Reverse Phosphotransfer from OmpR to EnvZ in a Kinase\(^-\)/Phosphatase\(^+\) Mutant of EnvZ (EnvZN347D), a Bifunctional Signal Transducer of Escherichia coli

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EnvZ of Escherichia coli is a transmembrane histidine kinase belonging to the family of two-component signal transducing systems prevalent in prokaryotes and recently discovered in eukaryotes. In response to changes in medium osmolality EnvZ regulates the level of phosphorylated OmpR, its conjugate response-regulating transcription factor for ompF and ompC genes. EnvZ has dual opposing enzymatic activities; OmpR-phosphorylase (kinase) and phospho-OmpR-dephosphorylase (phosphatase). The osmotic signal is proposed to regulate the ratio of the kinase to the phosphatase activities of EnvZ to modulate the level of OmpR phosphorylation. In this work we used a COOH-terminal fragment of a previously identified kinase/phosphatase\(^+\) EnvZ mutant (EnvZN347D) to demonstrate that the phosphorylation on the OmpR-OmpR is transferred back to EnvZ to the same histidine residue (His\(^{243}\)) that is utilized for the autokinase reaction by the wild type protein. Phospho-EnvZN347D thus formed could also transfer its phosphoryl group back to OmpR. The phosphotransfer reaction from phospho-OmpR to EnvZN347D was inhibited by ADP while Mg\(^{2+}\) ions stimulated the dephosphorylation reaction, resulting in release of inorganic phosphate. These results indicate that the energy levels of phosphoryl groups on OmpR and EnvZ are very similar and that the phosphatase reaction in the EnvZN347D mutant involves a reversal of the phosphotransfer reaction from EnvZ to OmpR using the identical His\(^{243}\) residue.

Phosphorylation and dephosphorylation of cellular proteins plays a critical role in signal transduction to regulate numerous cellular functions in both prokaryotes and eukaryotes. Protein histidine kinases and their response regulators, so-called "two-component" systems, constitute a large family (>50) of signal transducing systems that enable bacteria to adapt to the changing environment (reviewed by Parkinson and Kofoid (1992)). Classical eukaryotic protein kinases are tyrosine, serine, and threonine kinases. Recently, however, histidine kinases have been found not only in yeast (Maeda et al., 1994; Ota and Varshavsky, 1993) but also in plants (Chang et al., 1993) (reviewed by Alex and Simon (1994) and Swanson et al. (1994)) and in mammalian cells (Crovello et al., 1995). This represents a novel paradigm for eukaryotic cell signaling. Prokaryotic histidine kinases exhibit considerable homology in their COOH-terminal domains, and it is believed that they share a common mechanism of action.

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Exposure of bacteria to high osmolarity leads to dehydration, collapse of ion gradients over the cytoplasmic membrane, and decrease in cell viability. Therefore, the first response of bacteria to osmotic stress consists of changes in the activities of enzymes and transport systems so that the turgor pressure is restored and the cytoplasmic environment is optimized. Somewhat later, changes in gene expression provide additional flexibility in adapting cells to osmotic shock (for reviews see Csonka (1989), and Csonka and Hanson (1991)). One of the means that Escherichia coli adopts to handle osmotic stress is to modulate the type of diffusion pores that exist in the outer membrane (Csonka and Hanson, 1991). These pores are formed by homotrimERIC association of the porin proteins, OmpF and OmpC. These proteins are highly expressed (approximately 10\(^3\) molecules/cell), and the rate of diffusion through OmpF has been measured to be 10 times faster than through OmpC (Nikaido and Vaara, 1985). The expression of these two porins is differentially regulated through the members of the omp8 operon, ompR and envZ (Hall and Silhavy, 1981; Forst and Inouye, 1988). OmpF is preferentially produced at low osmolarity and OmpC at high osmolarity.

