Phosphatase activity of histidine kinase EnvZ without kinase catalytic domain

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Most histidine kinases are bifunctional enzymes having both kinase and phosphatase activities. The cytoplasmic kinase domain of EnvZ, a transmembrane histidine kinase functioning as an osmosensor in *Escherichia coli*, consists of two distinct functional subdomains: domain A [EnvZc(223–289)] and domain B [EnvZc(290–450)]. NMR studies demonstrated that domain A consists of a four-helix bundle serving as a dimerization and phosphotransfer domain, and domain B functions as the ATP-binding and catalytic domain. Here we demonstrate that domain A by itself has the phosphatase activity both *in vitro* and *in vivo*. This phosphatase activity is Mg^{2+} dependent but is not activated by ADP, ATP, or adenosine 5′-[β,γ-imido]triphosphate (AMPPNP), each of which may serve as a cofactor for the EnvZ phosphatase activity. Domain B showed a small but distinct effect on the domain A phosphatase activity only in the presence of ADP or AMPPNP. However, when domain B was covalently linked to domain A, dramatic cofactor-dependent enhancement of the phosphatase activity was observed. Extending domain A for another 75 residues at the C terminus or 44 residues at the N terminus did not enhance its phosphatase activity. Substitution mutations at His-243, the autophosphorylation site, demonstrate that the His residue plays an essential role in the phosphatase activity. The so-called X-region mutant L288P that is known to specifically abolish the phosphatase activity in EnvZ had no effect on the domain A phosphatase function. We propose that the EnvZ phosphatase activity is regulated by relative positioning of domains A and B, which is controlled by external signals. We also propose that the His-243 residue participates in both kinase and phosphatase reactions.

In prokaryotes the histidyl-aspartyl (His-Asp) phosphorelay signal transduction system plays a major role in cellular adaptation to various environmental stresses and growth conditions (1). EnvZ, the osmosensor in *Escherichia coli*, is a transmembrane histidine kinase consisting of 450 amino acid residues, of which the C-terminal cytoplasmic kinase domain possesses highly conserved regions (H box, N box, G1 box, F box, and G2 box) unique in histidine kinases (2, 3). OmpR, the cognate response regulator, is phosphorylated at Asp-55 by EnvZ. The phosphorylated product, OmpR-P, functions as a transcription factor and regulates the expression of the genes for porin proteins OmpF and OmpC. Most histidine kinases, including EnvZ, are bifunctional having both kinase and phosphatase activities (4–6). EnvZ, thus, has the OmpR kinase activity as well as OmpR-P phosphatase activity. It has been proposed that the cellular level of OmpR-P is regulated by the OmpR-P phosphatase activity, whereas the OmpR kinase activity is maintained at a constant level (7).

EnvZ consists of the periplasmic putative receptor domain (residues 48–162), two transmembrane regions (TM1, residues 16–47; TM2, residues 163–179), the linker region (residues 180–222), and the cytoplasmic kinase domain (residues 223–450) (8, 9). The kinase domain possesses both kinase and phosphatase function as the full-length EnvZ and can be further dissected into two distinct functional domains: A (residues 223–289) and B (residues 290–450) (10). It has been demonstrated that domain A, containing the autophosphorylation site His-243, forms a stable dimer and can be phosphorylated in the presence of ATP by domain B that exists as a monomer. The phosphorylated domain A subsequently transfers the high-energy phosphoryl group to OmpR. Because of their unique features, domains A and B are termed the DHp (dimerization and histidine phosphotransfer) domain and the CA (catalytic and ATP-binding) domain, respectively (11).

The NMR structures of both domains have been recently determined. One domain A homodimer comprises a four-helix bundle with twofold symmetry. Each subunit folds into a compact structure consisting of antiparallel helices connected by a turn (12). The three-dimensional structure of adenosine 5′-[β,γ-imido]triphosphate (AMPPNP)-bound domain B (13) reveals a protein kinase fold (α/β sandwich fold) with remarkably high homology with the ATP-binding domains of three ATPases: Hsp90, DNA gyrase B, and MutL (14). The x-ray crystal structure of another histidine kinase domain, that of the CheA, has recently been solved (15), demonstrating that the α/β sandwich fold is a prevailing feature among histidine kinases.

