The HAMP domain plays an essential role in signal transduction not only in histidine kinase but also in a number of other signal-transducing receptor proteins. Here we expressed the EnvZ HAMP domain (Arg<sup>180</sup>–Thr<sup>235</sup>) with the R218K mutation (termed L<sub>RK</sub>) or with L<sub>RK</sub> connected with domain A (Arg<sup>180</sup>–Arg<sup>235</sup>) (termed L<sub>RK</sub>) of EnvZ, an osmosensing transmembrane histidine kinase in <i>Escherichia coli</i>, by fusing it with protein S. The L<sub>RK</sub> and L<sub>RK</sub> proteins were purified after removing protein S. The CD analysis of the isolated L protein revealed that it consists of a random structure or is unstructured. This suggests that the EnvZ HAMP domain by itself is unable to form a stable structure and that this structural fragility may be important for its role in signal transduction. Interestingly the substitution of Ala<sup>193</sup> in the EnvZ HAMP domain with valine or leucine in Tez1A1, a chimeric protein of Tar and EnvZ, caused a constitutive OmpC phenotype. The CD analysis of L<sub>RK</sub>(A193L) revealed that this mutated HAMP domain possesses considerable secondary structures and that the thermostability of this entire L<sub>RK</sub>(A193L) became substantially lower than that of L<sub>RK</sub> or just domain A, indicating that the structure of the HAMP domain with the A193L mutation affects the stability of downstream domain A. This results in cooperative thermodenaturation of domain A with the mutated HAMP domain. These results are discussed in light of the recently solved NMR structure of the HAMP domain from a thermophilic bacterium (Hulkó, M., Berndt, F., Gruber, M., Linder, J. U., Truffault, V., Schultz, A., Martin, J., Schultz, J. E., Lupas, A. N., and Coles, M. (2006) <i>Cell</i> 126, 929–940).

Histidine kinases are the major signal transducer in bacteria, playing critical roles in adaptation to external stresses.EnvZ from <i>Escherichia coli</i> is one of the most extensively studied histidine kinases in terms of the functional and structural aspects (1). EnvZ is a transmembrane histidine kinase located in the inner membrane and functions as an osmosensor. It consists of a periplasmic receptor domain; two transmembrane domains, TM1 and TM2; and a cytoplasmic domain. The cytoplasmic domain has the bifunctional histidine kinase activity of EnvZ and can be further divided into three functional domains: the linker domain, domain A, and domain B. Domain A and domain B form the catalytic core of bifunctional histidine kinase EnvZ, having both kinase and phosphatase functions (2). Biochemical and structural studies of both domain A and domain B revealed that domain A contains the autophosphorylation site of a conserved His<sup>243</sup> and that it is a dimerization domain of EnvZ forming a four-helical bundle, whereas domain B contains an ATP-binding pocket (2–4). Thus, domain A and domain B are generally termed DHp and CA domains, respectively (5).

The linker domain of EnvZ connects TM2 and the cytoplasmic domain. Although it is not directly involved in the enzymatic activities of EnvZ, it is known that the linker domain plays an important role in signal transduction. This is supported by the fact that a number of point mutations within the linker domain of EnvZ caused perturbation of the proper response against osmotic stresses (6–9). The analysis of signal-transducting proteins from histidine kinases, adenyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases revealed that like EnvZ, these proteins also contain linker-like domains connecting their transmembrane domains to the cytoplasmic domains (10, 11). Importantly secondary structure prediction of these domains showed that they share a highly conserved helix-turn-helix fold even though their amino acid sequences have low homology. Therefore, these domains are called HAMP domains because this common motif was shared with histidine kinases, adenyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases. Mutational studies of HAMP domain of BarA (12), Aer (13), or NarX (14) also demonstrated that the HAMP domains play a crucial role in their signal transduction, supporting the notion that the HAMP domains are required for the proper signal transduction of histidine kinases, adenyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases (10, 11).