EnvZ protein is a bifunctional histidine kinase/phosphatase. It is a trans-inner membrane osmosensor consisting of 450 amino acids. It contains two transmembrane domains (16–46 and 163–179), a 115-amino acid residue periplasmic domain (47–162), a 270-amino acid residue COOH-terminal cytoplasmic domain, and a short (1–15) NH\(_2\)-terminal cytoplasmic region. Conserved amino acid residues that EnvZ shares with other histidine kinases include His\(^{243}\), the autophosphorylation site, Asn\(^{347}\) (function unknown), and two glycine-rich segments DXGXG (373–377) and GXG (403–405) (putative ATP-binding domains). EnvZ autophosphorylates His\(^{243}\) using ATP and then transfers the phosphoryl group to a transcriptional factor, OmpR (Igo and Silhavy, 1988; Forst et al., 1989; Aiba et al., 1989). OmpR is a cytoplasmic protein of 239 amino acid residues. It has an NH\(_2\)-terminal regulatory domain that bears significant homology to other response regulators of the two-component system and a COOH-terminal DNA-binding domain. EnvZ phosphorylates OmpR on Asp\(^{55}\) (Delgado et al., 1993). EnvZ also dephosphorylates phospho-OmpR (Igo et al., 1989; Aiba et al., 1989a). The concentration of phosphorylated OmpR in the cell differentially regulates the transcription of ompF and ompC genes for outer membrane porins, OmpF and OmpC (Sarma and Reeves, 1977; Hall and Silhavy, 1981; Mizuno and Mizushima, 1987; Slauch and Silhavy, 1989). Of the kinase and phosphatase activities of EnvZ, the latter is considered to be regulated by the osmotic signal, which thereby controls the levels of phosphorylated OmpR in the cell (Yang and Inouye, 1991; Russo and Silhavy, 1991; Yang et al., 1993; Jin and Inouye, 1993).

Taz1 is a hybrid receptor consisting of the NH\(_2\)-terminal (256-residue) ligand-binding domain of Tar (a chemoreceptor
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for aspartate) and the COOH-terminal (228-residue) signaling domain of EnvZ (Utsumi et al., 1989). This chimeric receptor induces ompC-lacZ expression in response to aspartate, thus enabling one to study EnvZ function in response to a well-defined ligand (the natural ligand for EnvZ is unknown). It has been shown that all substitution mutants at the conserved Asn residues used for substitution (D, E, Q, H, A, T, and C). The Asn substitution mutants could complement the H277V (the autophosphorylation site) mutant of Tacl in vivo to restore wild type levels of osmoregulation. For a finer analysis of the role of the conserved Asn in EnvZ, the 271-residue (Arg180–Gly450) cytoplasmic domain of the mutant EnvZ-N347D(C) was purified, and its enzymatic activity was characterized. We note how the previous report that the phosphatase reaction of EnvZ-N347D(C) involves the reverse phosphotransfer of the phosphoryl group from OmpR to EnvZ to the identical His243 residue which is the site of autophosphorylation. The dephosphorylation of phospho-OmpR by the mutant protein was found to be inhibited by ADP but stimulated by Mg2+ ions. These results indicate that the phosphatase activity of EnvZ-N347D(C) involves the reverse phosphotransfer of the phosphoryl group from EnvZ to OmpR using the identical His243, the autophosphorylation site.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

Nitrocellulose membranes were from Schleicher & Schuell. Alkaline phosphatase-conjugated goat anti-rabbit antibody was from Bio-Rad. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt was from Life Technologies, Inc. [γ-32P]ATP was obtained from Amersham. Endoproteinase Lys-C (sequencing grade) was purchased from Boehringer Mannheim.

