The membrane-located osmosensory kinase, EnvZ, that contains a leucine zipper-like motif functions as a dimer in *Escherichia coli*

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Abstract The *Escherichia coli* EnvZ protein is a membrane-located osmosensor, which is a typical member of histidine kinases involved in His-Asp phosphotransfer signaling. We found that EnvZ has a leucine zipper-like motif in its presumed periplasmic domain. The functional importance of this leucine zipper-like sequence was assessed by introducing a number of appropriate amino acid substitutions. The results collectively suggest that certain leucine residues in the leucine zipper-like structure play an important role in the osmotic signal transduction mediated by EnvZ. When cysteine was substituted for the crucial leucine residues, the EnvZ dimer with disulfide bridge was detected in the cytoplasmic membrane. It was thus demonstrated that the EnvZ osmosensor exists and exerts its signaling ability as a dimer.

Key words: EnvZ osmosensor; Leucine zipper; Signal transduction; Osmoregulation; *Escherichia coli*

1. Introduction

The ability of cells to adapt to external osmotic stress is a fundamental biological process that protects unicellular microorganisms against direct fluctuations in water activity and solute content of their environment. In fact, many types of microorganisms have developed mechanisms to adapt to severe osmotic changes of their environment (for reviews, see [1,2] and references therein). In this respect, they most likely possess an 'osmosensor' that propagates external osmotic information across the cell membranes. In general, however, little is known about the nature of such putative osmosensors, nor the underlying molecular mechanism by which an external physical force couples to internal genetic regulation.

Among the putative osmosensors in prokaryotes, the best characterized is the *Escherichia coli* EnvZ protein, which is involved in the osmotic regulation of the major outer membrane proteins, OmpC and OmpF [3]. Furthermore, EnvZ is a typical member of widespread histidine kinases that belongs to the so-called two-component regulatory systems (or His-Asp phosphorelay) (see Fig. 1) [4–6]. In the light of extensive studies from our and other laboratories, the following scenario can be proposed for the molecular mechanisms underlying the EnvZ-mediated signal transduction in response to medium osmolarity [7–9]. EnvZ in the cytoplasmic membrane somehow senses an external osmotic stimulus. This protein is capable of undergoing autophosphorylation at its particular histidine residue (His-243). It is also capable of transferring a phosphoryl group to OmpR, which is a DNA-binding transcriptional activator specific for the *ompC* and *ompF* genes. OmpR is phosphorylated at a certain aspartate residue (Asp-55), and upon phosphorylation it becomes active in DNA binding. EnvZ is also capable of stimulating dephosphorylation of phospho-OmpR, thus this osmosensor can apparently be envisaged as a kinase/phosphatase specific for OmpR. It is postulated that the relative activities of EnvZ are modulated in response to medium osmolarity. As a whole, OmpR is able to trigger *ompC* and *ompF* transcription, depending on medium osmolarity.

EnvZ has two membrane-spanning segments (TM1 and TM2, see Fig. 1), and also has both the extracellular periplasmic and intracellular cytoplasmic domains [10–12]. The cytoplasmic domain obviously serves the catalytic function, while the functions of the periplasmic and membrane-spanning domains are not fully understood. Recently, the functional importance of a linker region between TM2 and the catalytic domain was also recognized [13]. In any event, based on several lines of indirect evidence, EnvZ is postulated to function in a homodimer [14,15], yet this view has not been proved directly. Furthermore, it is largely unknown how the EnvZ osmosensor propagates information across the membrane. To address these issues, we recently inspected the primary sequence of EnvZ more closely, and noticed that it has a leucine zipper-like motif in the presumed periplasmic domain (see Fig. 1). In this study, we examined the functional importance of this newly found motif, which might be responsible for the presumed EnvZ dimerization. Here we report for the first time not only the functional importance of the EnvZ periplasmic domain containing a leucine zipper-like motif, but also direct evidence that EnvZ functions as a dimer in the cytoplasmic membrane.

