JOURNAL OF BACTERIOLOGY, Feb. 1997, p. 1413–1416 Vol. 179, No. 4
0021-9193/97/$04.00+0
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Role of His243 in the Phosphatase Activity of EnvZ in *Escherichia coli*

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Received 25 September 1996/Accepted 2 December 1996

EnvZ undergoes autophosphorylation at His243 and subsequently transfers the phosphate group to OmpR. EnvZ also possesses an OmpR-phosphate phosphatase activity. We examined the role of His243 in the phosphatase function by replacing His with either Val, Tyr, Ser, Asp, or Asn. EnvZH243V and EnvZH243Y were both shown to possess phosphatase activity in vitro. In addition, the mutant proteins were able to reduce the high level of OmpR-phosphate present in the *envZ473* strain. These results indicate that His243 of EnvZ is not essential for stimulating the dephosphorylation of OmpR-phosphate.

In *Escherichia coli*, the sensor molecule EnvZ and the regulatory protein OmpR control expression of the genes encoding the outer membrane porin proteins OmpF and OmpC (8, 11, 13, 14). The *ompF* and *ompC* genes are differentially regulated in response to changes in the osmolarity of the growth environment. In cells grown under low-osmolarity conditions, EnvZ functions to maintain lower levels of OmpR-phosphate, leading to the preferential expression of *ompF*. When cells are grown under high-osmolarity conditions, the level of OmpR-phosphate in the cell increases, which, in turn, stimulates expression of *ompC* and repression of *ompF* (1, 7, 10, 19, 20). In addition, OmpR has been shown to regulate genes other than *ompF* and *ompC* (9) and appears to occupy a central position in the regulation of flagellum synthesis in *E. coli* (18). EnvZ undergoes autophosphorylation at His243 (16), and the phosphate group is subsequently transferred to Asp55 of OmpR (4). EnvZ also possesses a phosphatase activity that stimulates the dephosphorylation of OmpR-phosphate (1, 10). The relative activities of the kinase and phosphatase functions of EnvZ have been proposed to control the level of OmpR-phosphate in the cell (8, 13, 14, 17). While the autokinase and phosphotransfer reactions of EnvZ have been extensively studied, the phosphatase activity of EnvZ has not been as carefully characterized. In this report, the role of His243 in the phosphatase reaction was investigated.

Purification of the soluble cytoplasmic domain of EnvZ. We had previously isolated a cytoplasmic fragment of EnvZ ($EnvZ^c$; 16) by first solubilizing an inclusion body form of the protein and subsequently cleaving at Arg214 to liberate soluble EnvZ. Since in this study we were interested in purifying mutant EnvZ fragments, we wanted to circumvent problems that might arise during the solubilization, refolding, and cleavage of the inclusion body forms of the mutant proteins. To this end, the DNA fragments encoding the His222-to-Gly450 region of wild-type or mutant EnvZ proteins were cloned into the *Nde*I-*Hin*dIII sites of the pET16b His-tag (Novagen, Inc.) vector system (see Table 1). The overproduced proteins were soluble and could be purified in a single step with a Ni^{2+}-affinity column (Fig. 1).

To induce the wild-type EnvZ cytoplasmic fragment, $EnvZ^H$, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 50 ml of cells during early exponential growth. After induction for 3 h, $EnvZ^H$ was found to be produced at high levels (Fig. 1, lane 2). Subsequent centrifugation (390,000 × *g* for 14 min) resulted in the removal of the major outer membrane proteins, while $EnvZ^H$ remained in the soluble fraction (lane 3). The $EnvZ^H$-containing solution was applied to the Ni^{2+}-affinity column, and after the column was washed with 3 volumes of 100 mM imidazole-containing buffer (lane 4), pure $EnvZ^H$ was eluted from the column with 500 mM imidazole. Lane 5 shows that the peak fraction of the eluted $EnvZ^H$ was >95% pure. The $EnvZ^H$ that eluted after the peak fraction was also highly purified (lane 6), while earlier fractions contained very small amounts of contaminating protein. To replace His243 with other residues, we used the Sculptor in vitro mutagenesis kit (Amersham). By this approach, His243 was replaced with either Val, Tyr, Ser, Asp, or Asn. Figure 1 shows that the production and purification of EnvZH243V (lanes 7 to 12) were identical to those of $EnvZ^H$. The other mutant EnvZ proteins were also purified by the same procedure (data not shown).