Cell Strain and Construction of Plasmids

E. coli B strain BL21-DE3 (FompR rB mB) was used for the expression of the envZ(C) and envZ-N347D(C) gene fragments cloned into the pEt11a vector. The 1.5-kilobase pair EcoRI/EcoRI DNA fragment corresponding to the 271-amino acid residue (Arg180–Gly450) cytoplasmic domain of EnvZ (EnvZ(C)) was recloned from the ompC-envZ plasmid previously constructed in the laboratory (Forst et al., 1989) into a pEl11a T7 expression system to construct pEt11aE-Z(C). The EnvZ(C) protein thus expressed contains four extra amino acids (Met, Ala, Gly, Ile) at the amino-terminal end. The mutagenesis of envZ was performed by site-directed mutagenesis using M13 as described previously (Forst et al., 1989). The envZ-N347D(C) mutant was then subcloned into pEl11aE in the same manner as the wild type gene.

Purification of EnvZ(C) and EnvZ-N347D(C) Protein Fragments

EnvZ(C) was purified from E. coli B strain BL21-DE3 (FompR rB mB) transformed with pEt11aE-Z(C) plasmid by a modification of the procedure described previously (Forst et al., 1989). The protein was precipitated using 30% ammonium sulfate saturation instead of 40%. The Aff-Gel blue affinity column was substituted with a Green A affinity column from which the protein was eluted with a linear gradient 0–1.5 M KCl in buffer A (20 mM Tris-Cl (pH 7.8) containing 5% glycerol, 10 mM β-mercaptoethanol, and 1 mM EDTA). Protein fractions were pooled and centrinic-concentrated.

Preparation of Membrane Fractions

PDR 200 cells harboring wild type EnvZ was grown in LB medium. Mid-logarithmic-phase cells were harvested by centrifugation and washed once and sonicated in buffer B (0.1 M sodium phosphate (pH 7.2) containing 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA, 5 mM 1,10-phenanthroline, 0.3 mM p-hydroxymercuribenzoate, and 1 μM leupeptin). Cell debris was removed by centrifugation at 10,000 x g for 15 min. The supernatant was then ultracentrifuged at 180,000 x g for 90 min at 4°C. The pellet was washed with buffer C (20 mM Tris-Cl (pH 7.8), 5% glycerol, 1 mM EDTA, 10 mM β-mercaptoethanol) containing 2 mM KCl and sonicated in the same buffer and then centrifuged at 393,000 × g for 14 min. This washing step was repeated. The resulting membrane fraction (EnvZ(M)) was resuspended in buffer C. The concentration of total protein in the membrane preparation was estimated by the Bio-Rad assay.

In Vitro Autophosphorylation and pH Stability Assays

EnvZ membrane preparations or soluble purified cytoplasmic proteins were incubated in 0.1 M Tris-Cl buffer (pH 8.0) containing 50 mM KCl, 5 mM CaCl2, 1 mM PMSF, and 10% glycerol with 0.4 μM[γ-32P]ATP (1,100 cpm/pmol) at 25°C for 10 min. The reaction was stopped by adding 5× SDS gel loading buffer containing 10% (w/v) SDS, 3 mM β-mercaptoethanol, and 40% glycerol. The reaction mixture was then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis protein bands were transferred to a BA85 nitrocellulose membrane (0.45 μm). The membrane was exposed for autoradiography.

Dephosphorylation of Phosphorylated OmpR by EnvZ Cytoplasmic Proteins

The phosphatase activity of EnvZ-soluble cytoplasmic proteins was measured as follows.

Step 1: Autophosphorylation of EnvZ(M) for 20 Min—EnvZ(M) (50 μg) was autophosphorylated with [γ-32P]ATP (100 μCi, specific activity: 3,000 Ci/mmol) in 200 μl of 0.1 M Tris-HCl buffer (pH 8.0) containing 50 mM KCl, 5 mM CaCl2, 1 mM PMSF, and 10% glycerol) at 25°C for 20 min. The reaction mixture was centrifuged at 393,000 × g for 14 min at 4°C using a Beckman TL 100 ultracentrifuge. The membrane pellet was washed three times with 0.1 M Tris-HCl buffer (pH 8.0) containing 50 mM KCl, 5 mM CaCl2, 1 mM PMSF, and 10% glycerol, sonicated, and then resuspended in the same buffer. The suspended membrane fraction was centrifuged and washed six more times. The removal of [γ-32P]ATP and P, in the membrane fraction was monitored by thin-layer chromatography. The final membrane fraction containing phosphorylated EnvZ(M) was resuspended in 200 μl of the same washing buffer.