2. Materials and methods

2.1. Bacteria and plasmids

E. coli K-12 strain AT142 (*ΔenvZ lacU169 araD rpsL flbB relA thiA*) was mainly used [16]. Cells were cultivated in Luria broth unless otherwise noted. If necessary, ampicillin (50 μg/ml) was added. Plasmid pAT2005S was mainly used, which carries the intact *envZ* gene under the control of the *lpp/lac* promoter/operator [17]. Note that properties of the EnvZ product from this particular plasmid have previously been characterized extensively [11,17]. It should be mentioned here that AT142 carrying this plasmid produced an appropriate amount of EnvZ in the absence of any inducer for the *lpp/lac* promoter, and exhibited the wild-type phenotype with regard to the EnvZ function (see Fig. 1) [17].

2.2. Recombinant DNA techniques and oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis was carried out with plasmid pAT2005S according to the method of Kunkel [18], with a set of appropriate synthetic oligonucleotides. Nine derivatives of

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pAT2005S were thus constructed, each of which carries a mutant *envZ* gene encoding the EnvZ protein with each different amino acid substitution, as shown in Fig. 1B.

2.3. Preparation of outer membrane proteins

For analyses of osmoregulatory profiles as to OmpC/OmpF expression in the outer membrane, cells were grown in medium A with or without sucrose (15% w/v) [16]. Outer membrane proteins were prepared by extraction of cell envelopes with sodium *N*-lauroyl sarcosinate, and then the outer membrane protein compositions were analyzed by means of urea-SDS-polyacrylamide gel electrophoresis (PAGE), followed by staining with Coomassie brilliant blue, as described previously [16].

2.4. Preparation of urea-treated cytoplasmic membranes containing EnvZ

Strain AT142 carrying plasmid pAT2005S and its mutant derivatives was grown in Luria broth. From these cells, urea-treated cytoplasmic membranes containing the intact form of EnvZ were prepared, as described previously [17].

2.5. Dithiothreitol treatment

The urea-treated membrane containing EnvZ, prepared as described above, was originally suspended in a buffer comprising 50 mM Tris-HCl (pH8.0), 0.35 mM EDTA, and 10% glycerol. Dithiothreitol (DTT, up to a final concentration of 10 mM) was added to the suspension, and then the samples were boiled in 1% sodium dodecyl sulfate (5 min), prior to analysis by SDS-PAGE, followed by immunoblotting.

2.6. Immunoblot analysis

Protein samples were subjected to SDS-PAGE (10% acrylamide), according to the method of Laemmli [19]. Proteins on the gels were transferred to nitrocellulose filters, as described previously [17]. The filters were treated with an anti-EnvZ antiserum and then treated with alkaline phosphatase-coupled goat anti-rabbit immunoglobulin G to detect cross-reacting proteins.

3. Results

3.1. EnvZ contains a leucine zipper-like motif in the periplasmic domain

To gain new insight into the structure and function of EnvZ, we inspected the primary amino acid sequence of EnvZ more closely (Fig. 1). In the EnvZ sequence of 450 amino acids, the well characterized histidine kinase domain is located in the C-terminal region. In the N-terminal region, there are two stretches of hydrophobic amino acids, both of which serve most likely as membrane-spanning segments, designated TM1 and TM2. A region between them is envisaged to be extruded into the external periplasmic space. We found that the first membrane-spanning segment, TM1, is immediately followed by an intriguing stretch of amino acids (Fig. 1A), which appears to have a feature characteristic of the well-documented leucine zipper motif [20,21]. As shown in Fig. 1A, in this sequence leucine is the first residue in four of the four contiguous heptad repeats. If this particular sequence is supposed to adopt an α-helical structure, it may create an amphipathic helical structure, as illustrated in Fig. 1B. In general, a typical leucine zipper is known to serve as a DNA-binding motif, which is frequently found in eukaryotic DNA-binding regulatory proteins, including GCN4 of yeast and c-Fos/c-Jun of mammals, etc. [20,21]. It is also present in membrane proteins that do not bind to DNA [22,23]. It is worth mentioning that several precedents of bacterial leucine zipper-like motifs are known [24–26]. In any case, it is clear from previous studies that such a motif is involved in homo- or heterodimer formation through a coiled-coil structure.

