the Thr\textsuperscript{247} mutants in RU1012 cells expressing \textit{ompC-lacZ}, we demonstrate that the external signal primarily down-regulates the phosphatase activity of EnvZ. Of the nine EnvZc(Thr\textsuperscript{247} mutant) constructs expressing \textit{ompC-lacZ}, we demonstrate that the external signal primarily down-regulates the phosphatase activity of EnvZ.

One of the most extensively studied His-Asp phosphorelay signal transduction systems is the EnvZ/OmpR-mediated osmosensory pathway in Escherichia coli (8–10). EnvZ, located in the inner cytoplasmic membrane, undergoes ATP-dependent \textit{trans-autophosphorylation} (11, 12) on the conserved His\textsuperscript{243} residue (13). It subsequently transfers this phosphoryl group to the conserved Asp\textsuperscript{535} residue on OmpR (14–17). Phosphorylated OmpR (OmpR-P)\textsuperscript{1} functions as a transcription factor for \textit{ompF} and \textit{ompC} genes, encoding the outer membrane porin proteins OmpF and OmpC, respectively (18, 19). The level of OmpR-P in the cell reciprocally regulates the production of these porins, with OmpF and OmpC being predominantly produced under low and high osmolar conditions, respectively (18, 20–22).

Structurally, EnvZ consists of an N-terminal short cytosolic segment, the first membrane-spanning segment (TM1), a periplasmic domain, the second transmembrane segment (TM2), and a C-terminal cytosolic domain. The cytosolic domain has the highly conserved regions for histidine kinases: the H, N, F, G\textsubscript{1}, and G\textsubscript{2} boxes (3) and the recently recognized G\textsubscript{3} box (23). Domain dissection studies revealed that the enzymatically active kinase/phosphatase segment (residues 223–450) of EnvZ consists of two complementary functional domains (26): the 67-residue dimerization and histidine phosphotransfer domain A (the DHp domain, residues 223–289) and the 161-residue catalytic and ATP-binding domain B (the CA domain, residues 289–450) (27). The linker domain (residues 180–222) connects the kinase/phosphatase segment to TM2 and is responsible for transducing the signal (see Fig. 1A) (24, 28). Recently, the NMR solution structures of domain B (29) and domain A (30) of EnvZ have been solved.

The NMR solution structure of domain A reveals that the dimerization core of EnvZ is formed by a parallel association of helical hairpins (helixes I and II), resulting in a four-helix bundle (30). The invariant His\textsuperscript{243} residue is located in the middle of helix I of each subunit, completely exposed to the solvent. His\textsuperscript{243} is involved in both the kinase and phosphatase activities of EnvZ (31–33). No other amino acid residues have been implicated in the enzymatic functions of EnvZ or any other histidine kinase/phosphatase.

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1 The abbreviations used are: OmpF-P, phosphorylated OmpR; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis; AMP-PNP, adenosine 5′-(\(\beta,\gamma\)-imino)triphosphate.
In this study, we report the identification of Thr247 in the H box on helix I of domain A as a critical active-site residue in EnvZ. Using EnvZc(T247X) mutant proteins, generated by site-directed mutagenesis, we provide clear evidence that Thr247 plays an important role, affecting all EnvZ catalytic activities, most importantly, the phosphatase function.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids Expressing EnvZc(T247X) Mutants—** Plasmid pET11a-EnvZc (28) was used to express the cytoplasmic domain of EnvZ (EnvZc, residues 180–450) and the plasmid pET11a-EnvZ(T247R) (EnvZ11) was used to express the EnvZ11 mutant of Thr247, EnvZ(T247R) (EnvZ11), was first reported as a TP-1-resistant mutant (38), and the pTazT247X plasmids and the vector plasmid pTTII (24) were each transformed into the E. coli host, BL21(DE3), and then transformed into TG-1 competent cells. The cells harboring different plasmids were cultured to mid-log phase when 1 mM isopropyl-

β-D-thiogalactopyranoside was added. After another 3-h incubation, the cells were harvested and broken with a French press. The EnvZc and EnvZc(T247X) mutant proteins were purified by 40% ammonium sulfate fractionation and subsequent Sephacryl S-100HR gel-filtration chromatography (15). The purified proteins were quantified using the Bio-Rad protein assay reagent.