Step 2: Isolation of Phosphorylated OmpR Protein—Purified OmpR protein (approximately 15 μg) was incubated with the membrane fraction containing phosphorylated EnvZ protein in a total volume of 140 μl for 20 min at 25°C. After incubation the reaction mixture was centrifuged at 393,000 × g for 14 min to remove the EnvZ-containing membrane. The 120-μl supernatant containing phosphorylated OmpR was collected and immediately used as substrate for the phosphatase assay.

Step 3: OmpR Phosphatase Assay—Phosphorylated OmpR protein (1.7 μM) was mixed with purified cytoplasmic fragment of EnvZ (EnvZ(C) or EnvZ-N347D(C) (8.7 μM) and ADP (final concentration: 1 mM) in phosphate buffer (0.1 M Tris-HCl buffer (pH 8.0) containing 50 mM KCl, 5 mM CaCl2, 1 mM PMSF, and 10% glycerol) for the required length of time at 25°C. The reaction was stopped by adding 5× SDS gel loading buffer and analyzed by SDS-PAGE, transferred to nitrocellulose, and then subjected to autoradiography as described above.

1 J. Delgado and M. Inouye, unpublished data.

2 The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
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Fig. 1. Autophosphorylation and phosphotransferase activity of EnvZ(N347D(C) and EnvZ(C)). Two micrograms of purified cytoplasmic proteins EnvZ(C) (lane 1) and EnvZ(N347D(C) (lane 3) were incubated in 0.1 M Tris-HCl buffer (pH 8.0) containing 50 mM KCl and 5 mM CaCl₂ with 0.4 mM [γ-32P]ATP (1,000 cpm/pmol) at 25 °C for 10 min. The reaction was stopped by the addition of 5 × SDS gel loading buffer. In a duplicate set of reactions (lanes 2 and 4) purified OmpR protein (0.6 μg) was added to the reaction mixture at the end of 5 min and further incubated for 15 min before stopping the reaction with 5 × SDS sample buffer. The reaction mixtures were then subjected to 17.5% SDS-PAGE analysis. After electrophoresis protein bands were transferred to a nitrocellulose membrane and exposed for autoradiography. Protein bands were visualized with anti-EnvZ polyclonal antiserum as described under “Experimental Procedures.” A, SDS-PAGE autoradiogram. B, immunoblot of the same gel as in A.

Reverse Transfer of Phosphoryl Group from EnvZ(N347D(C) to OmpR Protein

Phosphorylated OmpR protein was used as substrate for the phosphatase assay for EnvZ(N347D(C) as described above. For the study of the reverse transfer of the phosphoryl group from the mutant protein, purified non-phosphorylated NH₂-terminal fragment of OmpR (1 μg, obtained from S. Harlocker (Delgado et al., 1993)) was added to the reaction mixture containing EnvZ(N347D(C) and incubated for an additional 10 min under the same conditions. The results were analyzed in the same manner as described above.

Endoproteinase Lys-C Digestion of Phospho-EnvZ(C) and Phospho-EnvZ(N347D(C)

EnvZ(C) protein (8 μg) was autophosphorylated as described above. EnvZ(N347D(C) protein was phosphorylated using phosphorylated OmpR following the protocol described above except that all the reactions were carried out in 25 mM Tris-HCl (pH 8.5) containing 1 mM EDTA and 1 μg of endoproteinase Lys-C. After incubation SDS loading buffer was added to the reaction mix and further incubated for 30 min at 40 °C. The digestion products were separated on a Tricine-SDS-PAGE gel (Schagger and von Jagow, 1987). The separating gel composition was 16.5% T and 6% C, while the stacking gel was 4% T and 3% C. Insulin B chain was used as a marker. The protein bands were transferred to nitrocellulose and exposed for autoradiography.