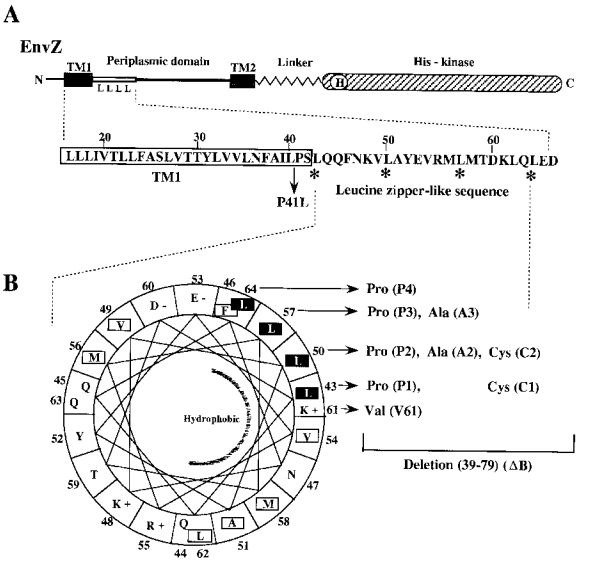

Fig. 1. Structural features characteristic of the EnvZ osmosensor. A: The structure of the EnvZ protein is schematically shown. TM1 and TM2 indicate the membrane-spanning segments, and the periplasmic domain is flanked by these two transmembranous regions. The C-terminal region consists of two distinct domains, the linker and histidine kinase domains, both of which are located in the cytoplasmic space. The amino acid sequence of TM1 is shown in a box, which is followed by a newly found leucine zipper-like sequence, in which the first leucine residue in four of the four heptad is indicated by an asterisk. B: The amino acid sequence of the leucine zipper-like motif is also plotted in an α-helical wheel, in order to emphasize its amphipathic nature, in which hydrophobic residues are boxed and the four leucine residues are highlighted by black boxes. Each single amino acid substitution characterized in this study is summarized in the figure, in which the previously characterized EnvZ mutants, Pro-41 to Leu and ΔB (Δ39–79), are also included.

Although EnvZ is a prokaryotic non-DNA-binding protein, this finding encouraged us to examine the functional importance of this putative motif, particularly in terms of dimer formation and signal propagation in EnvZ.

3.2. Functional importance of the leucine residues in the putative leucine zipper

To examine the function of the four leucine residues in the putative leucine zipper motif, a proline residue was substituted for each corresponding leucine, by site-directed mutagenesis of plasmid pAT2005S carrying the wild-type *envZ* gene. This yielded four types of mutant plasmids, named pAT2005S-P1, -P2, -P3, and -P4 (see Fig. 1B), each of which was expected to produce a mutant EnvZ protein having a single amino acid substitution, Leu-43 to Pro, Leu-50 to Pro, Leu-57 to Pro, and Leu-64 to Pro, respectively. These mutant plasmids were introduced into strain AT142 carrying the *ompR*, *ompC*, and *ompF* genes, but lacking the *envZ* gene, in order to examine the function of each EnvZ mutant with regard to the osmotic regulation of OmpC and OmpF production. Since EnvZ is the cytoplasmic membrane protein, it was critical to first examine whether or not the set of EnvZ mutants were produced at a reasonable level, and more importantly, whether or not they were incorporated into the membrane as normally as in the case of the wild-type. Thus, the membrane fractions were prepared from each transformant, and then the EnvZ protein in the isolated membrane was detected by immunoblotting analysis with an anti-EnvZ antiserum, as