**Trypsin Digestion—** The EnvZc and EnvZc(T247X) mutant proteins (30 μg) were digested with 0.3 μg of trypsin (Trypsin Type XI, Sigma) in digestion buffer (50 mM Tris-HCl (pH 8.0) containing 150 mM KCl, 20 mM β-mercaptoethanol, 20% glycerol, and 1 mM EDTA) at room temperature for 30 and 60 min. The reaction was stopped by adding 2× Tricine-SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (0.9 mM Tris-HCl (pH 8.45), 24% glycerol, 8% (v/v) SDS, and 0.005% protein red) and boiling for 5 min. The products were analyzed by Tricine-PAGE (9.6% spacer gel and 16.5% separation gel) (34) after staining with Coomassie Blue.

**Autophosphorylation Reaction—** The EnvZc and EnvZc(T247X) mutant proteins (2 μM) were each incubated at room temperature in autokinase reaction buffer (50 mM Tris-HCl (pH 8.0) containing 50 mM KCl, 5 mM CaCl₂, 5 mM β-mercaptoethanol, and 5% glycerol) with 50 μM ATP and 0.25 μl of [γ-32P]ATP (3000 Ci/mmol, 10 mCi/ml) by guest on July 24, 2018http://www.jbc.org/Downloaded from
Glu, Lys, Cys, Ser, Tyr, and Arg) at Thr247 in EnvZ did not significantly affect the conformation of the protein. These results indicate that the substitutions we made (Ala, Glu, Lys, Arg, Cys, Ser, or Tyr) by site-directed mutagenesis.

**Construction and Purification of EnvZc(T247X) Mutant Proteins**—We mutated plasmid pET11a-EnvZc, which expresses the cytoplasmic domain of EnvZ (residues 180–450), and replaced Thr247 with Ala, Glu, Lys, Arg, Cys, Ser, or Tyr by site-directed mutagenesis. The various plasmids were transformed into E. coli BL21(DE3) cells. The proteins were overexpressed and subsequently purified by a two-step procedure involving 40% ammonium sulfate precipitation and Sephacryl S-100 gel-filtration chromatography. All the mutant proteins were soluble and stable upon storage at −20 °C.

**EnvZc(T247X) Mutant Proteins Have Trypsin Digestion Profiles Similar to That of EnvZc**—To check whether the mutation of Thr247 in EnvZ resulted in any major alteration of conformation in the mutant proteins, they were subjected to trypsin digestion. 30 μg of each protein was digested with 0.3 μg of trypsin at room temperature. Aliquots were removed at 30 and 60 min and analyzed by Tricine/SDS-PAGE. No remarkable change was observed in the digestion profiles of the EnvZc and EnvZc(T247X) mutant proteins (Fig. 2). Whereas the EnvZc (T247E), EnvZc(T247K), and EnvZc(T247A) mutant proteins were more susceptible to trypsin digestion (in decreasing order) than EnvZc, the EnvZc(T247R) protein was more resistant. These results indicate that the substitutions we made (Ala, Glu, Lys, Cys, Ser, Tyr, and Arg) at Thr247 in EnvZc did not significantly affect the conformation of the protein.

**Mutation of Thr247 in EnvZ Variably Affects Its Autokinase Activity**—The autophosphorylation activities of the EnvZc(T247X) mutant proteins were compared with that of EnvZc in an *in vitro* biochemical assay using [*γ*-*32*P]ATP as described under “Experimental Procedures.” The time course of incorporation of the phosphoryl group into the proteins was followed and is shown in Fig. 3 (A and B). Interestingly, the incorporation of the phosphoryl group into the mutant protein was variable, depending on the specific substitution (Tyr > Arg > Ser > Cys > Ala > Lys > Glu). Densitometric analysis revealed that the maximum level of incorporation of the phosphoryl group into EnvZc(T247Y) and EnvZc(T247R) was ~1.6 times higher than that into EnvZc (Fig. 3B), whereas the incorporation into the EnvZc(T247E), EnvZc(T247K), and EnvZc(T247A) mutant proteins was significantly lower that that into wild-type EnvZc. Of the mutant proteins, EnvZc(T247S) followed by EnvZc(T247C) exhibited levels of autokinase activity that were close to that of wild-type EnvZc (Fig. 3B). Since the autokinase levels varied significantly with the nature of the substituting amino acid, it is unlikely that Thr247 plays a catalytic role in the autokinase activity of EnvZc. It is clear though that Thr247 significantly influences the autophosphorylation reaction.