Fig. 2. Phosphorylation of EnvZ(N347D(C) by phospho-OmpR. Phosphorylated OmpR was prepared as described under “Experimental Procedures.” Lane 1, EnvZ(N347D(C) obtained by autophosphorylation with [γ-32P]ATP. Lane 2, EnvZ(N347D(C) incubated with OmpR. Lanes 3 and 4, EnvZ(N347D(C) incubated in phosphatase buffer with EnvZ(C) or EnvZ(N347D(C) (2 μg in each case) and ADP (final concentration: 1 mM) for 1 and 20 min, at 25 °C. The reaction was stopped by adding 5 × SDS gel loading buffer and analyzed by SDS-PAGE, transferred to nitrocellulose, and then subjected to autoradiography as described in the legend to Fig. 1. Lanes 1 and 6, EnvZ(N347D(C), 1 and 20 min, respectively; Lanes 7 and 8, EnvZ(C), 1 and 20 min, respectively.

RESULTS

The COOH-terminal Fragment of EnvZ(N347D Is Kinase / Phosphatase—The purified EnvZ(N347D(C) was tested for its ability to autophosphorylate in the presence of [γ-32P]ATP and thence to transfer the phosphoryl group to OmpR (kinase activity). Fig. 1A shows that EnvZ(C) was autophosphorylated (lane 1) and could concomitantly transfer the phosphoryl group to OmpR (lane 2). EnvZ(N347D(C), however, was deficient in autophosphorylation (lane 3), and therefore subsequent transfer of the phosphoryl group to OmpR was also not detected (lane 4). Equivalent quantities of purified COOH-terminal pro-
and incubated for a further 10 min (molar ratio of OmpR to OmpR(N) was 1:5). On incubation with OmpR(N) reverse transfer of the phosphoryl group was detected from EnvZ(N347D(C)) to OmpR and EnvZ(N347D(C)) at a molar ratio of 1:2. Aliquots were taken at 0.5, 1, 10, 30, and 45 min and analyzed by SDS gel electrophoresis (Fig. 4, lanes 1–5). By 30 min (lane 4), the ratio of \(^{32}\)P in OmpR to that of EnvZ(N347D(C)) reaches a constant (by densitometry), indicating that the phosphoryl transfer reaction between the two proteins has reached an equilibrium. At 45 min, three aliquots were taken. To one, additional non-phospho-OmpR was added (lane 6), to the second non-phospho-OmpR(N) was added (lane 7), and to the third both OmpR and OmpR(N) was added (lane 8). These reaction mixtures were incubated for another 10 min. From lanes 6–8, it is clear that on the addition of OmpR or OmpR(N) the phosphoryl group is transferred to the added component. On the basis of the distribution of \(^{32}\)P among these proteins, it appears that all three compete for the phosphoryl group. Also, OmpR(N) was not a better substrate than OmpR for the phosphoryl transfer from EnvZ(N347D(C)). It is to be noted that when phospho-OmpR(N) was used instead of phospho-OmpR for the phosphorylation of EnvZ(N347D(C)), it was at least 95% less efficient than that with phospho-OmpR (data not shown). This may suggest that the COOH-terminal domain of OmpR has some role in the reverse transfer of phosphoryl group from OmpR to EnvZ(N347D(C)).

Effects of ADP and Metal Ions on the Reverse Phosphotransfer—Since ADP, ATP, and non-hydrolyzable analogues of ATP are known co-factors that enhance the rate of the phosphatase reaction (Aiba et al., 1989a; Igo et al., 1989), we investigated the effect of ADP on the phosphatase reaction with EnvZ(N347D(C)). Interestingly, as the ADP concentrations increased from 0 to 10 mM, a progressive inhibition in the phosphoryl group transfer from phospho-OmpR to EnvZ(N347D(C)) was observed (Fig. 3, lanes 2–5). At 10 mM ADP the phospho-

transferring was completely blocked.