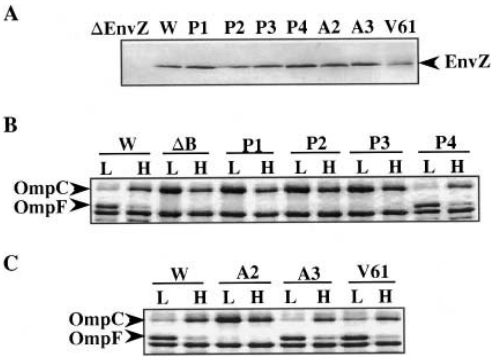

Fig. 2. Immunoblot detection of the EnvZ protein with an anti-EnvZ antiserum, and urea-SDS-PAGE showing the osmoregulatory phenotypes exhibited by the cells carrying the set of mutant *envZ* genes. A: The urea-treated cytoplasmic membranes were prepared from strain AT142, which was transformed with each plasmid producing the indicated EnvZ mutants (see Fig. 1). These samples were analyzed by immunoblotting with an anti-EnvZ antiserum. B and C: Strain AT142 was transformed with each plasmid producing the indicated EnvZ mutants. The cells were grown in medium A with or without 15% sucrose (L, low osmolarity medium; H, high osmolarity medium). Outer membrane proteins were prepared and analyzed by urea-SDS-PAGE. The positions of the OmpC and OmpF proteins are indicated.

shown in Fig. 2A. The results showed that all of the mutant EnvZ proteins, as well as the wild-type, were stably incorporated into the membrane at equivalent amounts relative to each other. Based on this rationale, the osmoregulatory profiles of the OmpC and OmpF outer membrane proteins were examined (Fig. 2B). Expression of the OmpC and OmpF proteins in cells transformed by the wild-type *envZ* gene exhibited a typical osmoregulatory profile, as demonstrated previously [27] (i.e. lane L, a large amount of OmpF in the low osmolarity medium; lane H, a large amount of OmpC in the high osmolarity medium containing 15% sucrose). However, the cells carrying the EnvZ mutants, EnvZ-P1, -P2, and -P3, exhibited a markedly altered profile (i.e. no OmpF production, and constitutive production of OmpC, irrespective of the medium osmolarity). The EnvZ-P4 mutant appeared to function as normally as in the case of the wild-type. A previously characterized EnvZ mutant (named EnvZ-ΔB), lacking the amino acids from position 39 to 79, was also re-examined as a reference (Fig. 2B) [11]. Note that this EnvZ mutant lacks the entire leucine zipper-like sequence (see Fig. 1B). This mutant also exhibited an altered osmoregulatory profile very similar to those exhibited by EnvZ-P1, -P2, and -P3.

To address this issue further, alanine (instead of proline) was substituted for some of the leucine residues in question, particularly, Leu-50 and Leu-57 (note that although we attempted extensively to substitute Leu-43 with Ala, we did not succeeded in doing so). Also, when inspecting the presumed amphipathic helix in EnvZ, a positively charged lysine residue was found in the hydrophobic surface at position 61. A hydrophobic amino acid, valine, was thus substituted for this particular lysine residue. After confirming that these EnvZ mutants were also stably produced in the membrane (Fig. 2A), their properties were examined (Fig. 2C). It was found that when Leu-50 was changed to Ala (i.e. EnvZ-A2), the osmoregulatory profile was markedly affected in a fashion similar to EnvZ-P1, -P2, and -P3, while others (EnvZ-A3 and -V61) were not.

These results collectively suggest that certain leucine residues in the putative leucine zipper-like motif in EnvZ appear to play a role in osmotic signal transduction. Among the four leucine residues, those proximal to TM1 (i.e. Leu-43, Leu-50) seem to be more crucial. Leu-57 appears to be less important, but the reason for this is not clear. In any event, these results are compatible at least partly with the idea that the leucine zipper-like motif is important for EnvZ to mediate normally osmotic signal transduction.