On the basis of the structural information and our recent study on a synthetic monomeric histidine kinase, EnvZc[AAB], we have proposed that the three-dimensional structure of the EnvZ kinase domain dimer is composed of two domains A forming a four-helix bundle at the center and two domains B flanking either side of the bundle, with the ATP-binding pocket of one EnvZ monomer facing His-243 on helix I of domain A from another EnvZ monomer (16). Of the two enzymatic functions in EnvZ, the kinase has been associated with domain B, which was able to phosphorylate domain A by using ATP, whereas the phosphatase activity was barely detected in either domain or in the mixture of the two domains (10). Here, we reexamined the phosphatase activity and demonstrated that domain A is indeed a phosphatase domain. Domain B can significantly enhance the phosphatase activity only when it is covalently linked with domain A. We also found that His-243 is essential for the domain A phosphatase activity. We propose that the physical linkage between domains A and B is important for the phosphatase activity and enables the two domains to maintain their correct spatial arrangement in a dimer configuration, and that His-243 plays a critical role in both kinase and phosphatase reactions.

Materials and Methods

Strains and Plasmids. *E. coli* B BL21-DE3 was used for expression and purification of all of the proteins in this paper. LE513 was derived from MH513 {MC4100 *ara*$^+$, Φ(*ompF′-lacZ*)16–23 [λp1(209)]} with *envZ* deletion (Δ*envZ*:Km^r) (17).

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Abbreviations: AMPPNP, adenosine 5′-[β,γ-imido]triphosphate; IPTG, isopropyl β-D-thiogalactoside; A-P, phosphorylated domain A.

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PET11a-EnvZc(223–289) encoding domain A and pET11a-EnvZc(290–450) encoding domain B were constructed previously (10). By using PCR with pET11a-EnvZ(C) as template, A′ and A″ fragments were amplified in such a way that they were flanked by an *Nde*I site at the 5′ end and a *Bam*HI site at the 3′ end. The sequence of the 5′-end primer for both is primer 8470, 5′-TACATATGGCGGCTGGTGTT-3′. The sequences of the 3′-end primer for A′ and A″ are primer 9337, 5′-GCGGATCCTTAGCCGGGGTAAAGCGCGG-3′, and primer 9340, 5′-CAGGATCCTTACGGCTCCGTTCCGCT-3′, respectively. The sequences of the PCR fragments were confirmed by DNA sequencing before they were digested by *Nde*I and *Bam*HI and ligated to the *Nde*I/*Bam*HI-digested pET11a vector to construct pET11a-EnvZc(223–326) (A′ fragment) and pET11a-EnvZc(223–364) (A″ fragment). PET11a-EnvZc(223–289) H243V, H243Y, H243N, H243S, and H243K mutants were constructed by site-directed mutagenesis using pET11a-EnvZc(223–289) as template.

For *in vivo* phosphatase assay, A, A′, and A″ fragments were detached from the pET11a vectors by *Nde*I/*Bam*HI digestion, whereas A L288P fragment was obtained by PCR amplification using primer 8470 and primer 9663, 5′-CTGGATCCTTAGCGCGGGTAGTCGATAAAC-3′, and subsequently *Nde*I/*Bam*HI digestion. All fragments were ligated into the *Nde*I/*Bam*HI-digested pINIII(Amp) vector (18) to construct pINIII-A, A′, A″, and A L288P, respectively.

Protein Purification. All proteins were purified from 1.5 liters of isopropyl β-D-thiogalactoside (IPTG)-induced culture grown in M9–Casamino acid medium. Cells were harvested by centrifugation and disrupted by French press. After unbroken cells and the membrane fraction had been removed by centrifugation, the proteins were precipitated by 50% saturation with $(NH_4)_2SO_4$. The pellets were collected by centrifugation and dissolved into buffer A (50 mM Tris·HCl, pH 8.0/150 mM KCl/10 mM 2-mercaptoethanol/1 mM PMSF) and purified by a Sephacryl S-100HR gel filtration column (Pharmacia). Protein domain A and mutated protein domain A were further purified by a DEAE-Sephadex column (Pharmacia), and proteins A′ and A″, by a Q-Sephadex column (Pharmacia). The concentration of the purified proteins was measured by Bio-Rad protein assay.