Analysis of the autokinase and phosphatase activities of the various EnvZ proteins. The autokinase activity of $EnvZ^H$ was assayed under standard phosphorylation conditions (6), as shown in Fig. 2. Maximal phosphate incorporation into $EnvZ^H$ was reached by 2 min (lane 3), and EnvZ-phosphate was stable for over 120 min. The stoichiometry of incorporation was found to be approximately 0.2 mol of phosphate incorporated/mol of EnvZ. Both the level of incorporation and the initial rate of autophosphorylation were significantly greater for $EnvZ^H$ than for the $EnvZ^c$ used previously (16). These results show that the autokinase activity of $EnvZ^H$ was high. The EnvZ molecules in which His243 was replaced with either Val, Tyr, Ser, Asp, or Asn were also assayed for autokinase activity. As expected, none of these molecules were able to undergo autophosphorylation (data not shown).

$EnvZ^H$ and the mutant proteins were next analyzed for their ability to stimulate the dephosphorylation of OmpR-phosphate. Radiolabeled OmpR-phosphate was prepared by incubating purified OmpR and radiolabeled ATP with membrane vesicles derived from cells carrying a multicopy *envZ* plasmid (15). The membrane vesicles were subsequently removed by high-speed centrifugation, and the radiolabeled OmpR-phosphate was applied to a G-25 column equilibrated in 50 mM HEPES (pH 8.0) to remove unincorporated ATP. As shown in Fig. 3, $EnvZ^H$ stimulated rapid dephosphorylation of OmpR-phosphate. In the presence of $EnvZ^H$, 50% of the OmpR-phosphate was dephosphorylated by 1 min. The optimal rate of dephosphorylation occurred at 50 μM ATP, the amount used

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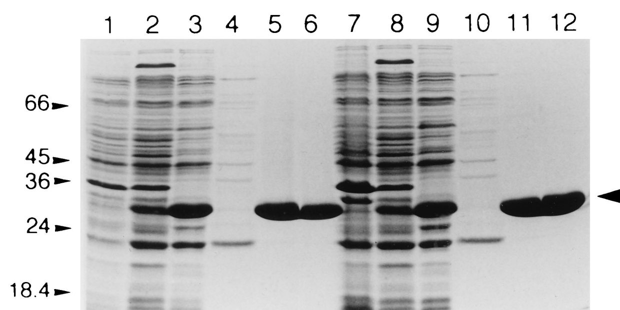

FIG. 1. Purification of EnvZ^H and EnvZH243V. The expression and purification of the cytoplasmic fragments of EnvZ were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes 1 to 6 depict the purification of EnvZ^H and lanes 7 to 12 depict the purification of EnvZH243V. Lanes 1 and 7 each contain 20 μg of total cellular extracts prior to induction, and lanes 2 and 8 each contain 30 μg of total cellular extracts after induction with 1 mM IPTG. The remaining lanes are described in the text. The positions of molecular size markers (in kilodaltons) are shown to the left of the gel, and the position of purified EnvZ is indicated by the arrowhead to the right of the gel.

in this study, while the rate decreased slightly in the presence of ADP. In addition, we found that even in the absence of adenine nucleotide, EnvZ was able to stimulate OmpR-phosphate dephosphorylation, albeit at a lower rate. Figure 3 also shows that both the EnvZH243V and EnvZH243Y proteins retained significant levels of phosphatase activity. In the presence of either EnvZH243V or EnvZH243Y, 50% of the OmpR-phosphate was dephosphorylated by 22 or 35 min, respectively. EnvZH243S was found to retain detectable levels of phosphatase activity, stimulating 50% dephosphorylation of OmpR-phosphate in 52 min. EnvZH243D possessed residual phosphatase activity, while EnvZH243N was unable to stimulate the dephosphorylation of OmpR-phosphate. OmpR-phosphate was stable for several hours in the absence of added EnvZ (data not shown). These results indicate that His243 was not essential for the dephosphorylation of OmpR-phosphate. However, substitution of His243 did result in a reduced rate of dephosphorylation, suggesting that it may have some role in the phosphatase activity of EnvZ.

In vivo analysis of the phosphatase activity of the wild-type and mutant proteins. We next addressed the question of whether the various EnvZ mutant proteins retained phosphatase activity in vivo as well. In a previous study, *envZ* alleles which possessed phosphatase activity but were defective for autokinase activity were isolated by introducing mutant *envZ* genes into the *envZ473* strain of *E. coli* (17). This strain produces elevated amounts of OmpR-phosphate, resulting in high-level production of OmpC and repression of OmpF production, even under low-osmolarity conditions. Introduction of *envZ* alleles that possess phosphatase activity into the *envZ473* strain results in a decrease in OmpC production and an increase in OmpF production. We used this approach to analyze the in vivo phosphatase activity of the various H243 *envZ* alleles (Table 1). Introduction of the plasmid containing wild-type *envZ* (Fig. 4, lane 2) stimulated OmpF production and caused the high level of OmpC normally produced in the *envZ473* strain (lane 1) to be reduced. Introduction of the plasmid containing *envZH243V* also stimulated OmpF production and reduced the production of OmpC (lane 3). The amount of OmpF produced and the reduction of OmpC were not as pronounced in the *envZH243V*-containing cells as in the cells containing wild-type *envZ*. This result was consistent with the observation that the EnvZH243V protein possessed some phosphatase activity. In cells containing *envZH243Y*, OmpF production was also detectable (lane 4), indicating that EnvZH243Y retained phosphatase activity. The other *envZ* alleles did not possess detectable phosphatase activity in vivo (lanes 5 to 7).

