BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **231,** 68–72 (1997)
ARTICLE NO. RC966007

Purification and Characterization of the Periplasmic Domain of EnvZ Osmosensor in *Escherichia coli*

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Received December 18, 1996

The EnvZ-OmpR histidyl-aspartyl phosphorelay system in *E. coli* responds to osmolarity by differentially modulating the expression of the major outer membrane porins OmpF and OmpC. To date, the natural ligand that activates EnvZ, a transmembrane histidine kinase, has not been identified and the role of the periplasmic domain of EnvZ is unclear. We now report on the purification and characterization of the periplasmic domain of EnvZ (Lys48-Arg162) which has been expressed as a soluble protein in fusion with the maltose-binding protein. Overexpression of the fusion protein did not compete for a signal that activates EnvZ. By amylose affinity chromatography and affinity blotting, interacting proteins could not be detected. The periplasmic domain was released by factor Xa and purified to homogeneity. From circular dichroism analysis, the periplasmic domain was estimated to consist of 35% α-helices and 16% β-sheets.

The EnvZ-OmpR system in *E. coli* is a member of a family of proteins involved in the histidyl-aspartyl phosphorelay (also described as two component regulatory systems) in bacteria that respond to environmental cues through a histidine protein kinase and a response regulator. While histidine kinases were thought to be present only in prokaryotes, they have recently been identified in both plants and yeast (1, 2). In addition, two eukaryotic osmosensors have been recently characterized in yeast (3). EnvZ is an inner membrane protein (450 aa) consisting of an N-terminal tail (residues 1-15), two transmembrane domains (16-47 and 163-179), a periplasmic receptor domain (48-162), and a cytosolic C-terminal signaling domain (4). EnvZ is responsive to environmental changes such as osmolarity, and once activated becomes autophosphorylated at a conserved cytoplasmic histidine residue His243. EnvZ has the ability to act as both kinase and phosphatase to regulate levels of phosphorylated OmpR. OmpR is a cytoplasmic phosphotransferase (239 aa) which receives the phosphate from EnvZ onto a conserved aspartate residue Asp 55 (5). Once phosphorylated, OmpR serves as a transcription factor to bind upstream sites on porin promoters to differentially modulate the expression of the outer membrane porins OmpF and OmpC (6, 7). In response to low medium osmolarity, OmpF levels increase while OmpC levels decrease, and reciprocal regulation is seen during high osmolarity (8, 9).

While it is clear that EnvZ responds to a variety of environmental stresses including: changes in medium osmolarity, pH, temperature, and chemical composition of the medium to regulate the level of phosphorylated OmpR (10), the primary activation signal has not been identified. In the absence of a defined ligand for EnvZ, a chimeric receptor, Taz1, was constructed to study the signal transduction pathway of EnvZ. Using Taz1 (N-terminal Tar chemoreceptor; C-terminal EnvZ) which is activated by aspartate, it has been shown that ligand binding to the receptor domain regulates the ratio of kinase to phosphatase activities of the EnvZ signaling domain (11). Molecular manipulations including *envZ* missense mutations in the cytoplasmic domain (i.e. *envZ11*, Thr247Arg), and deletions of portions of the periplasmic domain of EnvZ can induce the high osmolarity response (12, 13). Treatment with local anesthetics such as procaine, a membrane perturbant, can also activate EnvZ (14). Transmembrane *envZ* mutants that contain single amino acid replacements within or near the transmembrane domain lead to either OmpC or OmpF constitutive expression and provide evidence that the transmembrane domain has a role in responding to osmotic stimulation to modulate EnvZ activity (15). In addition, an N-terminal truncation of 38 amino acids that prevents proper localization of EnvZ but retains its ability to be autophosphorylated, cannot respond to osmolarity changes. This implies that the stimulus is derived from the periplasm or the membrane rather that the cytoplasm (16).