To elucidate the mechanism of the signal transduction, functional hybrid histidine kinases between EnvZ and chemosensor Tar have been constructed by fusing the receptor domain and HAMP domain of Tar to the cytoplasmic kinase domain of EnvZ (15). The binding of aspartate to the receptor of this hybrid histidine kinase, termed Taz, modulates the enzymatic activities of the cytoplasmic domain of EnvZ. When Tar HAMP domain of Taz was replaced with EnvZ HAMP domain, this new construct, termed Tez1A1, was also able to respond to...
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Aspartate in the media (16). These results demonstrated that
the HAMP domains of Tar and EnvZ share not only their struc-
ture but also their function in signal transduction. A similar
approach has been taken to construct hybrid signal-transduc-
ing proteins using a number of other histidine kinases and sig-
nal-transducing proteins: EnvZ and Trg (17), NarQ and NarX,
NarX and CpxA (18), Tar and Tsr (19), Tap and Tar (20), Trg
and Tsr (21), and Aer and Tsr (22). All these hybrid signal-
transducing proteins were able to properly respond to their
specific ligands, and importantly, their HAMP domains can be
shared by two different signal-transducing proteins. This fur-
ther supports the concept that a common mechanism for signal
transduction appears to prevail and that the HAMP domains
among signal-transducing proteins probably share a similar
structure as well as function in signal propagation across the
transmembrane to the cytoplasmic domains.

Recently it has been shown that a heterodimer of Taz1 and
Tez1A1 can respond to aspartate in the media, showing that the
signal can be asymmetrically transduced through only one of
two HAMP domains even though this heterodimer contains
two different HAMP domains, one from Tar in Taz1 and the
other from EnvZ in Tez1A1 (23). Furthermore it has been de-
monstrated that the intersubunit interaction between the HAMP
domains within the dimers of signal-transducing proteins is
important to symmetrically propagate a signal to both the
downstream subunits.

Because the nature of the HAMP domains have not well
characterized, here we characterized the biochemical proper-
ties of the HAMP domain using purified EnvZ HAMP domain
(Arg180-Thr285) with R218K mutation (LARK) and LARK con-
struct, the CD analysis of the purified mutated LARK carry-
ing the A193L mutation showed a significantly higher helical
content than LARK. This result indicates that this mutated
OmpC phenotype (7), was introduced into the LRK or LARK
EnvZ HAMP domain, which is known to cause a constitutive
structure as well as function in signal propagation across the
CD analysis of the purified EnvZ HAMP domain.

Thrombin Cleavage—The purified protein was treated with
thrombin to cleave the His6-tagged PrS tag. The protein was
dialyzed against the cleavage buffer (20 mM Tris-HCl (pH 8.0),
150 mM NaCl, and 5 mM β-mercaptoethanol) containing 1 mM phenylmethylsulfonyl
fluoride and 5% glycerol. The cells were centrifuged at
20,000 × g for 20 min at 4 °C, and the supernatant was ultra-
centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant
was precipitated with ammonium sulfate. The pellets of His6-PrS-L
and -LA were then dissolved in 20 ml of the washing buffer (20
mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM phenylmethylsulfonyl
fluoride, and 10 mM imidazole) and mixed with Ni-NTA
agarose (Qiagen). The protein was eluted with imidazole elu-
tion buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM phenylmethylsulfonyl
fluoride, and 100 mM imidazole) and precipitated with ammonium sulfate. The resultant
pellet was dissolved in the gel filtration buffer and further purified with
Sephacyrl S-100 size-exclusion gel chromatography.

Experimental Procedures

Strains and Plasmids—E. coli DH5α and BL21(DE3) were
used for the cloning and expression of the proteins, respec-

tively. Strain RU1012 (MC4100 ara+, Φ (omp-lacZ) 10–25
ΔenvZ::Km) (11) was used for the in vivo study.