3.3. *EnvZ exists as a homodimer in the membrane*

The above finding suggested that the leucine zipper-like motif in EnvZ plays a functional role for transmembrane signal propagation. Nonetheless, this does not necessarily prove that EnvZ exists as a homodimer. To address this issue critically, cysteine was needed to be substituted for appropriate leucine residues in the motif. If the leucine residues of the two EnvZ molecules in the presumed dimer are located closely enough to form a coiled-coil interface, the two EnvZ molecules should be ligated via a disulfide bridge, provided that cysteine was substituted for the leucines. This was indeed the case, as demonstrated below.

We constructed another set of plasmids (named pAT2005S-

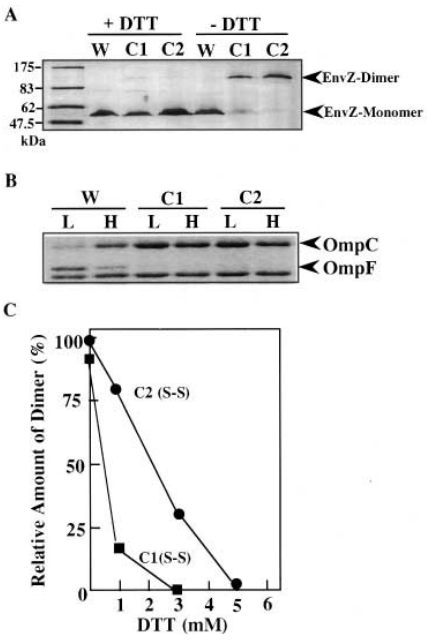

Fig. 3. Immunoblot detection of the EnvZ proteins with an anti-EnvZ antiserum, and urea-SDS-PAGE showing the osmoregulatory phenotypes exhibited by the cells carrying the set of mutant *envZ* genes. A: The urea-treated cytoplasmic membranes were prepared from strain AT142, which was transformed with each plasmid producing the indicated EnvZ mutants (see Fig. 1). These samples were analyzed by immunoblotting with an anti-EnvZ antiserum. Note that samples were pretreated with and without dithithreitol (DTT, 25 mM). The positions of the EnvZ monomer and dimer are indicated. B: Strain AT142 was transformed with each plasmid producing the indicated EnvZ mutants. The cells were grown in medium A with or without 15% sucrose (L, low osmolarity medium; H, high osmolarity medium). Outer membrane proteins were prepared and analyzed by urea-SDS-PAGE. The positions of the OmpC and OmpF proteins are indicated. C: Essentially the same experiment as in A was conducted. However, the samples were pretreated with various concentrations of DTT. Relative amounts of EnvZ dimer, detected by immunoblotting for EnvZ-C1 and EnvZ-C2, are plotted against the concentrations of DTT used.

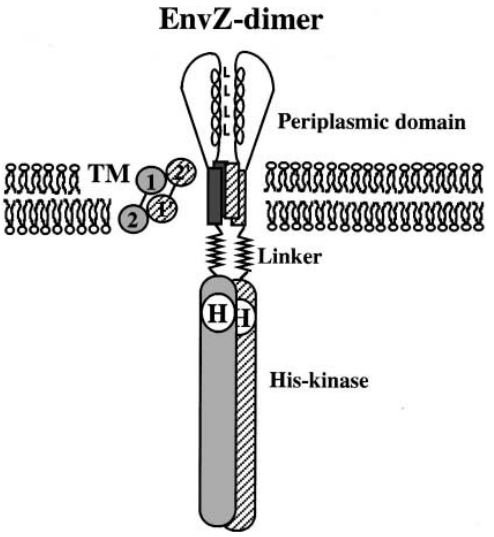

Fig. 4. Proposed model of the structure of the EnvZ dimer in the cytoplasmic membrane. With regard to the structural features of the EnvZ-monomer, see Fig. 1. In the periplasmic domain, a leucine zipper-like motif was found, which may form a coiled-coil interface in the EnvZ dimer, as schematically shown (L, leucine residue). The four membrane-spanning segments of the two EnvZ molecules (TM1-TM2 and TM1′-TM2′) may form a four-helix bundle in the lipid bilayer. Relative orientations of the four helices were also predicted, as schematically shown (a view from the top). Other details are given in the text.