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description	Reference
Strains		
MC4100	F$^-$ Δ(*argF-lac*)*U169 rspL relA flhD deoC ptsF rbsR*	7
MH1471	MC4100 *envZ473*	17
Plasmids		
pYK12	Amp^r; pBR322 vector carrying *ompB*; Δ*Aat*II-*Hin*dIII of pBR322	6
pKS10RB	pYK12 carrying *ompR envZ*$^+$	This study
pKS10RB derived		
pRB173-A5	pKS10RB carrying *envZ473*	This study
pRB112-4	pKS10RB carrying *envZH243V*	This study
pRB317-12	pKS10RB carrying *envZH243Y*	This study
pRB324-3	pKS10RB carrying *envZH243S*	This study
pRB324-11	pKS10RB carrying *envZH243D*	This study
pRB2-1	pKS10RB carrying *envZH243N*	This study
pET16b derived		
pETA-1	pET16b carrying cytoplasmic *envZ*	This study
pET112-2	pET16b carrying cytoplasmic *envZH243V*	This study
pET317-4	pET16b carrying cytoplasmic *envZH243Y*	This study
pET324-2	pET16b carrying cytoplasmic *env243S*	This study
pET324-4	pET16b carrying cytoplasmic *envZH243D*	This study
pET2-5	pET16b carrying cytoplasmic *envZH243N*	This study

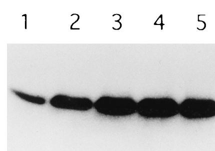

FIG. 2. Analysis of the autokinase activity of EnvZ^H. Autoradiograph of the time course of autophosphorylation of EnvZ^H. The proteins were incubated under standard phosphorylation conditions (6) and with 2 μM EnvZ and 5 mM $CaCl_2$. Reactions were initiated by the addition of [γ-^{32}P]ATP, and reaction mixtures were incubated at 25°C for 0.5, 1, 2, 5, and 10 min (lanes 1 to 5, respectively). Reactions were terminated by the addition of sodium dodecyl sulfate (SDS) sample buffer, and radiolabeled EnvZ was subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, and autoradiography was performed to visualize the radiolabeled bands.

Based on these results, we conclude that His243 is not directly involved in the dephosphorylation of OmpR-phosphate. Thus, the phosphatase reaction does not proceed via the reversal of the phosphotransfer reaction. In addition, H243S and H243D retained partial activity in vitro, but this activity was not detectable in vivo, while the H243N molecule was completely inactive. Similar results have been obtained with two other histine kinase sensors, NarX (3) and NRII (2, 12). Cavicchioli et al. (3) have shown that NarX containing the H399E substitution retained phosphatase activity both in vitro and in