Next, we examined the effect of divalent cations on the phosphatase reaction. We carried out the reaction in 25 mM Tris-HCl (pH 8.5) in the absence of ADP and in the presence of 5 mM EDTA, 5 mM EGTA, 5 mM MgCl\(_2\), and 5 mM CaCl\(_2\). As shown in Fig. 5, lane 3, 5 mM EDTA inhibits the reaction. The EDTA inhibition can be reversed by addition of 5 mM MgCl\(_2\) (data not shown). In contrast, EGTA does not inhibit the reaction (Fig. 5, lane 4). These results indicate that Mg\(^{2+}\) is a necessary co-factor of the phosphatase reaction. Since very little phosphoprotein is detected in the presence of Mg\(^{2+}\), it clearly enhances the phosphatase reaction. Ca\(^{2+}\) on the other hand stabilizes phospho-OmpR and retards the phosphotransfer (lane 6).

Involvement of the Conserved Autophosphorylation Site, His\(^{243}\), in the Phosphatase Reaction of EnvZ(N347D(C))—To identify the residue phosphorylated in EnvZ(N347D(C)) by phospho-OmpR, we first examined its pH stability. Phospho-EnvZ(N347D(C)) was alkali stable (pH 13) but acid-labile (pH 1) (data not shown), which is characteristic of histidyl phosphate. To determine which histidine residue(s), of the six histidine residues in EnvZ(N347D(C)) was phosphorylated, both wild-type and mutant phosphoproteins were subjected to endoproteinase Lys-C digestion. Non-phosphorylated EnvZ(C) was also digested. Note that EnvZ(C) was autophosphorylated with \([\gamma-^{32}\text{P}]\text{ATP}\), while EnvZ(N347D(C)) was phosphorylated using \([\gamma-^{32}\text{P}]\text{phospho-OmpR}\). The products of digestion of non-phospho-EnvZ(C), autophosphorylated EnvZ(C), and phospho-EnvZ(N347D(C)) were separated on a Tricine-SDS-PAGE gel (Fig. 6, lanes 2–4, respectively). Lanes 1 and 2 of the gel were stained with Serva blue dye. Lane 2 shows the seven predicted EnvZ(C) Lys-C digestion bands. Lanes 3 and 4 of the gel were subjected to autoradiography. For both phosphoproteins, a single band, migrating at the identical position corresponding to the fourth band in the stained gel, was phosphorylated. From its size the fragment was judged to be the peptide Gln\(^{229}\) to Asn\(^{272}\), containing His\(^{243}\), the only histidine residue present in that fragment. This result clearly demonstrates that the only residue (His\(^{243}\)) which was autophosphorylated by ATP in the wild type EnvZ(C) was phosphorylated in EnvZ(N347D(C)) by phospho-OmpR.

**DISCUSSION**

Previous work from this laboratory demonstrated that substitutions at the conserved asparagine residue blocks the kinase but maintains the phosphatase activity (Yang and Inouye, 1991; Yang et al., 1993). The present data show that although the N347D mutation (corresponds to the N381D mutation in
EnvZ severely impaired the ATP-dependent autophosphorylation, EnvZ(N347D(C)) can still receive the phosphoryl group from phospho-OmpR. Moreover, once phosphorylated it also retains its ability to transfer the phosphoryl group back to OmpR or OmpR(N). The phosphatase assay yielded unexpected results with the N347D(C) mutant protein. The mutant could efficiently dephosphorylate phospho-OmpR by removing the phosphoryl group from it onto its own histidine (His243) which is also the autophosphorylation site. Similar reverse phosphoryl transfer from phospho-OmpR to EnvZ was observed with purified EnvZ(N347H(C)). In the presence of 5 mM Mg2+ and absence of ADP very little phospho-protein is detected for both EnvZ(N347D) and OmpR. The phosphoryl group is released as inorganic phosphate under these conditions (not shown). Therefore the “phosphatase” reaction by EnvZ(N347D(C)) involves a phosphotransfer reaction from phospho-OmpR to the mutant protein. It is important to point out that the in vivo complementation experiments (Yang et al., 1993) clearly demonstrated that the asparagine substituent mutants can complement the H277V mutant (confers null phenotype) to restore functional signal transduction, indicating that the phosphatase activity of the conserved asparagine substituent mutants is functionally equivalent to the wild type phosphatase activity.