***EnvZc(T247R) Mutant Protein (EnvZ11) Has a Lower Requirement for Divalent Metal Ions than EnvZc for Its Autokinase Activity***—The autophosphorylation reaction of EnvZc is metal ion-dependent (39). To further investigate the higher rate of autophosphorylation observed in EnvZc(T247R) and EnvZc(T247Y), we compared the divalent metal ion dependences of the autokinase activities of these two mutant proteins with those of EnvZc and EnvZc(T247S). Each protein was autophosphorylated in autokinase reaction buffer containing either 10 mM EDTA or 10 mM MgCl2. Aliquots were removed at 1 and 3 min, and the course of the reaction was followed as shown in Fig. 4. EnvZc(T247R) was unique in being able to autophosphorylate well not only in the presence of Mg2+ ions (Fig. 4, lanes 9–12), but also in the absence of divalent metal ions (10 mM EDTA). Interestingly, EnvZc(T247Y), which also has a higher rate of autophosphorylation compared with wild-type EnvZc, did not show a similar divalent metal ion independence (Fig. 4, lanes 13–16). This aspect is being separately investigated.

***EnvZc(T247X) Mutant Proteins Are Impaired in Their Phosphotransferase Activity***—Phosphorylated EnvZ transfers its phosphoryl group from the phosphorylated EnvZ donor protein was followed by phosphotransferase experiment was conducted on ice (Fig. 5A). In all the mutant proteins with the exception of EnvZc(T247S) and EnvZc(T247A), the transfer of the phosphoryl group from the phosphorylated EnvZ donor protein was distinctly impaired. The EnvZc(T247K) mutant protein displayed the least phosphotransferase activity, with very little OmpR-P detectable even 5 min after the reaction (Fig. 5A). These differences became much more pronounced when the phosphotransferase experiment was conducted on ice (Fig. 5B). Following the decrease in phosphorylated EnvZc with time, it was evident that at the end of 30 s, hardly any loss of the phosphoryl group from the phosphoryl group from the EnvZc(T247K)Y/C/R/E) mutant proteins had occurred. Only the conservative mutations EnvZc(T247S) and EnvZc(T247A) exhibited phosphotransferase activities that were close to wild-type levels (Fig. 5, A and B). These results clearly indicate that residue 247 in EnvZ strongly influences the phosphotransfer reaction between EnvZ and OmpR.
son of the in vitro phosphatase activities of the mutant proteins was performed. OmpR-P purified of free ATP was incubated with each EnvZc(T247X) protein at room temperature in phosphate buffer. Aliquots were removed at 1, 2.5, 5, and 10 min, and the time course of the reaction was followed as shown in Fig. 6 (A and B). Most significantly, of all the mutant proteins, only EnvZc(T247S) displayed phosphatase activity comparable to that of EnvZc. The phosphatase activities of EnvZc(T247A) and EnvZc(T247Q) were negligible compared with that of wild-type EnvZc. Furthermore, no dephosphorylation of OmpR was detected with the EnvZc(T247Y) mutant protein. Interestingly, EnvZc(T247K), EnvZc(T247E), and EnvZc(T247R) displayed a reverse phosphotransfer reaction, where part of the phosphoryl group was transferred from OmpR-P back to the respective EnvZ mutant (Fig. 6A).

Comparison of the Enzymatic Activities of EnvZc(T247N) and EnvZc(T247Q) Mutant Proteins with That of EnvZc—The biochemical characterization of the EnvZc(T247A/E/K/C/S/R) mutant proteins strongly suggested that the conserved Thr<sup>247</sup> residue is critical for the phosphatase activity of EnvZ. Interestingly, the H<sup>+</sup> residue in the H box of the nitrogen sensor NtrB is replaced with asparagine. NtrB is the only bifunctional histidine kinase/phosphatase other than EnvZ whose phosphatase activity has been localized to an isolated domain equivalent to domain A of EnvZ (42, 43). Therefore, we also investigated two other substitution mutations of Thr<sup>247</sup>, T247N and T247Q.