DNA fragments containing the DNA sequence of protein S
(PrS)2 tag, the N-terminal domain of protein S (amino acid
residues 1–92), and EnvZ HAMP domain with various lengths
of domain A were amplified by PCR. The PCR parameters were
30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min
using the following primers. The DNA fragment for PrS tag was
amplified with 5′-CCAGGGATATGGGCAAACATTACC-3′
and 5′-GATCAGGATGTCTGAACCTCGAGGTACCAACAT-
GGTTGCAGCGGACCGACATGAC-3′. The PCR prod-
ucts were digested with Ndel and BamHI (GGATCC) and
ligated with the pET system (Novagen) to construct pET-PrS.
DNA fragments for various constructs of EnvZ HAMP domain
were amplified with 5′-GTTGGCCTGGTACCGTAT-
ATCAGAAAC-3′ and 5′-CCATCAAGATCTTACGGTG-
CATCCGCCG-3′ for construct a, 5′-GCCATGATCTTACG-
CTGCCGATCCTGAC-3′ for construct b, 5′-GCCATGATCTTACG-
CTGCGGGATCCTACCCGGG-3′ for construct c, 5′-ATCAGGATATTACCGCCAGCGCATC-3′ for construct d, or 5′-GATCAGGATCTTACGGTGAGTTGAC-
TAAAA-3′ for construct e. The PCR products were digested with
KpnI (GGTACC) and BamHI and ligated with pET-PrS.

Expression of the Proteins—Transformants were cultured in
M9-CAA medium supplemented with 50 μg/ml ampicillin at
37 °C and induced at the midexponential phase at room tem-
perature by the addition of 0.05 mM isopropyl 1-thio-
β-D(-)-
galactopyranoside. After incubation for 18 h at room tempera-
ture, the cells were harvested, and the expression level of the
protein was detected by SDS-PAGE.

Purification of the Fusion Proteins—After expression of the
protein, the cells were harvested and subsequently disrupted
using a French press in 40 ml (per 1 liter of cultured cells) of
gel filtration buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 5
mM β-mercaptoethanol) containing 1 mM phenylmethylsulfonyl
fluoride and 5% glycerol. The cells were centrifuged at
20,000 × g for 20 min at 4 °C, and the supernatant was ultra-
centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant
was precipitated with ammonium sulfate. The pellets of His6-PrS-L
and -LA were then dissolved in 20 ml of the washing buffer (20
mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM phenylmethylsulfonyl
fluoride, and 10 mM imidazole) and mixed with Ni-NTA
agarose (Qiagen). The protein was eluted with imidazole elu-
tion buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM phenylmethylsulfonyl
fluoride, and 100 mM imidazole) and precipitated with ammonium sulfate. The resultant
pellet was dissolved in the gel filtration buffer and further purified with
Sephacyrl S-100 size-exclusion gel chromatography.

The abbreviations used are: PrS, protein S; L, linker; LA, linker plus domain A;
Ni-NTA, nickel-nitritotriacetic acid; Tricine, N-(2-hydroxy-1,1-bis(hydroxy-
methyl)ethyl)glycine.

2 The abbreviations used are: PrS, protein S; L, linker; LA, linker plus domain A; Ni-NTA, nickel-nitritotriacetic acid; Tricine, N-(2-hydroxy-1,1-bis(hydroxy-
methyl)ethyl)glycine.