Preparation of OmpR-P and *in Vitro* Phosphatase Assay. OmpR-P was prepared and purified as described previously (10) with the following modifications: the membrane fraction containing EnvZ11 T247R known as "superkinase" was phosphorylated with 50 μCi (1 μCi = 37 kBq) of [γ-^{32}P]ATP in buffer B (50 mM Tris·HCl, pH 8.0/50 mM KCl/5% glycerol/5 mM $CaCl_2$) for 20 min at room temperature. After centrifuging at 393,000 × *g* for 14 min at 4°C in a Beckman TL100 ultracentrifuge, the membrane pellet was washed twice with buffer B. Purified OmpR protein was then mixed with the membrane fraction, which contained ^{32}P-labeled EnvZ11, for 20 min at room temperature. The reaction mixture was centrifuged at 393,000 × *g* for 14 min at 4°C to remove the membrane fraction. The supernatant containing OmpR-P was washed seven times with buffer C (50 mM Tris·HCl, pH 8.0/50 mM KCl/5% glycerol/1 mM EDTA) by using a 1.5-ml concentrator (molecular weight cut-off 10,000; Millipore) to remove free [γ-^{32}P]ATP and Ca^{2+} ions. The final protein preparation was analyzed by TLC to confirm that there was no free [γ-^{32}P]ATP or $^{32}P_i$ in the preparation. The phosphatase activity was determined as described previously (19). To measure the half-life of OmpR-P, each phosphatase reaction was repeated at least three times. The results were reproducible within a 1-min range.

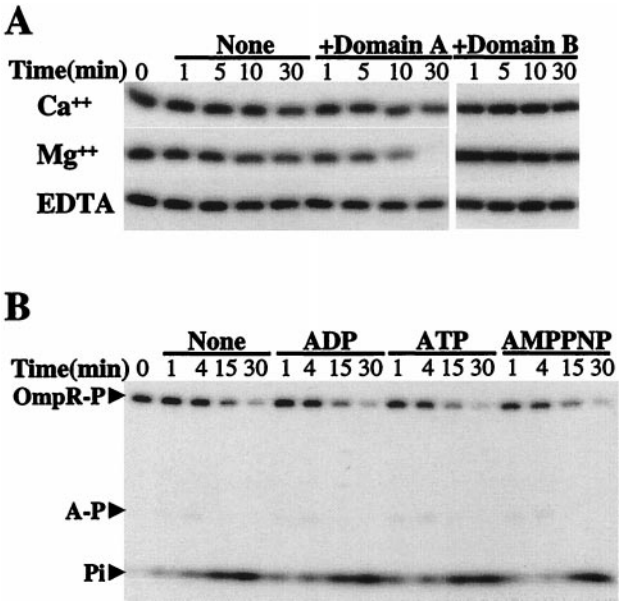

Fig. 1. Phosphatase activity of domain A. (*A*) Metal ion-dependent phosphatase activity of domains A and B. Purified OmpR-P (1.67 μM) was incubated with or without 3.34 μM domain A or B protein in phosphatase reaction buffer (50 mM Tris·HCl, pH 8.0/50 mM KCl/5% glycerol) containing 5 mM $CaCl_2$ (*Top*), 5 mM $MgCl_2$ (*Middle*), or 5 mM EDTA (*Bottom*) at room temperature. Aliquots were removed at the indicated time points and the reactions were stopped by 5× SDS gel loading buffer. The reaction mixtures were then analyzed by SDS/PAGE followed by autoradiography as well as PhosphorImager quantification. (*B*) Effect of cofactors on the phosphatase activity of domain A. The phosphatase reactions were carried out in Mg^{2+}-containing buffer described for *A* in the presence of 1 mM ADP, ATP, or AMPPNP. The positions of phosphorylated domain A and P_i are indicated by arrows.

***In Vivo* Phosphatase Assay.** β-Galactosidase activities of LE513 cells transformed with various pINIII vectors were determined as previously described (20).