The EnvZc(T247N) and EnvZc(T247Q) mutant proteins were overexpressed and purified following the same protocol used for the other EnvZc(T247X) proteins. The purified proteins were both stable upon storage at −20 °C. Limited trypsin digestion of the two proteins showed no significant difference in the digestion patterns (Fig. 7A). EnvZc(T247Q) appeared to be slightly more resistant to digestion by trypsin than wild-type EnvZc (Fig. 7A). The two proteins were then analyzed for their autokinase, phosphotransferase, and phosphatase activities using the same methods and protein concentrations as were used for the other EnvZc(T247X) mutants. Comparison of these activities with those of wild-type EnvZc is shown in Fig. 7 (B–E). Fig. 7B shows the comparison of the autophosphorylation activities; both EnvZc(T247N) and EnvZc(T247Q) exhibited very similar abilities to utilize ATP. The maximum level of incorporation of the phosphoryl group into EnvZc(T247N) and EnvZc(T247Q) was, however, 60% of that of wild-type EnvZc. Fig. 7C represents the phosphotransferase activities: whereas replacement of threonine with glutamine did not affect the ability of EnvZc to transfer its phosphoryl group to OmpR, the transfer was slow in the EnvZc(T247N) mutant. Importantly, the results of the phosphatase assays of the EnvZc(T247N) and
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EnvZ(T247Q) proteins revealed that both mutants exhibited low but detectable phosphatase activities (Fig. 7, D and E). Whereas the half-life of OmpR-P was ~30 s in the presence of EnvZ and 50 s in the presence of EnvZ(T247S), it was prolonged to 2 min with EnvZ(T247N) and still further to ~4 min with EnvZ(T247Q). Thus, EnvZ(T247N) exhibited 16.7% and EnvZ(T247Q) only 8.3% of the wild-type EnvZ phosphatase activity (Fig. 7E). Interestingly, the phosphatase activity of NtrB, in which the H-4 residue is asparagine, is negligible in the absence of the accessory protein PII (41).

Phosphatase Activity of the T247R Mutant in Domain A of EnvZ (Domain A(T247R))—We have recently demonstrated that the isolated domain A of EnvZ (residues 223–289) itself exhibits OmpR-P phosphatase activity (33). Domain B of EnvZ modulates this activity in the presence of cofactors like ADP and AMP-PNP. Since the EnvZ(T247X) mutants appear to be severely impaired in this function, we decided to test whether replacing threonine at position 247 in domain A would also cause a similar reduction in phosphatase function. As shown in Fig. 8, indeed, the domain A(T247R) mutant was also impaired in its ability to dephosphorylate OmpR-P.

Comparison of Kinase/Phosphatase (ATPase) Activities of EnvZ and EnvZ(T247X) Mutants—The net result of the autophosphorylation, phosphotransferase, and phosphatase activities of EnvZ can also be termed as EnvZ/OmpR-dependent ATPase activity, the final products of which are inorganic phosphate and ADP. We followed the course of the EnvZ/OmpR-dependent ATPase reaction for the wild-type EnvZ and EnvZ(T247S) (Fig. 8). The EnvZc and EnvZc(T247R) proteins were each incubated with 4 μM OmpR at room temperature (A) or at 4 °C (B). A, aliquots were removed at 20 and 40 s and 2 and 5 min. The reaction was stopped with 5× SDS loading buffer. The products were separated by 17.5% SDS-PAGE. The dried gel was exposed for autoradiography. B, aliquots were removed at 15 and 30 s, and the reaction was stopped and analyzed as described for A. EnvZc-P, phosphorylated EnvZc.

DISCUSSION

EnvZ, a histidine kinase/phosphatase, displays three enzymatic activities: autokinase, phosphotransferase (OmpR kinase), and OmpR-P phosphatase. The results presented in this study indicate that a balance of these activities is critical in modulating the cellular level of OmpR-P, which in turn reciprocally regulates the ompF and ompC genes encoding outer membrane porins (Figs. 9 and 10). Mutations of the conserved Thr^{247} residue in EnvZ tested in this study variously affect the three enzymatic activities of EnvZ, resulting in changes in the OmpR-P level (Fig. 9).

Substituting Thr^{247} alters the autokinase activity, from negligible (e.g., EnvZ(T247E)) to 1.6-fold higher than that of wild-type EnvZ (EnvZ(T247R) and EnvZ(T247Y)) (Fig. 3). EnvZ(T247R) is unique in that its autokinase is highly active even in the absence of Mg^{2+}, whereas wild-type EnvZc and all the other EnvZc(T247X) mutants proteins, including EnvZc-