While the C-terminal domain of EnvZ retains kinase

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0006-291X/97 $25.00

and phosphatase activities, the function of the periplasmic domain of EnvZ has not been addressed (17, 18). In addition, the primary activation signal and critical domain for initiating the signal transduction pathway in other osmosensors such as the yeast osmosensor, Sln1P which responds to osmolarity by modulating cytoplasmic glycerol content, also remains elusive (2,3). To assess the role of the periplasmic domain of EnvZ in signal detection, this report describes the soluble expression, purification and characterization of the periplasmic domain of EnvZ. This analysis will provide insight into our understanding of how the activation signal may be sensed by prokaryotic as well as eukaryotic osmosensors.

MATERIALS AND METHODS

Antibodies. Rabbit α-$EnvZ_{peri}$ polyclonal antibodies were generated against the periplasmic domain of EnvZ which was purified from inclusion bodies using a pET-11a/EnvZ-N expression vector. Protein was gel purified, electroeluted and used to inject rabbits at 100μg/injection using freund's complete adjuvant followed by freund's incomplete adjuvant at 2 week intervals.

Plasmids and oligonucleotides. pMalp2 (New England Biolabs) was used to construct the MBP::$EnvZ_{peri}$ (Lys48-Arg162) fusion protein. The EnvZ periplasmic domain was generated by PCR from pDR200, an EnvZ plasmid (4), to introduce MfeI and BamHI restriction sites and a C-terminal stop codon using PCR primers (upper primer 5′GCCAATTGAAAGTCCTCGCGTACGAA3′, lower primer 5′CGGGATCCTAGCGGAACAGCGGAG3′). The PCR product was cloned into the pCRII TA-cloning vector (Invitrogen), excised by MfeI/BamHI and subcloned into an EcoRI/BamHI digested pMAL-p2 vector after the factor Xa cleavage site and designated pLE119. The above construct containing MBP::(ISEL) $EnvZ_{peri}$ (K48-R162) was verified by sequencing (Sequenase) to ensure that mutations were not introduced by PCR.

Expression/purification. The *E. coli* TG-1 strain (19) was used to express pLE119 in LB broth with 75μg/ml ampicillin using a 2 hr IPTG induction (0.4 mM). Periplasmic extracts were prepared from a 2.5 liter culture according to the New England Biolabs suggested protocol. Periplasmic extracts were neutralized by the addition of 20 mM Tris-Cl pH 7.4 and directly loaded onto the amylose affinity resin. After washing in column buffer (20 mM Tris-Cl pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM β-ME) proteins were eluted in column buffer containing 10 mM maltose and collected as 3.0 ml fractions. Factor Xa (New England Biolabs) was used for subsequent cleavage at 25°C using a 1:10 molar ratio for 160 hrs. Cleaved proteins were further purified on a 595 ml bed volume S-100 Sephacryl gel filtration column (Pharmacia) with a flow rate of 16 ml/hr. Fractions containing periplasmic proteins were identified by 20% SDS-PAGE gels that were transferred to nitrocellulose and detected by immunoblotting using α-MBP at 1:5000 and/or α-EnvZperi at 1:500. Goat anti-rabbit IgG alkaline phosphatase conjugate was used at 1:7000 as the secondary antibody (BioRad) followed by NBT/BCIP detection (Gibco).

Circular dichroism (CD) spectroscopy. The CD spectrum (190-260 nM) of purified proteins (0.05 mg/ml in 20 mM K phosphate pH 7.2, 100 mM KCL) was measured with an Aviv Model 62DS spectropolarimeter at 25°C in a cuvette with a 0.1 cm path length. Protein concentration was determined by A_{280} nm and calculated based on protein molar extinction coefficients (20). Secondary structure was estimated by computer analysis (21) using both the SELCON and k_2D neural network analysis programs.

