Identification of the Site of Phosphorylation on the Osmosensor, EnvZ, of Escherichia coli*

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EnvZ is a membrane-bound histidine kinase that functions as an osmotic sensor capable of phosphorylating the regulator protein OmpR in *Escherichia coli*. To characterize the site of phosphorylation biochemically, we overexpressed a 36-kDa truncated EnvZ protein (Glu-106 to Gly-450) that formed inclusion bodies in the cell. After solubilization, the inclusion body form of EnvZ was cleaved into two major fragments with molecular weights of 25,000 and 10,000. The 25-kDa fragment, EnvZ', was purified and found to exist as a dimer. N-terminal sequence analysis established that cleavage had occurred at Arg-214, indicating that EnvZ' contained most of the cytoplasmic domain of EnvZ. After labeling EnvZ' with [γ-32P]ATP, the protein was proteolytically digested, and the resulting peptides were separated by reverse phase chromatography using high performance liquid chromatography. One major radioactive peptide containing greater than 90% of the recovered peptide-associated radioactivity was isolated. Amino acid analysis of this purified peptide indicated that the composition was consistent with a peptide that contained His-243. The amino acid sequence of this peptide was determined to be MAGVSEDLRTP (residues 238-248). These results indicate that the site of phosphorylation on EnvZ and represents the first biochemical characterization of the site of phosphorylation of a membrane histidine kinase of the two-component regulatory family of molecules in bacteria.

The pores in the outer membrane of *Escherichia coli* K-12 are formed by the porin proteins OmpF and OmpC. These pores are essential for the passive diffusion of small hydrophilic molecules through the outer membrane. In most *E. coli* strains, both OmpF and OmpC are expressed when the bacterium is cultured at 37 °C in minimal medium, whereas OmpC is preferentially expressed and OmpF is repressed when the cells are grown under conditions of high osmolarity (1, 2). EnvZ, an integral membrane protein, and OmpR, a DNA-binding protein, transfer this phosphate moiety to OmpR (8, 10-12), the DNA-binding protein that binds the regulatory regions upstream of the ompF and ompC promoters (13-16). EnvZ has also been suggested to be involved in the dephosphorylation of OmpR phosphate (7, 17, 18). It is currently thought that during growth under low osmolarity conditions, EnvZ functions to maintain lower levels of OmpR phosphate (7, 17, 19). This level of OmpR phosphate stimulates the expression of ompF as well as ompC (7, 17, 19). When the bacteria are shifted to high osmolarity conditions or to growth in the presence of proline, the levels of OmpR phosphate are elevated, and ompC is preferentially expressed while ompF is repressed (7, 17-20). This mechanism is supported by the results obtained from the in vivo phosphorylation of OmpR in parenteral cells grown in different osmolarity environments and with several envZ strains (19, 21).

The EnvZ/OmpR molecules belong to a family of conserved signal-transducing proteins, referred to as two-component regulatory systems, which are involved in adaptive responses to diverse environmental stimuli in prokaryotes. In the EnvZ family of molecules, many of which are integral membrane proteins, the histidine residue at position 243 on EnvZ represents an invariant histidine residue found in all members of the so-called histidine kinase class of molecules (22, 23). It has been proposed previously that His-243 was the site of phosphorylation on EnvZ (7-9). The site of phosphorylation of two other members of the histidine kinase family of molecules has been determined. In the soluble chemotaxis modulator protein CheA, His-48 was shown to be the site of phosphorylation (24). In the soluble nitrogen sensor molecule NtrB (NR₁), the site of phosphorylation was determined to be His-139 (25). Since EnvZ is a membrane-associated histidine kinase, it has been difficult to obtain sufficient quantities of pure protein to identify the site of phosphorylation biochemically. In this report we used a soluble, cytoplasmic fragment of EnvZ to determine biochemically that His-243 is the major site of phosphorylation on EnvZ.