C1 and -C2, see Fig. 1B), which specify the EnvZ mutants having the Leu-43 to Cys and Leu-50 to Cys substitutions, respectively. These plasmids were transferred into AT142, and then the membrane fractions were prepared from the transformants. The isolated membranes were suspended in a buffer containing no reducing reagent (e.g. DTT; dithiothreitol). The membrane proteins were directly analyzed by SDS-PAGE, followed by immunoblotting of EnvZ with an anti-EnvZ antiserum (Fig. 3A, lanes denoted −DTT). Both EnvZ mutants, EnvZ-C1 and -C2, were stably incorporated into the membrane, and more importantly, they existed predominantly as a dimer under these conditions. It may be noted that a pretreatment of the isolated membrane with *N*-ethylmaleimide did not affect the profile (data not shown). However, when treated with DTT, EnvZ-C1 and -C2 migrated to the position at which the wild-type EnvZ monomer was detected on the gel (lanes denoted +DTT). It was also of interest to examine the function of these particular EnvZ mutants (Fig. 3B). Both of the cells carrying these mutants were revealed to exhibit the same altered osmoregulatory profiles as those observed for EnvZ-P1 and -P2. This altered osmoregulatory phenotype, observed for EnvZ-C1 and -C2, strengthens our conclusion in the above section.

Then, the urea-treated cytoplasmic membranes were isolated from the cells producing each one of the mutant EnvZ proteins (i.e. EnvZ-P1, -P2, -C1, and -C2). These samples were assessed in terms of their in vitro ability to phosphorylate OmpR (i.e. kinase activity). The results showed that all of these mutant proteins fully retained the kinase activity, and they were assumed to be defective in their phosphatase activity (data not shown). It should thus be emphasized that these mutant proteins had not lost their function, but rather had gained a 'locked active function', irrespective of the external osmotic stimulus (see Section 4). These results collectively demonstrate that EnvZ exists and exerts its catalytic ability as a dimer, and a certain region of the periplasmic domain encompassing the leucine zipper-like motif must be physically in close proximity to the EnvZ dimer. In this context, the relative sensitivities toward the reducing reagent (DTT) were compared between the disulfide bonds of EnvZ-C1 and -C2 (Fig. 3C). It was found the the EnvZ-C1 disulfide bond was more easily cleaved with a relatively low concentration of DTT. This may suggest that the amino acid position 50 is buried more inside in the presumed interface of the EnvZ dimer, as compared with position 43.

4. Discussion

By incorporating the findings of this study into those from previous extensive studies on the EnvZ osmosensor, one can now reasonably envisage a hypothetical view as to the EnvZ structure in the cytoplasmic membrane, as illustrated in Fig. 4.

EnvZ is a member of a large family of bacterial sensory kinases in prokaryotes, most of which are located in the cytoplasmic membrane (for a review, see [28] and references therein). These bacterial sensors often have two membrane-spanning segments, which flank a periplasmic domain. Their C-terminal region contains a common histidine kinase domain. They are thus assumed to have a topology similar to EnvZ: the C-terminal catalytic domain protruding into the cytoplasmic space, and the presumed N-terminal signal-input domain projecting into the periplasmic space [10]. In this respect, EnvZ should offer a general model for exploring transmembrane signaling mechanisms at the molecular level. In fact, a number of studies on the structure and function of the EnvZ osmosensor have been reported ([13,15,29] and references therein). However, what remained unclear was whether the EnvZ molecules in the cytoplasmic membrane exist as a monomer or a dimer, although several lines of indirect evidence supported the latter view (see below). In this study, our results provided direct evidence that EnvZ functions as a dimer.