RESULTS AND DISCUSSION

Soluble expression. The periplasmic domain of EnvZ(K48-R162) was expressed as a fusion protein with the maltose-binding protein (MBP) using pMalp2 (Fig. 1A) (22). The fusion protein allows soluble expression of the periplasmic domain of EnvZ which was directed towards the periplasmic space (Fig. 1B). The periplasmic domain is relatively hydrophobic (52%) which may have contributed to the difficulties during previous attempts to express this domain as a soluble protein when using a non-fusion approach. Increased solubility when expressing the periplasmic domain of EnvZ in fusion with the MBP has been attributed to the co-translational transport of MBP through the inner membrane. When comparing the cytoplasmic extract to the periplasmic extract (Fig. 1B, lanes 3 and 5) it was clear that osmotic shock fluid provides a significant purification step, and 45% of the total fusion protein was contained within the periplasmic compartment. Initial purification of the fusion protein was achieved by amylose affinity purification of the osmotic shock fluid (Fig. 1B). Expression was confirmed by immunoblotting (Fig. 1C).

Functional analysis. MBP:$EnvZ_{peri}$ fusion protein was then used to probe for specific periplasmic proteins that may interact with the periplasmic domain of EnvZ. For this purpose, ^{35}S-labeled periplasmic extracts prepared from low and high osmolarity cultures were examined by amylose affinity chromatography (reviewed in 23). MBP or fusion proteins bound to the amylose resin were treated with ^{35}S extracts, and maltose eluted proteins were analyzed by Coomassie Brilliant Blue staining of SDS/PAGE followed by autoradiography. Using amylose affinity chromatography, no specific interacting periplasmic proteins were identified (data not shown). Affinity blotting was also carried out (24) using α-$EnvZ_{peri}$, but again specific interacting proteins were not identified (data not shown). These results support a model in which there is no protein ligand for EnvZ. Alternatively, the protein ligand may be unstable and coupling of the fusion to the amylose resin may disrupt its ability to bind a ligand. Currently, there is no assay to assess the activity of $EnvZ_{peri}$. While there are numerous examples of purified ectodomains such as human growth factor receptor, human insulin receptor, epidermal growth factor receptor, platelet derived growth factor receptor, cytokine receptors, and bacterial chemoreceptor Tar that can bind ligand in a similar fashion as their full length counterparts, this may not apply to EnvZ (reviewed in 25, 26), and the transmembrane and/or cytoplasmic domains may also be required for the function of the periplasmic domain.

Next, overexpression of the MBP::$EnvZ_{peri}$ fusion protein was examined *in vivo* to determine whether it could compete for a signal which activates EnvZ. Fol-