MATERIALS AND METHODS

**Bacterial Strains and Plasmids**—The *E. coli* strain SB21 (pp, hsdR, trpE5, leuB6, lacY, recA, F'laclac-pro*) containing pSF102 was used throughout this study (8).

**Purification of EnvZ**—*E. coli* containing pSF102 was incubated at 37 °C in L broth supplemented with 50 μg/ml ampicillin and induced with 0.1 mM isoamyl-l-thio-β-d-galactopyranoside (4). The cells were harvested and resuspended in 20 mM phosphate buffer, pH 7.0, containing 4 mM dithiothreitol. After passage through a French press, the resulting insoluble 36-kDa fragment of EnvZ (EnvZ') was isolated by centrifugation at 2,000 x g for 30 min at 4 °C. EnvZ' was solubilized by incubation of the pellet in solubilization buffer at 4 °C for 48 h with constant stirring. During incubation, EnvZ' was specifically cleaved into two major fragments: a soluble 25-kDa fragment (EnvZ) and a 10-kDa fragment. The 25-kDa EnvZ' was purified using a Mono Q HR 5/5 fast protein liquid chromatography column (Pharmacia LKB Biotechnology, Inc.) equilibrated in 20 mM Hepes, pH 7.2, and 4 mM dithio-
threetol, and elution was achieved using a linear gradient of KCl. EnvZ' eluted at 0.35 m KCl and was shown to be greater than 95% pure as determined by SDS-polyacrylamide gel electrophoresis analysis. The final protein concentration of the purified EnvZ' was 2 mg/ml as determined by the rose bengal protein assay (26).

**Solubilization, Purification, and Characterization of EnvZ' and EnvZ**—EnvZ is an integral membrane protein that is present at exceedingly low levels in E. coli (27). The envZ expression vector, pSF102 (8), encodes a 36-kDa fragment of EnvZ which is comprised of a truncated periplasmic domain (Glu-106 to Arg-162), a transmembrane domain (Tyr-163 to Ile-179), and a large cytoplasmic domain (Arg-180 to Gly-450; see Fig. 1B). Growth of E. coli containing pSF102 in the presence of isopropyl-1-thio-β-D-galactopyranoside resulted in the production of an insoluble EnvZ fragment (Glu-106 to Gly-450; Fig. 2A). This 36-kDa fragment, EnvZ', was purified to near homogeneity by low speed centrifugation (Fig. 2A, lane 4). During purification, we observed that EnvZ' was cleaved into two major fragments: a soluble 25-kDa fragment of EnvZ (EnvZ'), and a 10-kDa fragment (Fig. 2A, lane 4). EnvZ' was purified using a Mono Q column and was determined to be greater than 95% pure by SDS-polyacrylamide gel electrophoresis analysis (Fig. 2A, lane 5). N-terminal sequence analysis of EnvZ' revealed that proteolysis specifically occurred between residues Arg-214 and Ser-215, presumably because of a trypsin-like activity (Fig. 1). The amino acid composition of EnvZ' was consistent with that of the EnvZ fragment Ser-215 through Gly-450 (data not shown). The sequence analysis also revealed that the purified EnvZ' peak contained a minor peptide generated by cleavage at Arg-218.

In previous studies, larger forms of EnvZ extending from Arg-180 to Gly-450 were used to demonstrate the autophosphorylation function of the cytoplasmic domain of this histidine kinase (8). To determine whether a smaller form of EnvZ retained the ability to undergo autophosphorylation by ATP, EnvZ was phosphorylated in vitro. Fig. 2B shows that although EnvZ' and EnvZ (lanes 1 and 3, respectively) were both capable of undergoing autophosphorylation, EnvZ' incorporated 6.0 × 10⁻² mol of phosphate/mol of EnvZ' monomer, whereas EnvZ incorporated 6.0 × 10⁻² mol of phosphate/mol of monomer (see "Materials and Methods"). These results indicated that the cytoplasmic portion of EnvZ located between Ser-215 and Gly-450 was highly active and was sufficient for the autophosphorylation function of EnvZ. As expected, the 10-kDa fragment could not be phosphorylated by ATP in vitro (data not shown).