Results

Phosphatase Activity of EnvZ Domain A. The phosphatase activity of each of the two subdomains of EnvZ, domains A and B, was undetectable in the Ca^{2+}-containing buffer normally used in our laboratory for the phosphatase assay (ref. 10; Fig. 1*A Top*). The half-life of OmpR-P by itself is about 90 min in the Ca^{2+}-containing buffer. The addition of either domain A or domain B did not cause any detectable change in the half-life. Furthermore, the mixing of domains A and B did not restore the phosphatase activity (data not shown). Because Mg^{2+} ions are known to be more effective for the phosphatase activity of EnvZ (21), in the present paper the effect of Mg^{2+} on the phosphatase activities of domains A and B was re-examined. As shown in Fig. 1*A Middle*, a significant level of phosphatase activity was observed with domain A, but not with domain B, in the presence of 5 mM Mg^{2+}. The half-life of OmpR-P alone or in the presence of domain B in the Mg^{2+}-containing buffer was approximately 90 min, similar to that in the presence of Ca^{2+}. Upon the addition of domain A, however, the half-life decreased to about 8.7 min. This activity was absolutely Mg^{2+} dependent, as no phosphatase activity was detected in the presence of EDTA (Fig. 1*A Bottom*). This result is consistent with the previous report that the EnvZ kinase domain functions as phosphatase in the presence of either Mg^{2+} or Ca^{2+}, but not in the presence of EDTA (21).

It is known that ADP, ATP, or AMPPNP (a nonhydrolyzable analogue of ATP) each can be a cofactor for the EnvZ phosphatase reaction. NMR studies demonstrated that one AMPPNP molecule binds to one domain B (13). ADP required for

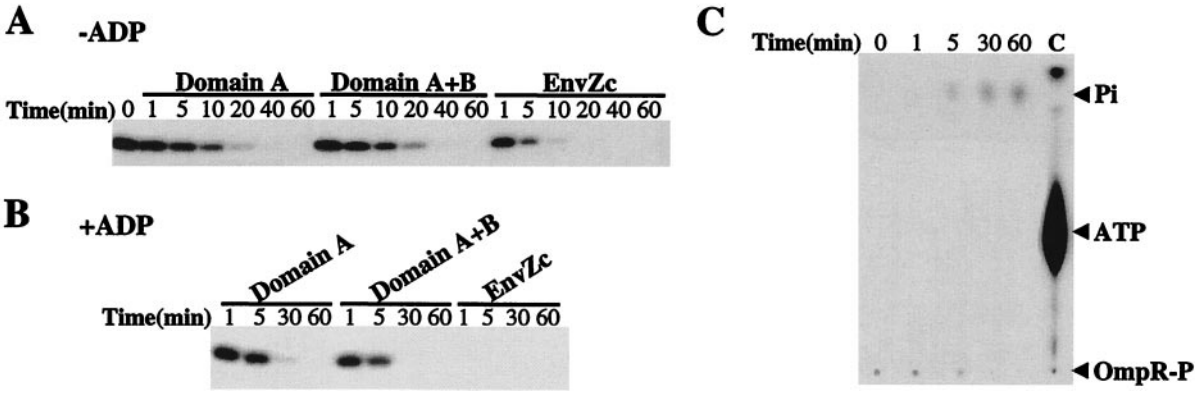

Fig. 2. Effect of domain B on the domain A phosphatase activity. OmpR-P (1.67 μM) was incubated with following proteins: 3.34 μM domain A, 3.34 μM domain A plus 3.34 μM domain B, or 3.34 μM EnvZc in the absence (*A*) or in the presence (*B*) of 1 mM ADP. The products of the phosphatase reaction in the presence of domains A and B and 1 mM ADP were analyzed by TLC followed by autoradiography (*C*). Lane C, [γ-^{32}P]ATP marker. The positions of P_i, ATP, and OmpR-P are indicated by arrows.

phosphatase activation is assumed to bind to the same site. Consistent with this idea, the phosphatase activity of domain A was not affected by the addition of 1 mM ADP, ATP, or AMPPNP in the reaction mixture (Fig. 1*B*). Increasing the concentrations of these cofactors to 10 mM had no effect on the phosphatase activity (data not shown).

It is important to note in Fig. 1*B* that the reduction of the amount of OmpR-P accompanies simultaneous release of P_i, and that the formation of a small amount of phosphorylated domain A (A-P) is observed only in the first 4 min of the reaction. At 30 min, the phosphoryl group of OmpR-P is almost completely released as P_i. These results demonstrated that domain A indeed functions as a phosphatase for OmpR-P. At present it is unknown whether A-P is an obligatory intermediate for the phosphatase reaction.