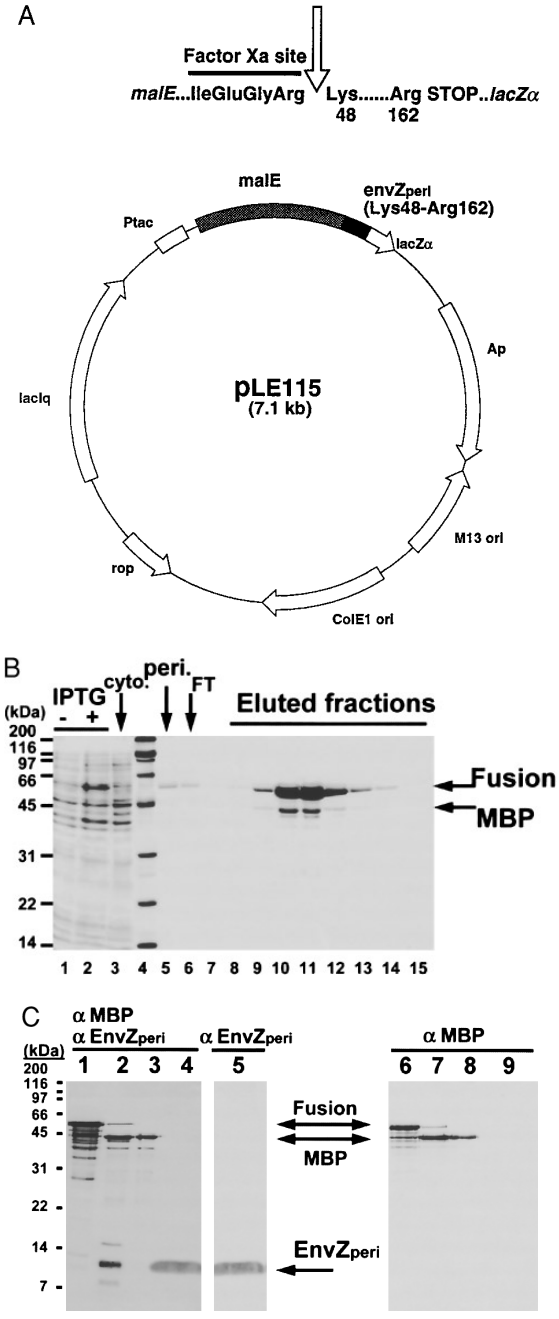

FIG. 1. Soluble expression and purification. (**A**) pLE119 was used to express the MBP:(ISEL)EnvZ$_{peri}$(K48-R162) fusion protein. The tetrapeptide recognition sequence for the Factor Xa cleavage site is indicated above the plasmid map. (**B**) During purification of the soluble fusion protein, the following samples were analyzed by 10% SDS-PAGE and subjected to Coomassie Brilliant Blue staining: total cell extract before and after IPTG induction (lanes 1, 2); cytoplasmic fraction (lane 3), molecular weight std. in kDa (lane 4), periplasmic extract (lane 5), flow through (lane 6), wash (lane 7), and 3.0 ml fractions eluted by 10 mM maltose (lanes 8-15). The 56 kDa fusion protein and 42 kDa MBP are indicated by arrows. The size of molecular weight markers in kDa is indicated beside lane 1. (**C**) Amylose affinity purified fusion protein was cleaved with factor Xa and further purified by gel filtration. The following samples were analyzed by immunoblot with both α-MBP (NE Biolabs) at 1:5000 and α-EnvZperi at 1:500 (lanes 1-4), only α-EnvZperi (lane 5) and only α-MBP (lanes 6-9): amylose affinity purified protein before factor Xa cleavage (lane 1, and 6), after factor Xa cleavage (lane 2, and 7), 500 ng MBP standard (NE Biolabs) (lane 3, and 8), and purified periplasmic domain of EnvZ (lane 4,5, and 9).

lowing a hyperosmotic shift from 5 to 50 min., strains induced to overexpress the fusion protein were ^{35}S pulse-labeled, and OmpF and OmpC porin profiles were examined by Coomassie Brilliant Blue staining of SDS-PAGE/urea gels followed by autoradiography. Assuming that the activation signal for EnvZ was limiting, effective competition would be measured as a delayed block in ^{35}S-labeled OmpF. Overexpression of the periplasmic domain in TG-1 (19) or MC4100 (27) strains, however, did not affect OmpF expression indicating that the high osmolarity signal may be present in excess which cannot be titrated by competition with the periplasmic domain of EnvZ (data not shown). In addition, porin profiles prepared at both low and high osmolarity were not affected by overexpression of the periplasmic domain. Collectively, these results argue against the presence of a specific protein that interacts with the periplasmic domain of EnvZ.

Structural analysis. Circular dichroism (CD) spectroscopy was used initially to demonstrate that the fusion proteins examined in the above functional analysis retained secondary structure. By computer analysis of the CD spectra, the fusion protein and a MBP protein standard both contained 37% α-helices, 26% β-sheets, and 38% random coils. This CD analysis was in agreement with the three-dimensional crystal structure of MBP (28). While this technique could assess the structural composition of the fusion protein, it could not accurately determine the structural contribution of the periplasmic domain which comprised 24% of the fusion protein.