**RESULTS**

**Solubilization, Purification, and Characterization of EnvZ' and EnvZ**—EnvZ is an integral membrane protein that is present at exceedingly low levels in E. coli (27). The envZ expression vector, pSF102 (8), encodes a 36-kDa fragment of EnvZ which is comprised of a truncated periplasmic domain (Glu-106 to Arg-162), a transmembrane domain (Tyr-163 to Ile-179), and a large cytoplasmic domain (Arg-180 to Gly-450; see Fig. 1B). Growth of E. coli containing pSF102 in the presence of isopropyl-1-thio-β-D-galactopyranoside resulted in the production of an insoluble EnvZ fragment (Glu-106 to Gly-450; Fig. 2A). This 36-kDa fragment, EnvZ', was purified to near homogeneity by low speed centrifugation (Fig. 2A, lane 4). During purification, we observed that EnvZ' was cleaved into two major fragments: a soluble 25-kDa fragment of EnvZ (EnvZ'), and a 10-kDa fragment (Fig. 2A, lane 4). EnvZ' was purified using a Mono Q column and was determined to be greater than 95% pure by SDS-polyacrylamide gel electrophoresis analysis (Fig. 2A, lane 5). N-terminal sequence analysis of EnvZ' revealed that proteolysis specifically occurred between residues Arg-214 and Ser-215, presumably because of a trypsin-like activity (Fig. 1). The amino acid composition of EnvZ' was consistent with that of the EnvZ fragment Ser-215 through Gly-450 (data not shown). The sequence analysis also revealed that the purified EnvZ' peak contained a minor peptide generated by cleavage at Arg-218.

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Proteolysis of EnvZ and Purification of the Radiolabeled Peptide—During the initial studies to proteolyze EnvZ', we inadvertently discovered that trypsinogen extensively cleaved EnvZ' (see Fig. 4A). This result indicated that trypsinogen either contained residual trypsin activity or a contaminating protease, possibly chymotrypsin, which cleaved EnvZ' into a large number of small peptides. We took advantage of this extensive cleavage in an attempt to obtain a radiolabeled peptide that would contain a single histidine residue.

EnvZ' was radiolabeled with [γ-32P]ATP, and unincorporated [γ-32P]ATP was removed by gel filtration chromatography. The radiolabeled EnvZ' was digested with trypsinogen, and the resulting peptides were separated using a C8 reverse phase column. Fig. 4A shows the peptide elution profile (dashed line) and the radioactivity elution profile (solid line) of the peptides generated from digestion of the phosphorylated EnvZ'. Of the large number of peptides generated, only two major radioactive peaks, with retention times of 8 and 34 min, were identified. These peaks accounted for 98% of the total injected radioactivity. Thin layer chromatography indicated that the radioactive peak that eluted at 5 min consisted of inorganic phosphate (data not shown). We also found that the 215 nm absorbance peak at 5 min was a reproducible injection peak that was observed even when only solvent was injected into the gradient system and therefore did not represent peptide absorbance. These results suggested that greater than 90% of the recovered peptide-associated radioactivity was contained within one peptide peak.