room temperature. Aliquots were removed at 30 and 60 min; reactions were stopped; and products were analyzed as described under “Experimental Procedures.” As shown in Fig. 9A, OmpR-P was barely detectable at the end of 1 h of incubation for EnvZc and EnvZc(T247S) protein reactions. This indicates that under the conditions used, the rate of dephosphorylation of OmpR-P was equal to or higher than the rate of OmpR phosphorylation in these reactions. In contrast, high levels of OmpR-P accumulated in the reaction mixtures of the EnvZc(T247N/Q/R) mutants. The highest accumulation of OmpR-P was observed for the EnvZc(T247R) mutant protein, which has enhanced autokinase activity, but negligible phosphatase activity. Note that the EnvZc(T247N) protein, which has 60% autokinase activity and only 16.7% of the wild-type phosphatase activity, accumulated appreciable amounts of OmpR-P. Although both EnvZc(T247S) and EnvZc(T247Q) reaction mixtures showed low levels of OmpR-P accumulation at 30 min, very little OmpR-P was detectable at 60 min in the case of EnvZc(T247S), whereas in the EnvZc(T247N) reaction, higher OmpR-P levels were maintained. The relative level of OmpR-P accumulation was estimated by PhosphorImager analysis by taking the OmpR-P value of the EnvZc(T247R) reaction at 30 min as 100 (Fig. 9B). When the amount of OmpR-P produced in the wild-type EnvZc reaction was taken as 1, the relative OmpR-P levels were calculated to be 1, 4, 8, 6, 159, and 365 at 30 min and 1, 2, 8, 7, 116, and 393 at 60 min for EnvZc, EnvZc(T247S), EnvZc(T247A), EnvZc(T247N), EnvZc(T247Q), and EnvZc(T247R), respectively.

ompC-lacZ Expression of Taz1-1(T247X) Mutants—Taz1-1 is a derivative of the hybrid receptor Taz1, in which the receptor domain of Tar, an aspartate chemoreceptor, is fused with the cytoplasmic signaling domain of EnvZ (24, 35). It has been previously demonstrated that Taz1-1 responds to aspartate in E. coli RU1012 cells (24). In the absence of a known ligand for osmolarity, the Taz constructs have been successfully employed to study the regulation of EnvZ function in vivo by monitoring the production of β-galactosidase.

We took advantage of this system to investigate the phenotypic effect of introducing the T247X mutations in Taz1-1. The plasmids carrying mutant taz1-1 genes were transformed into E. coli RU1012 cells. The transformed cells were incubated in the presence of 0, 1, 2, 5, and 7 mM aspartate in M9 medium until they reached mid-log phase and then were assayed for β-galactosidase activity. As shown in Fig. 10, Taz1-1(T247S) was the only aspartate-regulatable mutant. All other Taz1-1(T247X) mutants resulted in an OmpC^{-} (LacZ') constitutive phenotype. It is important to note that both Taz1-1(T247N) and Taz1-1(T247Q) also exhibited OmpC^{-} constitutive phenotypes.

**Fig. 5.** Phosphotransfer from phosphorylated EnvZc and EnvZc(T247X) mutant proteins to OmpR. The EnvZc and EnvZc(T247X) mutant proteins were each first autophosphorylated and purified of free ATP as described under “Experimental Procedures”. 2 μM phosphorylated wild-type EnvZc (WT) or EnvZc(T247X) mutant proteins were each incubated with 4 μM OmpR at room temperature (A) or at 4 °C (B). A, aliquots were removed at 20 and 40 s and 2 and 5 min. The reaction was stopped with 5× SDS loading buffer. The products were separated by 17.5% SDS-PAGE. The dried gel was exposed for autoradiography. B, aliquots were removed at 15 and 30 s, and the reaction was stopped and analyzed as described for A. EnvZc-P, phosphorylated EnvZc.
require Mg$^{2+}$ ion for optimal autokinase activity (Fig. 4). It seems plausible that the guanidino group of arginine replaces the Mg$^{2+}$ ion of the MgATP complex that binds wild-type EnvZc. Such a replacement has been proposed for histidyl-tRNA synthetases, which harbor an arginine at a position where the other aminoacyl-tRNA synthetases bind a catalytic Mg$^{2+}$ ion (44). This interesting possibility for the EnvZc-(T247R) mutant is currently under investigation.