Effect of Domain B on the Phosphatase Activity of Domain A. Next we examined the effect of domain B on the Mg^{2+}-dependent phosphatase of domain A. When domain B was added to the reaction mixture containing domain A in the absence of ADP, the half-life of OmpR-P was found to be 8.2 min (Fig. 2*A*), which is almost identical to that with domain A alone (8.7 min). This finding implies that a separated domain B has hardly any effect on the phosphatase activity of domain A. It should be noted that if the two domains are covalently linked as seen in EnvZc (the cytoplasmic region of EnvZ, residues 180–450), the half-life of OmpR-P is shortened to 2.5 min under the same conditions (Fig. 2*A*). Note that N-terminal extension with the so-called linker region (residues 180–222) has been shown to have no effect on either kinase or phosphatase activity of the kinase domain (9).

Because domain B has an ATP-binding site, we further studied the effect of ADP on the phosphatase activity of domain A plus domain B. As shown in Fig. 2*B*, the addition of 1 mM ADP to the reaction mixture containing both domains A and B enhanced the phosphatase activity, as the half-life of OmpR-P was reduced from 9.1 to 5.8 min. Note that 1 mM ADP activates the EnvZc phosphatase activity with a half-life of OmpR-P less than 30 sec (Fig. 2*B*). The observed ADP stimulation of the phosphatase reaction is not due to the enhancement of the reverse reaction to form ATP from A-P and ADP, as the phosphoryl group of OmpR-P was quantitatively converted to P_i without the formation of any detectable amount of ATP (Fig. 2*C*). Furthermore, AMPPNP showed a stimulatory effect on the phosphatase activity similar to that of ADP in the presence of domain B (data not shown), demonstrating that ADP functions as a cofactor for the phosphatase reaction as in the case of EnvZc. Therefore, one may speculate that ADP binding to domain B causes conformational changes, which allow domain B to interact with domain A and stimulate the phosphatase activity. It has been shown that ATP binding to MutL, Hsp90, and DNA gyrase B, which are structurally similar to the EnvZ domain B, changes the conformation of their ATP-binding domains (14).

Phosphatase Activity of Domain A *in Vivo*. It was shown that in the absence of EnvZ, OmpR can still be phosphorylated by acetyl phosphate in the cell. A sensitive *in vivo* phosphatase activity assay has been carried out based on this phenomenon (22). In an *envZ*-null strain (LE513) carrying the *ompF′-lacZ* gene and vector alone, β-galactosidase is expressed at a significant basal level (157 units without IPTG and 133 units with IPTG), likely due to the OmpR-P generated by acetyl phosphate (Fig. 3). If domain A is able to exhibit the phosphatase activity *in vivo*, OmpR-P should be dephosphorylated in cells expressing domain A, resulting in reduced β-galactosidase activity. For this experiment, we constructed strain LE513, which carries the ORF for domain A under the control of the *lpp* and *lac* promoters. In the absence of IPTG the cells expressed 117 units of β-galactosidase activity, which was reduced to 29.2 units after IPTG induction. This result indicates that domain A functions as phosphatase *in vivo* (Fig. 3).

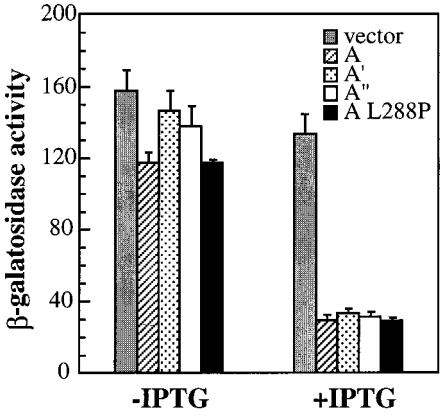

Fig. 3. The *in vivo* phosphatase activities of A, A′, A″, and A L288P. β-Galactosidase activities were measured in the cells containing pINIII vector alone or with pINIII A, pINIII A′, pINIII A″, or pINIII A L288P, with or without IPTG induction. Error bars indicate standard deviations.