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RESULTS

Expression of the HAMP Domain of EnvZ—Our earlier effort to express the EnvZ HAMP domain consisting of 43 residues failed because it formed inclusion bodies in the cells. To overcome this problem, we constructed a fusion of EnvZ HAMP domain with protein S, which is a major spore coat protein from Myxococcus xanthus. Its NMR solution structure showed that protein S mostly consists of -sheets and was highly stable in solution (31). Previously we have used the N-terminal domain of protein S to fuse to OmpR, a response regulator protein and a transcription factor in E. coli, to increase its molecular weight for the analysis of OmpR binding to promoter regions (32). In this experiment, two tandem, repeated fragments of the N-terminal domain of PrS (92 residues) were fused to the N-terminal end of OmpR, resulting in a 20-kDa larger molecular mass of PrS2-OmpR. During the course of these experiments, we noticed that fusing the two tandem, repeated PrS fragment (PrS2) to OmpR resulted in substantial increase of yields of purified PrS2-OmpR (∼5-fold) and significant improvement of its solubility (up to 20-fold of purified OmpR itself, 40 mg/ml). On the basis of this observation, various PrS fusions with HAMP domain of EnvZ containing five different lengths of domain A were constructed with His6 tag at the N-terminal end (Fig. 1). Of these five constructs, PrS-L (PrS + linker + 13 residues from domain A) (construct b) and PrS-LA (PrS + linker + domain A) (construct e) were successfully expressed as soluble forms (Fig. 1), whereas the other three constructs were either expressed poorly or expressed as inclusion bodies (not shown). Note that the LA (linker + domain A) protein itself was previously expressed as a soluble protein using the pET expression system and then characterized (33).

Purification of EnvZ HAMP Domain—When PrS-L and PrS-LA were treated with thrombin to cleave and remove the His6-tagged PrS, thrombin cleaved not only its cleavage site, (both using a polypeptide reference set (29)); and CD deconvolution based on neural networks (30), a neural network analysis program. All methods were in good agreement with each other. The thermal denaturation studies were carried out by measuring the change in the ellipticity at 222 nm over the temperature range of 4–70 °C at 0.2 °C intervals with an equilibration of 18 s at each temperature. Data points were collected at each temperature interval for 5 s. Each melting temperature was determined by fitting the thermal unfolding data for a two-state transition between a folded dimer and an unfolded monomer. The protein concentrations were determined using the BCA Protein Assay kit (Pierce).
Leu-Val-Pro-Arg ↓ Gly-Ser, after PrS (Fig. 1) but also unexpectedly the EnvZ HAMP domain at Arg218. The sequence of Ser-Val-Thr-Arg ↓ Ala-Phe in EnvZ HAMP domain was apparently recognized and was cleaved by thrombin. Therefore, to prevent this internal cleavage, Arg218 was substituted with Lys, which still maintains the basic nature of the residue. The effect of R218K mutation was examined by introducing this point mutation into Tez1A1, which is a chimeric protein consisting of the periplasmic and transmembrane region of Tar, aspartate chemosensor, and the HAMP domain and cytoplasmic kinase domain of EnvZ. Tez1A1 responds to aspartate in the medium, resulting in the induction of β-galactosidase. This result demonstrates that R218K mutation did not affect the function of Tez1A1 as well as the function of the EnvZ HAMP domain (see Fig. 4A).

Therefore, an R218K mutation was introduced into PrS-L and PrS-LA proteins, and the resultant PrS-LRK and PrS-LARK mutants were purified using Ni-NTA resin followed by size-exclusion gel chromatography. When the purified PrS-LRK and PrS-LARK mutants were treated with thrombin, both LRK and LARK fragments were detected as a single polypeptide as judged by SDS-PAGE (Fig. 2). The cleaved products were then passed through a Ni-NTA column to remove the His6-tagged PrS tag, and both the LRK and LARK proteins thus purified were soluble and used for further characterizations.

Circular Dichroism Analysis of LRK and LARK—The secondary structure of purified LRK and LARK proteins was estimated using CD spectroscopy. The CD spectrum of LARK showed two minima at 208 and 222 nm, which are characteristic of an α-helical structure (Fig. 3). However, we were unable to detect any secondary structure for the LRK (Fig. 3). The covalent linking of the LRK fragment to domain A (LARK) shows that the presence of the HAMP domain increased the negative ellipticity of domain A. This difference can be better visualized by determining the difference spectrum of LARK and domain A, which exhibits significant secondary structure as compared with isolated domain LRK. According to the secondary structure prediction of the HAMP domain from various signal-transducing proteins including EnvZ, the structure of the HAMP domain consists mostly of a helix structure (34). Additionally a recent NMR study using the HAMP domain from a hyperthermophilic archaeon revealed that it consists of two α-helices connected by a long loop structure (24).