Mutating Thr 247 in EnvZc had more severe effects on its phosphotransferase activity. With the exception of EnvZc-(T247S), EnvZc(T247A), and EnvZc(T247Q), all the other EnvZc(T247X) mutant proteins were impaired in transferring their phosphoryl groups to OmpR (Figs. 5 and 7C). The consequence of mutations of Thr 247 was most remarkable for the phosphatase activity. Of the nine mutant proteins, only EnvZc(T247S) exhibited phosphatase activity comparable to that of wild-type EnvZc (Figs. 6 and 7D). The hierarchy of the activity was as follows: EnvZc(T247S), EnvZc(T247N), and EnvZc(T247Q), 40, 16.7, and 8.3% of the wild-type EnvZc activity, respectively. All others displayed negligible phosphatase activity (Figs. 6 and 7D). Interestingly, EnvZc(T247K), EnvZc(T247E), and EnvZc(T247R) showed some reverse phosphotransfer from OmpR-P (Fig. 6). Such reverse phosphotransfer activity from OmpR-P to EnvZ has been previously reported for the EnvZc(N347D) mutant protein (32). In each of these cases, a charged residue substitutes for a polar residue, suggesting that the charge environment at the active-site histidine plays a role in shifting the reaction equilibrium in favor of the back-reaction.

The isolated domain A of EnvZ carrying the T247R mutation (domain A(T247R) protein) exhibits autokinase and phosphotransferase activities similar to those observed for the C-terminal domain of EnvZ carrying the same T247R mutation (EnvZc(T247R) protein) (data not shown). Replacing Thr with Arg in domain A of EnvZ also abolished its intrinsic phosphatase function (Fig. 8), strongly supporting the notion that Thr$^{247}$ plays a critical role in EnvZ function. The role of domain A in EnvZ function has been analyzed recently (33). The results obtained with the domain A(T247R) protein strengthen the view that domain A of EnvZ is not only the dimerization and histidine phosphotransfer domain, but also the phosphatase domain. Although domain B was considered to be the catalytic and ATP-binding domain (27), the present evidence substantiates the notion that domain A is the true catalytic unit responsible for all three activities of EnvZ, whereas domain B is the ATP-binding and regulatory unit.

The present results demonstrating that Thr$^{247}$ can be replaced only with Ser to retain comparable levels of all three EnvZ activities clearly indicate that Thr$^{247}$ is a critical residue at the active center of EnvZ. However, the extent of involvement of Thr$^{247}$ in the three activities is likely to be different. It appears that Thr$^{247}$ might be directly involved in catalyzing the phosphatase reaction while facilitating the autokinase and phosphotransferase reactions. Whereas the exact mechanism of the phosphatase reaction is unknown, we speculate that the imidazole of the proximal His$^{243}$ residue could be functioning as an acid-base catalyst. It could enhance the nucleophilicity of the hydroxyl group of Thr$^{247}$, thereby enabling it to directly
attack the phosphorus of the phosphoryl moiety on Asp55 of OmpR-P, forming a highly reactive ester acyl-enzyme, which is rapidly hydrolyzed. Alternatively, the hydroxyl group of Thr247 could potentiate the oxygen of a bound water molecule to make a nucleophilic attack on the phosphorus atom on Asp55. The structure of the N-terminal domain of OmpR has not been solved. However, five water molecules are found in the active site of the Mg²⁺-bound structure of the homologous response regulator CheY (45). On the other hand, the use of the threonine hydroxyl rather than the direct attack of a water molecule on the substrate is considered to be more favorable as alcohols are often better nucleophiles than water molecules (46). However, it has been clearly demonstrated that His243 plays an important role in the phosphatase function of EnvZ (31, 33, 47).
Therefore, a model that involves both the invariant His\textsuperscript{243} residue and the highly conserved Thr\textsuperscript{247} residue in the phosphatase function of EnvZ seems to be more likely. Note that the structural dynamism of the H box segment (residues 242–248), which includes Thr\textsuperscript{247}, might also play an indirect role in this process.

The role of the histidine kinase in the phosphotransfer reactions between the kinase and the response regulator in the His-Asp signaling systems has been debated. Response regulators can use small molecule phosphodonor such as phosphohistidine and acetyl phosphate directly (48, 49). The half-lives of phosphoaspartate groups under denaturing conditions are generated and acetyl phosphate directly (48, 49). The half-lives of phosphoaspartate groups under denaturing conditions are generated. The ATP-binding domains of histidine kinases are being determined domain in this class of enzymes (30). On the other hand, as the nucleotide-binding CA domain (domain B) share extensive homology with the Hsp90, Mut1, and gyrase families of ATPases (25), antibiotics developed against domain B may have some cross-reactivities with mammalian enzymes.

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The Critical Role of the Conserved Thr\textsuperscript{247} Residue in the Functioning of the Osmosensor EnvZ, a Histidine Kinase/Phosphatase, in \textit{Escherichia coli}
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