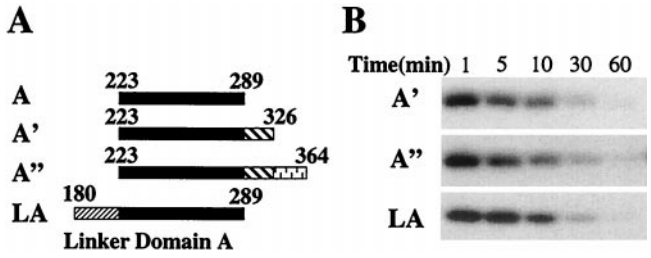

Fig. 4. Effect of N- and C-terminal extensions of domain A on the phosphatase activity. (*A*) Domain organization of A, A′, A″, and LA fragment. (*B*) Phosphatase activities of A′, A″, and LA. Experiments were carried out as described for Fig. 1*A*.

Effect of N- and C-Terminal Extensions of Domain A on phosphatase activity. In the cytoplasmic domain of EnvZ, domain A is connected at its N terminus with the linker region and at its C terminus with domain B. Because the phosphatase activity of domain A is substantially lower than that of EnvZc in both the absence and the presence of ADP, we examined whether extension of domain A at either the N or the C terminus could increase its phosphatase activity. For this purpose, the following three constructs were made: LA (linker region plus domain A, residues 180–289), A′ (residues 223–326), and A″ (residues 223–364) (Fig. 4*A*). NMR studies revealed that domain B has an α/β fold (13): one layer comprises three α-helices and the other layer consists of a five-stranded β-sheet. On the basis of this structure, A′ was designed to have a 37-residue C-terminal extension containing β1, α1, and β2 from domain B and A″ a 75-residue extension consisting of β1, α1, β2, β3, α2, and β4. All these proteins, LA, A′, and A″, were soluble when expressed in a T7-expression vector and purified to higher than 95% purity. As shown in Fig. 4*B*, all these proteins processed phosphatase activity at a similar level as domain A: the half-lives of OmpR-P mixed with A′, A″, and LA were 9.1, 10.8, and 8.5 min, respectively. An *in vivo* experiment confirmed that A′ and A″ proteins had a level of phosphatase activity similar to that of domain A as judged by the reduction of β-galactosidase activity in the presence of IPTG (Fig. 3). Clearly, the linker region has no effect on phosphatase activity, and partial extensions at the C terminus of domain A do not have any effect on phosphatase activity either.

His-243 Is Essential for the Phosphatase Activity of A Domain. EnvZc H243V, an autophosphorylation site mutant, was shown to have neither kinase nor phosphatase activity (23). Several other substitutions at His-243 also substantially decrease the phosphatase activity of EnvZ to different levels (22, 24). A number of mutants in the H box, such as T247R and P248S, have been shown to have very weak phosphatase activity *in vitro* and exhibit an $OmpF^-$ $OmpC^c$ phenotype *in vivo* (25, 26), suggesting that His-243 and other residues in the H box are involved in the phosphatase activity of EnvZ. To examine the role of His-243 in the domain A phosphatase function, several substitution mutations (Y, S, K, N, V) at the His-243 site were constructed. All proteins were expressed in BL21 cells under the control of T7 promoter and purified to higher than 95% purity. Fig. 5 depicts their phosphatase activities: replacing His-243 with any other amino acids tested almost abolished phosphatase activity of domain A. The half-lives of OmpR-P in the presence of these domain A mutants were all approximately 90 min, almost identical to that of OmpR-P alone. This result strongly suggests that the His-243 residue plays an essential role in the domain A phosphatase activity. Note that circular dichroism spectra of these mutant proteins were identical to the spectrum of wild-type domain A (data not shown).

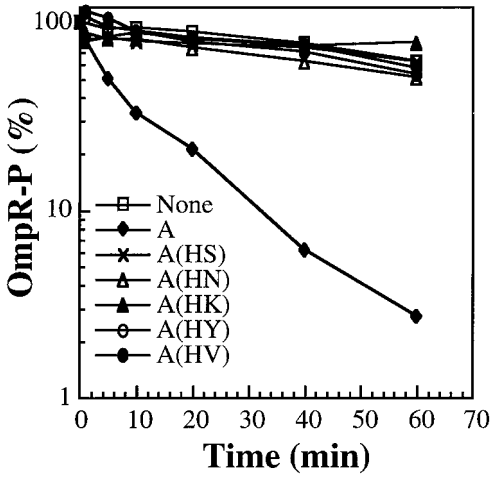

Fig. 5. The essential role of His-243 in the domain A phosphatase activity. The phosphatase activities of various domain A His-243 mutants (Y, K, N, V, S) were measured in Mg^{2+}-containing buffer described in the legend of Fig. 1. The OmpR-P amounts at indicated time points were quantified by PhosphorImager and represented graphically. The results are expressed as the amount relative to the amount of OmpR-P present before the addition of domain A.