The mechanism of the phosphatase reaction has not been elucidated for EnvZ. Also the role of the conserved asparagine is currently undefined. The results obtained with the EnvZ(N347D(C)) mutant protein offers some insights into the roles of the conserved Asn243 and the phosphatase function of EnvZ. Since EnvZ(N347D(C)) is deficient in autophosphorylation (Fig. 1), it is possible that the conserved asparagine is involved in ATP binding and/or in the subsequent transfer of the phosphoryl group to histidine. It has been postulated that EnvZ may have a modulator binding site which may or may not overlap with the putative ATP binding site. Contrary to what is observed with the wild type protein ADP serves as a negative effector for the phosphatase reaction of the N347D mutant protein (Fig. 3), suggesting that the putative modulator binding site has been modified in the EnvZ(N347D(C)) mutant. Therefore the conserved asparagine in EnvZ may be involved directly or indirectly in defining the putative ATP and/or modulator binding sites in EnvZ.

EnvZ(N347D(C)) is phosphorylated during the dephosphorylation of phospho-OmpR and in the presence of Mg2+ and the absence of ADP the phospho group is released very efficiently as inorganic phosphate. This raises the possibility that the phosphatase reaction of the mutant EnvZ(N347D(C)) proceeds in two steps. In the first step phosphoryl group transfer occurs from phospho-OmpR to His243 on EnvZ, which is the autophosphorylation site of EnvZ. In the second step this phosphorylated intermediate is hydrolyzed to inorganic phosphate which is a Mg2+-dependent reaction. Therefore the phosphotransferase reaction of the phosphoryl group from phospho-OmpR just represents the reverse phosphotransferase reaction of the the phosphorylation of OmpR by EnvZ. If His243 is involved in the phosphatase reaction of wild type EnvZ (as follows from the first possibility), an important implication would be that non-phosphorylatable substitutions at His243 should inactivate both the kinase and phosphatase functions of EnvZ. Known mutations at this position in EnvZ include H243V (Yang and Inouye, 1991; Yang et al., 1993) and H243R (Tokishita and Mizuno, 1994), all of which confer a null phenotype. This is in agreement with the prediction. It is of interest to note here that in the nitrogen regulatory system, mutating the conserved histidine at position 319 to asparagine (H319N) does not eliminate the phosphatase activity of NtrB (Atkinson and Ninfa, 1993; Kamberov et al., 1994). Although the Ntr system closely parallels the osmoregulatory system, they differ in many respects. EnvZ is a membrane-localized sensor kinase, while NtrB is a soluble cytoplasmic protein. Unlike EnvZ, NtrB requires an accessory protein, P_n, for its phosphatase activity (Ninfa and Magasanik, 1986; Keener and Kustu, 1988). Thus it is possible that the mechanism of the phosphatase reaction of NtrB may be different from that of EnvZ.

The present results suggest that the energy levels of the phosphoryl group is similar between the sensor, EnvZ, and the response regulator, OmpR, so that the phosphotransfer reaction is readily reversible. We are currently investigating whether the wild type EnvZ also dephosphorylates phospho-OmpR by the reverse reaction of the OmpR-phosphorylation reaction.

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