Yang and Inouye previously demonstrated that two co-expressed types of mutant EnvZ molecules, in which one of the mutants lacked the conserved histidine (His-243) and the other had a deletion in the C-terminal catalytic domain, exhibited significant autophosphorylation and OmpR phosphorylation abilities [14]. This suggested that the autophosphorylation in EnvZ molecules is an intermolecular phosphorylation event, requiring the formation of a dimer at least in the cytoplasmic domain. We and others have previously demonstrated that a number of mutations in the membrane-spanning segments of EnvZ (TM1 or TM2) resulted in a defect in transmembrane signaling in vivo and in vitro [12,30]. One of these EnvZ mutants with the amino acid substitution in TM1 (Pro-41 to Leu) exhibited the same OmpC-constitutive phenotype as those observed for the mutants characterized in this study (see Fig. 1) [15]. With regard to this, an intriguing finding was that the altered phenotype of this mutation in TM1 (Pro-41 to Leu) was suppressed by a certain mutation in TM2 (Arg-180 to Trp) through intermolecular complementation, when the respective mutant EnvZ molecules carrying each amino acid change were co-expressed in vivo [15]. In any case, these previous results indirectly supported the idea that EnvZ molecules function as a dimer. In this study, this

view was proven by direct experimentation (i.e. the demonstration of the dimer by chemical cross-linking between the newly introduced cysteine residues). In this context, it is also worth mentioning that an intermolecular interaction between TM1 in one EnvZ molecule and TM2′ in another EnvZ molecule seems to play a role in transmembrane signaling [15]. The results of this study predicted further that TM1 and TM1′ also appear to be in close proximity. Thus, the four TMs from two EnvZ molecules may form a transmembranous four-helix bundle, as illustrated in Fig. 4.

The functional importance of the EnvZ periplasmic domain has been the subject of debate [11,29]. From the analyses of a set of deletion mutants for the periplasmic domain, we previously suggested that this external domain is crucial for EnvZ function [11]. Based on similar analyses, others argued against this view [29]. They claimed also that, although the *envZ* gene has been well studied genetically, mutations have not been identified in the periplasmic domain (i.e. they mentioned that if a *specific* structure in the periplasmic domain was required for sensing medium osmolarity, it seems likely that mutations in this domain *could* be isolated). Indeed, in this study a set of single amino acid substitution mutants exhibiting an altered osmoregulatory phenotype *could* be isolated in the periplasmic domain containing a *specific* structure, namely, a leucine zipper-like motif. The altered phenotypes observed for these mutants are reasonably explained by assuming that certain amino acid substitutions in the periplasmic domain of the EnvZ dimer would induce a conformation analogous to the presumed high osmolarity form of EnvZ, but the conformational change was irreversible (or locked). In other words, the conformational change caused by the mutations no longer fluctuates in response to medium osmolarity, thereby resulting in the OmpC-constitutive high osmolarity phenotype. It is thus tempting to speculate that a proper interaction between the leucine zipper-like interface in the EnvZ dimer might be important for a reversible conformational change, which is presumably involved in the underlying mechanism of osmosensing. In any event, we would like to propose that the periplasmic domain (at least the presumed coiled-coil region) plays not only a structural but also a functional role in the EnvZ dimer.

Although a relatively clear picture has emerged with regard to the structural and functional relationship of the EnvZ dimer in the cytoplasmic membrane, what remains still unclear is how EnvZ can propagate the external osmotic signal across the membrane. In this context, the structural view proposed for EnvZ in this study should provide us with hints to address this crucial and general issue.

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