Effect of L288P Mutations on the Domain A Phosphatase Activity. The X-region, a weakly conserved motif, was identified among histidine kinases (27). Two mutations within this region of EnvZ, Y287D and L288P, were shown to affect the phosphatase activity both *in vivo* and *in vitro*. Indeed, we confirmed that when the L288P mutation was incorporated into EnvZc, no phosphatase activity was detected, while kinase activity was retained (data not shown). The NMR structure of domain A shows that Leu-288 is located in the unstructured C-terminal region on the top of four-helix bundle, whereas the middle and bottom parts of the four-helix bundle interact with OmpR (12). A secondary structure prediction suggests that a turn structure including Leu-288 exists at the junction region between domains A and B. Therefore, it is reasonable to assume that the A–B junction region plays an important role in properly orienting domain B toward domain A to stimulate its phosphatase activity and that distortion of this junction may thus affect the phosphatase activity.

On the basis of these considerations, we examined the effect of the L288P mutation on the domain A phosphatase activity. When tested in an *in vivo* system (Fig. 3), similar to domain A, the induction of domain A L288P caused a significant reduction of β-galactosidase activity from 117 units without IPTG to 28.8 units with IPTG. It appears that the L288P mutation does not affect the intrinsic phosphatase activity of domain A. The mutation impairs the phosphatase activity only when domain A is covalently linked to domain B, supporting the idea that the A–B junction region indeed plays an important role in regulating the EnvZ phosphatase activity.

Discussion

Here, we demonstrated that domain A, the central 67-residue dimeric domain, plays a key role in the EnvZ phosphatase activity. Although reduced compared to EnvZc, the phosphatase activity is still retained in an isolated domain A but not in an isolated domain B. Simple extensions of domain A at the N terminus by 43 residues or at the C terminus by 37 or 75 residues did not enhance its phosphatase activity. We know of no other identification of a structurally defined phosphatase domain within histidine kinases. Previously NtrB phosphatase activity

has been shown *in vivo* to be associated with a fragment termed H domain which corresponds to the A′ fragment of EnvZ (28).

It is important to note that OmpR-P is substantially more stable than CheY-P and NtrC-P (29): the half-life of OmpR-P in the presence of Mg^{2+} is 90 min, whereas the half-lives of CheY-P and NtrC-P are a few seconds and 4 min, respectively. This observation indicates that OmpR-P by itself has a very weak autophosphatase activity in comparison with CheY-P and NtrC-P. Therefore, in the EnvZ-OmpR system, EnvZ plays a major role in dephosphorylation of OmpR-P.

When covalently linked to domain A, domain B significantly increases the phosphatase activity, reducing the half-life of OmpR-P from 8.7 to 2.5 min in the absence of ADP, and to less than 30 sec in the presence of ADP. The following three possible mechanisms can be considered for the role of domain B in the phosphatase activity. First, domain B may have an allosteric effect on the function of domain A, affecting the conformation of domain A to stimulate its phosphatase activity. It is interesting to note that when detached from domain A, domain B can still stimulate the phosphatase activity only in the presence of ADP or AMPPNP, suggesting that ADP or AMPPNP binding to domain B further enhance the allosteric stimulatory affect. Second, domain B may facilitate the interaction between domain A and its substrate OmpR-P. Third, ADP-bound domain B directly interacts with the catalytic center on domain A as a cofactor to stimulate the phosphatase reaction.