Effect of A193L Mutation on the Structure of EnvZ HAMP Domain—It is a known fact that many point mutations in the EnvZ HAMP domain affect the phenotype of EnvZ (6–9). The A193V mutation in EnvZ HAMP domain is one of point mutations that cause a "constitutive on" phenotype (7). This Ala193 residue has been proposed to play a crucial role in the hydrophobic interaction between the two helices in the dimer of the HAMP domain (24). Therefore, the introduction of a hydro-
phobic amino acid residue that favors hydrophobic interactions may stabilize the hydrophobic interaction within the EnvZ HAMP domain in either an intramolecular or intermolecular manner, resulting in the stabilization of this flexible protein.

First Ala193 in Tez1A1RK was mutated to two hydrophobic residues, Val or Leu, to examine the effects of these mutations on the phenotype of Tez1A1RK. As expected, the cells expressing both Tez1A1RK(A193V) and Tez1A1RK(A193L) mutants showed constitutive on ompC-lacZ gene in the absence or presence of aspartate (Fig. 4A). The expression levels of these mutant proteins were similar to that of Tez1A1RK as judged by Western blot analysis (Fig. 4B). Next A193V or A193L mutation was introduced into PrS-LRK and PrS-LARK to characterize the effect of these mutations on the structural stability of EnvZ HAMP domain.

The far-UV CD spectra of LRK(A193V) and LRK(A193L) were similar to that of LRK and showed no discernible effect on the secondary structure of EnvZ HAMP domain (Fig. 5A). When both mutations were incorporated separately into LA RK, LA RK(A193V) mutant exhibited a CD spectrum similar to that of LA RK, whereas the LA RK(A193L) mutant showed a dramatic change in the negative ellipticity, especially at 222 nm minimum (Fig. 5B). This result indicates that the A193L mutation in the EnvZ HAMP domain of LARK(A193L) mutant protein.

Effect of Trypsin Digestion—To further examine the effect of A193L mutation in the structural stability of LA RK(A193L), limited trypsic digestion was carried using LA RK and LA RK(A193L) mutant proteins. As shown in Fig. 6, the LA RK(A193L) mutant was substantially more resistant against trypsic digestion at room temperature than LA RK. The LA RK(A193L) mutant protein was stably maintained even after 30 min, whereas most of the LA RK protein was digested by trypsin. These observations were the same even at lower temperature at 4 or 15 °C, indicating that lowering the temperature did not stabilize the EnvZ HAMP domain of LA RK (data not shown). These results support the notion that the A193L mutation in the EnvZ HAMP domain of LA RK(A193L) stabilized the structure of the EnvZ HAMP domain.
The HAMP domain in transmembrane signal-transducing proteins links the transmembrane domains with the enzymatic or functional cytoplasmic domains. Thus the HAMP domains play a critical role in signal transduction across their transmembrane domains to modulate the activities of the cytoplasmic domains shared among signal-transducing proteins.

When several constructs of the EnvZ HAMP domain containing partial or entire domain A were expressed with a PrS tag, the N-terminal domain of protein S from *M. xanthus*, the constructs of PrS-LARK (construct b), which includes 13 extra residues from domain A, and PrS-LARK(A193L) (construct e) were highly expressed as soluble proteins (Fig. 1). The EnvZ HAMP domain by itself forms inclusion bodies; however, the addition of the N-terminal PrS tag dramatically improved its expression as well as solubility in constructs b and e.