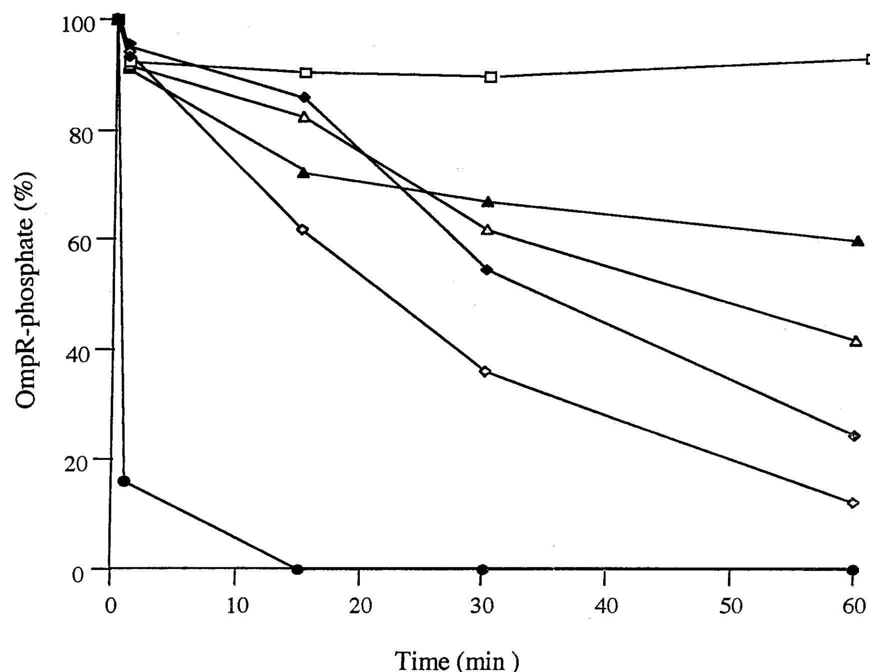

FIG. 3. Dephosphorylation of OmpR-phosphate by $EnvZ^H$ and the mutant proteins. Radiolabeled OmpR-phosphate (200 pmol) was incubated with 50 pmol of $EnvZ^H$ in dephosphorylation buffer containing 5 mM $MgCl_2$, 50 mM HEPES (pH 8.0), and 200 mM KCl. The dephosphorylation of OmpR-phosphate was stimulated by the addition of either $EnvZ^H$ (●), EnvZH243V (◇), EnvZH243Y (◆), EnvZH243S (△), EnvZH243D (▲), or EnvZH243N (□). The reactions were terminated at the indicated time points, and the reaction mixtures were applied to a SDS-polyacrylamide gel. Radiolabeled OmpR was transferred to a nitrocellulose membrane, and autoradiography was performed to visualize the radiolabeled bands. To determine the percentage of OmpR-phosphate remaining, autoradiographs were densitometrically scanned. The results were expressed as the percent decrease relative to the amount of OmpR-phosphate present under assay conditions in which EnvZ was not present. Each assay was performed at least three times with nearly identical results. A representative experiment is shown.

vivo, while the H399Q and H399K molecules were inactive. Similarly, NRII containing the H139N substitution was active both in vitro and in vivo, while the H139V mutant protein was inactive (2, 12). Thus, for NarX and NRII, the phosphatase reaction does not require the histidine residue involved in the autophosphorylation reaction. While it is not clear how the substitution of the active-site His with different amino acid residues differentially affects the phosphatase activity of a given histidine kinase molecule, the above results illustrate that numerous substitutions should be created before conclusions concerning the function of the His residue in the phosphatase reaction are made. While we found that EnvZ containing the H243V substitution had phosphatase activity, the Tar-EnvZ hybrid (Taz) containing the H243V substitution was found to be inactive (22, 23). In the latter case, the Taz mutant was studied in a membrane-bound form, contained within cytoplasmic membrane vesicles. Whether the different effect of the H243V substitution reflects differences in the form of the protein used (soluble versus membrane bound) or whether the Taz fusion protein may possess a slightly altered conformation relative to that of the wild-type EnvZ is not presently known. Finally, the phosphate group of OmpR-phosphate was recently shown to be reversibly transferred to His243 of the EnvZH347D mutant protein, while reversible phosphotransfer was not observed for wild-type EnvZ (5). Weiss and Magasanik (21) had previously shown that reversal of the phosphotransfer reaction, in which the phosphate moiety of NRI-phosphate was transferred to ATP in the presence of NRII and ADP, was possible. Future studies will need to address the possibility that more than one pathway exists for the dephosphorylation of OmpR-phosphate by EnvZ.

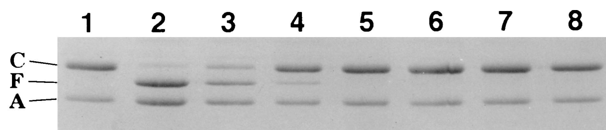

FIG. 4. Outer membrane protein profile of *envZ473* strain containing different *envZ* alleles. Outer membrane proteins were produced in the *envZ473* strain containing the following plasmids: pBR322 (lane 1), pKS10RB (*envZ*) (lane 2), pRB112-4 (*envZH243V*) (lane 3), pRB317-12 (*envZH243Y*) (lane 4), pRB2-1 (*envZH243N*) (lane 5), pRB324-3 (*envZH243S*) (lane 6), pRB324-11 (*envZH243D*) (lane 7), and pRB173-A5 (*envZ473*) (lane 8). Outer membrane proteins obtained from cultures grown in nutrient broth were prepared as described previously (20). The positions of OmpC, OmpF, and OmpA are indicated (C, F, and A, respectively).

This study was supported by Public Health Service grant GM44671 (to S.A.F.).

We are grateful to D. Roberts and J. Barberi for critically reading the manuscript.

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