To assess the secondary structure of the periplasmic domain of EnvZ, the fusion protein was purified by amylose affinity chromatography, cleaved by factor Xa, and isolated by gel filtration (Fig. 1C). The relative mobility of the cleaved periplasmic protein was 12.9 kDa by SDS-PAGE which agrees with the predicted mass of 13,780 Da. Using the purified periplasmic domain of EnvZ, CD spectra was collected from 260 to 185 nm and analyzed by several computer programs (Fig. 2) (21). SELCON analysis from 260 to 185 nm was used to determine that the periplasmic domain contained 34% α-helices, 14% β-sheets, 29% turns, 8% PP2 (poly-proline type structure), and 15% unordered structure. When the data set was truncated from 260 to 200 nm using SELCON, a similar composition of 37% α-helices, 18% β- sheets, 34% turns, 3% PP2, and 8% unordered structure was obtained. Using a neural network analysis, k$_2$d, the composition was 34% α-helices, 15% β-sheets, and 50% random structure. Based on the above computer analysis, the composition of the

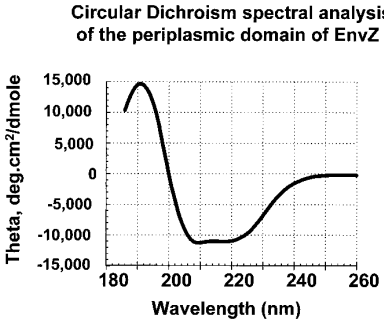

FIG. 2. Structural analysis by circular dichroism spectroscopy. The circular dichroism spectra of the purified periplasmic domain of EnvZ was measured from 185-260 nM and the secondary structure was determined by computer analysis (21).

periplasmic domain of EnvZ contains 35% α-helices and 16% β-sheets.

While the bacterial chemoreceptor Tar has a similar domain organization as EnvZ, and can function in the Taz1 chimeric protein (N-terminal Tar, C-terminal EnvZ), it is now clear that the structures of the periplasmic domains are distinct. Crystallization of the periplasmic domain of Tar has established that ligand binding occurs between two subunits in a dimer (26) and that each monomer is composed a four α-helical bundle. From the CD analysis presented in this report, the secondary structure of $EnvZ_{peri}$ also contains a significant contribution from β-sheets.

Comparison of EnvZ from *E. coli* with homologous proteins can also be used to evaluate the functional and structural roles of the periplasmic domain. Recently, an *envZ* homologue of 342 aa has been identified in *Xenorhabdus nematophilus* insect pathogenic bacteria (29). This homologue shares 50% identity with the C-terminal domain of *E. coli* EnvZ, but does not contain a periplasmic domain. Instead, the N-terminal 40-50 aa are highly hydrophobic. While *X. nematophilus envZ* can complement an *E. coli* Δ *envZ* strain to restore porin profiles and the response to osmolarity, the major porins in *X. nematophilus* do not respond to osmolarity. While this complementation approach provides evidence that the C-terminal domain of EnvZ is sufficient for porin regulation, this does not indicate that the periplasmic domain is not required for sensing an osmotic signal. Other approaches that could be used to determine the role of the periplasmic domain of EnvZ could involve domain replacement with a homologous sensor domain, such as a domain from the yeast osmosensor Sln1P to address the sequence specificity of this domain (3).

This report has addressed the function of the periplasmic domain, and provides evidence that this domain does not directly bind a protein ligand. The periplasmic domain in *E. coli* may sense a collective property of the medium such as macromolecular crowding or changes in cytoplasmic water activity. The recent identification of mechanosensitive ion channels may also be involved in mediating a conformational change in EnvZ (i.e. dimerization) (30). While *in vitro* and *in vivo* trans-complementation of EnvZ mutants and *in vitro* dimerization studies with purified proteins have demonstrated that the cytoplasmic domain of EnvZ can dimerize (31-34), it is unclear whether the periplasmic domain or transmembrane domain of EnvZ also play a role in dimerization and hence signal propagation.

ACKNOWLEDGMENTS

We thank Heiyoung Park for providing the pET-11/EnvZ-N plasmid and Norma Greenfield for assistance with the circular dichroism computer analysis. This work was supported by a NIH merit award to Masayori Inouye (GM 19043).

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