LARK was found not to have a well defined secondary structure, showing that it has a flexible and/or unstable folding (Table 1 and Fig. 5A). On the other hand, the CD analysis showed that LARK protein increases the negative ellipticity (Fig. 3 and Table 1) more than domain A by itself to a greater extent than domain A by itself, indicating that the HAMP domain attained significant secondary structure on being linked with domain A. This is consistent with the secondary prediction that the EnvZ HAMP domain has a tendency to fold into an $\alpha$-helical structure. These notions are supported by the fact that the A193L mutation in LARK significantly induced structural changes and further increased its $\alpha$-helical content by 16.5 or 11.3% when compared with LARK or domain A by itself, respectively (Table 1). Importantly without domain A this structural change in LARK(A193L) was not detected (Fig. 5A). In addition to the CD analyses, LARK(A193L) was significantly more resistant to tryptic digestion than LARK, supporting the notion that the A193L mutation substantially stabilized the HAMP domain structure (Fig. 6). It is worthwhile to mention that domain A was initially identified as a resistant and stable domain after treating the cytoplasmic domain of EnvZ (EnvZc) with trypsin (2). Therefore, it is likely that the structure of the EnvZ HAMP domain in LARK(A193L) is altered by the point mutation, attaining more helical structure and leading to resistance toward to tryptic digestion.

Recently the first NMR structure of a HAMP domain was published (11), their structural characterizations were not well documented. In the present study, we characterized the HAMP domain of EnvZ, an osmosensing histidine kinase, using CD to elucidate the molecular mechanism of signal transduction shared among signal-transducing proteins.

DISCUSSION

**Thermal Denaturation of the Linker**—To investigate further the effect of the A193V and A193L mutations on the stability of the EnvZ HAMP domain, the thermal unfolding was monitored between 4 and 80 °C by far-UV CD by measuring the change in ellipticity at 222 nm as a function of temperature. The spectra were recorded in 20 mM Tris-HCl (pH 8.0) containing 150 mM KCl, 2 mM CaCl$_2$, and 1 mM dithiothreitol between 4 and 70 °C. The $T_m$ values of each protein are summarized in Table 2. LARK, red circle; LARK(A193V), green circle; LARK(A193L), blue circle; domain A, pink circle.

**TABLE 2**

| Protein          | $T_m$ (°C) |
|------------------|------------|
| LARK             | 46.6       |
| LARK(A193V)      | 45.5       |
| LARK(A193L)      | 37.3       |
| Domain A         | 44.9       |

$^a$ The $T_m$ was determined by fitting the CD thermal transition data to the thermodynamic equation for two-state transition between a folded dimer and an unfolded monomer assuming the heat capacity of folded and unfolded states to be equal.

**FIGURE 6.** Tricine gel electrophoresis analysis of limited tryptic proteolysis of LARK and LARK(A193L) proteins. Purified LARK or LARK(A193L) proteins were incubated with trypsin at a ratio of 500:1 at room temperature in 20 mM Tris-HCl (pH 8.0) containing 150 mM KCl, 2 mM CaCl$_2$, and 1 mM dithiothreitol between 4 and 70 °C. The thermal unfolding of the LARK ($\alpha$-helical content by 16.5 or 11.3% when compared with LARK or domain A by itself, respectively (Table 1). Importantly without domain A this structural change in LARK(A193L) was not detected (Fig. 5A). In addition to the CD analyses, LARK(A193L) was significantly more resistant to tryptic digestion than LARK, supporting the notion that the A193L mutation substantially stabilized the HAMP domain structure (Fig. 6). It is worthwhile to mention that domain A was initially identified as a resistant and stable domain after treating the cytoplasmic domain of EnvZ (EnvZc) with trypsin (2). Therefore, it is likely that the structure of the EnvZ HAMP domain in LARK(A193L) is altered by the point mutation, attaining more helical structure and leading to resistance toward to tryptic digestion.