According to the NMR titration experiment, OmpR interacts with the middle and bottom regions of the four-helix bundle formed by domain A (12). Through this interaction, Asp-55 of OmpR is considered to be placed close to His-243 of EnvZ. The Leu-288 residue localizes at the assumed turn structure on the top of domain A, linking to domain B. The L288P mutation in EnvZc has been shown to abolish the phosphatase activity while retaining the kinase activity (27). However, the mutation has no effect on the phosphatase activity in domain A, suggesting that the L288P mutation alters the spatial arrangement between domains A and B rather than being directly involved in the phosphatase reaction. It appears that, as compared with the kinase activity, the phosphatase activity is more sensitive to distortion between domains A and B. It is interesting to note that EnvZ or EnvZc with the L288P mutation migrates abnormally in SDS/PAGE (ref. 27; unpublished data) and that tryptic digestion of EnvZc L288P shows a different pattern from that of wild-type EnvZc (unpublished data), suggesting that this mutation causes a distinct conformational change in EnvZ.

Using a Tar-EnvZ hybrid system (Taz1), we have demonstrated that the external signal regulates the ratio of kinase to phosphatase activity of EnvZ kinase domain mainly by inhibiting the phosphatase activity (7). On the basis of the above results, it is reasonable to propose a model that signals transduced across the membrane (osmolarity for EnvZ and aspartate for Tar and Taz1) alter the relative spatial arrangement between domains A and B to modulate the phosphatase activity but not the kinase activity. At a low osmolarity for EnvZ or in the absence of aspartate for Taz1, domains A and B are positioned in such a way that EnvZ or Taz1 exhibits both kinase and phosphatase activity. At high osmolarity for EnvZ or in the presence of a high concentration of aspartate for Taz1, the spatial arrangement between domains A and B is altered, resulting in negative regulation of the phosphatase function. Such a displacement of domain A of one subunit against domain B of the partner subunit within a dimer may be a consequence of a physical displacement of one helix in the four-helix bundle in the receptor domain upon ligand binding (30–32). It is likely that such a signal regulation model is applicable to other bifunctional histidine kinases as well.

In the present paper, we also demonstrated that the replacement of His-243 in domain A by five other amino acid residues (Tyr, Asn, Ser, Lys, or Val) almost abolishes the phosphatase activity, clearly indicating that His-243 plays an essential role in the domain A phosphatase function. Because His-243 is also the autophosphorylation site, from which the phosphoryl group is transferred to Asp-55 of OmpR, it plays the central role in both kinase and phosphatase reactions. In the EnvZ–OmpR complex, the phosphoryl group provided from either EnvZ-P or OmpR-P appears to be prone to form a phosphoester bond with Asp-55 rather than to form phosphoimidate bond with His-243, since phosphotransfer from EnvZ-P to OmpR is completing within 2 min (33), whereas the reverse transfer from OmpR-P to EnvZ is barely detectable under normal conditions (34). In this model the reversible transfer of the phosphoryl group to His-243 of EnvZ from OmpR-P may happen under certain conditions, although at present it is unknown whether it is an obligatory step for the phosphatase activity. Such reverse transfer was observed in the early period of the phosphatase reaction with domain A (see Fig. 1*B*) and in EnvZc kinase$^-$ phosphatase$^+$ mutant N347D (34). Reverse transfer can also be detected under some conditions with the wild-type EnvZc (unpublished data). Interestingly, EnvZ mutants with several amino acid substitutions at the His-243 site still retain a very low phosphatase activity (22, 24). In such mutants, the function of the His residue may be complemented by other residues in the H box together with the aid of ADP-bound domain B, albeit very poorly, to destabilize the phosphoryl group on Asp-55 of OmpR. We propose that kinase and phosphatase activities of histidine kinase are not independent but rather share the same active center containing His-243 of EnvZ and Asp-55 of OmpR. This center is arranged in such a way that a minor distortion caused by relative displacement between domains A and B results in severe effects on the phosphatase reaction but much less on the kinase reaction.

It is interesting to note that CheA, a histidine kinase required for chemotaxis, does not have phosphatase activity. This lack may be due to the fact that the domain arrangement in CheA is quite distinct from that of EnvZ, in which the CheA central dimerization domain does not contain the autophosphorylation sites. Instead, in a CheA dimer there are two extra asymmetrical four-helix bundles (Hpt domains), each of which contains only one His residue as the phosphorylation site (14, 15). In this arrangement, either because the water molecule required for CheY-P hydrolysis could not be coordinated around the His residue or because phosphorylated CheY may have poor affinity for CheA, the His residue may serve only for the kinase reaction, which is regulated by chemotaxis signals.

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