To purify the phosphorylated peptide that contained the site of phosphorylation of EnvZ, the peptides that eluted at 34 min were reapplied to the C8 column and separated using a trifluoroacetic acid/acetoni trile gradient. Fig. 4B shows that the radioactive peak isolated from the ammonium acetate gradient (Fig. 4A) contained several peptides. However, the recovered radioactivity still eluted as a single peak, which represented 95% of the injected radioactivity. This phosphorylated peptide was purified further by reapPLYing it to the same trifluoroacetic acid/acetoni trile elution conditions (Fig. 4C). The pure peptide was subjected to both amino acid analysis and primary sequence analysis. It is interesting to note that the histidine phosphate appeared to be stable to short exposures to low pH, as indicated by the initial purification using a trifluoroacetic acid gradient (Fig. 4B). However, repurification showed that
FIG. 4. HPLC analysis of the proteolyzed radiolabeled EnvZ. In each panel, the radioactivity elution profile is represented as a percentage of the total injected radiolabel (solid line). The peptide absorbance at 215 nm is also indicated (dashed line). Panel A, initial fractionation of the trypsinogen-generated peptides of EnvZ. Buffer A was 20 mM ammonium acetate, pH 6.8, and buffer B was 20 mM ammonium acetate, pH 6.8, in 60% acetonitrile (see "Materials and Methods"). TLC analysis of the radioactive peak with a retention time of 5 min indicated that it consisted of inorganic phosphate. Panel B, the radioactive peptide peak isolated in panel A was reapplied to a C8 reverse phase column equilibrated in buffer C (0.1% trifluoroacetic acid in water) and was eluted with buffer D (0.1% trifluoroacetic acid in acetonitrile) (see "Materials and Methods"). Because of the short exposure to trifluoroacetic acid, no inorganic phosphate was formed during this step. Panel C, purification of the radiolabeled peptide isolated from panel B. Chromatography was performed as outlined in panel B except the absorbance range was 0.05 arbitrary unit. Of the 78,000 cpm injected onto the column, 72,900 cpm eluted with a peptide at 33 min.

longer exposures to low pH caused slight hydrolysis of the imidazole phosphate (Fig. 4C). Previous reports have demonstrated that phosphorylated EnvZ could be precipitated using trichloroacetic acid and therefore indicated that the phosphate moiety on EnvZ was reasonably stable to short incubations to low pH at low temperatures (33).

Amino Acid Composition and Sequence Analysis of the Purified Peptide—The amino acid composition of the purified phosphorylated peptide is shown in Table I. The peptide contained 1 histidine residue, and the composition was consistent with that of the peptide extending from Met-238 through Leu-249 (Fig. 1C). This conclusion was confirmed by the amino acid sequence of this peptide, which was found to be $^{233}$M-A-G-V-S-H-D-L-R-T-P$^{248}$. During the sequence analysis, the cycle that contained the histidine residue gave a peak that had a retention time slightly less than native histidine, with no other peak being observed. This result suggests that all of the histidine in the purified peptide was modified, indicating that only the phosphorylated peptide was isolated. The yield of the Pro-248 cycle was low, and sequence information beyond Pro-248 could not be obtained. Since this peptide contained 2 leucine residues, it seems likely that it was generated by cleavage at Leu-237 and Leu-249 (Fig. 1C). The extra amino acids determined by the composition analysis were possibly a result of small
peptides that were generated during the extensive proteolysis procedure that was used. These very small peptides may have eluted during the first sequencing cycle and therefore were not detected. Based on these results, we can conclude that His-243 is the major site of phosphorylation on EnvZ by ATP and that no other modified site is detectable in vitro.

**DISCUSSION**

In this report we have biochemically confirmed that His-243 of EnvZ is the major site of phosphorylation of this membrane histidine kinase molecule. Most histidine kinase molecules in bacteria are membrane proteins (22, 23), and EnvZ represents the first molecule in this class in which the site of phosphorylation has been characterized both biochemically and genetically. Since we identified only one major radiolabeled peptide containing over 90% of the total peptide-associated radiolabel and did not detect significant amounts of other radiolabeled peptides, we conclude that EnvZ was not phosphorylated at secondary sites. It was reported previously that NtrB contained over 90% of the total peptide-associated radiolabel (7), whereas all other tryptic sites were resistant to cleavage. It is conceivable that the resistance to degradation reflects the structured domain organization of the cytoplasmic fragment of EnvZ and that the region located between the junction of the cytoplasmic membrane and the domains involved with the phosphotransfer of EnvZ represents a "hinge" region (residues 180–215) that was sensitive to proteolytic cleavage. Similar protease resistance of functional domains and sensitivity of hinge regions have been observed in many proteins (35). This region is likely to play a role in the regulation of the activity of EnvZ since numerous mutations have been identified in this location of the envZ gene (29, 36, 37).

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