Recently the first NMR structure of a HAMP domain was determined using the HAMP domain of a putative transmembrane receptor, Afi1503, from a hyperthermophilic archaeon,
Archeoglobus fulgidus (21). This HAMP domain consists of 56 residues and forms a stable dimer. Its NMR structure revealed that it comprises a homodimeric, four-helical, parallel coiled-coil structure. Although its amino acid sequence consists of the defined heptad repeating pattern of hydrophobic residues, its interhelical packing was unusual but similar to a knobs-to-knobs packing that is found in coiled-coil structures with no interhelical packing. The authors noticed that Ala291 is one of the highly conserved residues among the HAMP domains; this corresponds to Ala193 in EnvZ (Fig. 8). When this Ala residue in the A. fulgidus HAMP domain was mutated to Val to make the heptad repeat of hydrophobic residues stronger, it was observed that the A291V mutant protein oscillates between two different states, presumably between the knobs-to-knobs packing state and the knobs-to-holes packing state (24). Therefore, it was proposed that the smaller side chain of this Ala residue as compared with the other core hydrophobic residues likely contributes in the local flexibility of the hydrophobic interface to form knobs-to-knobs packing. Importantly the authors further proposed that a HAMP domain transmits a signal by rotation from the knobs-to-knobs packing state to the knobs-to-holes packing state.

In a manner similar to this structure, the EnvZ HAMP domain may form a helix-turn-helix structure with these packing states because it has a heptad repeating pattern similar to that of the HAMP domain of A. fulgidus (Fig. 8). Earlier a number of EnvZ mutants causing non-regulatable phenotypes were isolated, and some of them had different point mutations within the EnvZ HAMP domain such as the A193V mutation (6–9, 38). The effect of those mutations on the enzymatic activities of EnvZ was characterized using EnvZc, which consists of the EnvZ HAMP domain, domain A, and domain B. It was demonstrated that these mutations in the EnvZ HAMP domain did not affect any of the enzymatic activities of EnvZc (6). Therefore, it was concluded that the effect of these mutations becomes prominent only when the EnvZ HAMP domain is connected to the transmembrane domain of EnvZ. The CD analyses presented in the study clearly suggest that, although the covalent connection of the EnvZ HAMP domain to domain A helps to retain the helical structure in the HAMP domain, the EnvZ HAMP domain is still highly flexible and mostly unstructured. This is likely the reason why the effect of mutations at the EnvZ HAMP domain in EnvZc was not detectable. Therefore, these data suggest that only when the EnvZ HAMP domain is connected to the transmembrane domain can the EnvZ HAMP domain form a helix-turn-helix with proper packing state as observed in the HAMP domain of A. fulgidus.

Furthermore, Leu at 193 further extends the Leu patch of the hydrophobic interface within the predicted α1 (Fig. 8), which forms a better heptad repeat of hydrophobic residues. Hence it stabilizes the structure of the EnvZ HAMP domain but significantly loses the local flexibility of the hydrophobic interface, resulting in disrupting its packing states. In the case of the A193V mutation at the EnvZ HAMP domain of EnvZ, it likely disrupts the packing states of this domain as observed from the NMR studies of the A. fulgidus HAMP domain and its A291V mutant protein. These changes in the packing states of the EnvZ HAMP domain result in loss of its critical function to properly modulate the enzymatic activities of EnvZ by propagating a signal from the transmembrane domain to the downstream histidine kinase domain.

It is also interesting to note that the A193L mutation caused a significant decrease in the thermal stability of LARK(A193L) compared with that of LARK and even domain A itself (Fig. 7). LARK(A193L) has a well folded secondary structure, which was highly resistant to tryptic digestion, but yet the conformational change of the EnvZ HAMP domain clearly influenced the thermal stability of downstream domain A. At present, it is not certain how the thermal stability was affected by connecting the mutated HAMP domain to domain A. Nevertheless this is the first direct indication that a structural change within the EnvZ HAMP domain influences its downstream histidine kinase domain, reflecting the possible concerted rotation of the coiled-coil structure of the EnvZ HAMP domain in the normal osmosensing signal transduction in EnvZ.

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