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A monomeric histidine kinase derived from EnvZ, an *Escherichia coli* osmosensor

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Summary

Histidine kinases function as dimers. The kinase domain of the osmosensing histidine kinase EnvZ of *Escherichia coli* consists of two domains: domain A (67 residues) responsible for histidine phosphotransfer and dimerization, and domain B (161 residues) responsible for the catalytic and ATP-binding function. The individual structures of these two domains have been recently solved by NMR spectroscopy. Here, we demonstrate that an enzymatically functional monomeric histidine kinase can be constructed by fusing in tandem two domains A and one domain B to produce a single polypeptide (A–A–B). We show that this protein, EnvZc[AAB], is soluble and exists as a stable monomer. The autophosphorylation and OmpR kinase activities of the monomeric EnvZc[AAB] are similar to that of the wild-type EnvZ, while OmpR-binding and phosphatase functions are reduced. V8 protease digestion and mutational analyses indicate that His-243 of only the amino proximal domain A is phosphorylated. Based on these results, molecular models are proposed for the structures of EnvZc[AAB] and the kinase domain of EnvZ. The present results demonstrate for the first time the construction of a functional, monomeric histidine kinase, further structural studies of which may provide important insights into the structure–function relationships of histidine kinases.

Introduction

Signal reception, transduction and response are vitally important for cell survival and growth. Bacteria rely primarily on the His–Asp signal transduction system to adapt to environmental changes (Inouye, 1996). A sensor–transducer histidine kinase and its cognate response–regulator protein, which is phosphorylated on a conserved aspartate residue, constitute the two essential components in this regulatory system (reviewed by Stock *et al*., 1989; 1990; Parkinson, 1993; Hoch and Silhavy, 1995). Signalling by multiple histidine kinases and response regulators, such as the one involved in the initiation of sporulation in *Bacillus subtilis* (Burbulys *et al*., 1991), or by hybrid signalling proteins, such as ArcB (Ishige *et al*., 1994), which combine both histidine kinase and response regulator modules in a single polypeptide is known (reviewed by Appleby *et al*., 1996). Currently more than 250 such signalling systems have been reported in prokaryotes (Hsing *et al*., 1998) and, recently, they have been discovered in some lower eukaryotes (see Loomis *et al*., 1997 for a review of eukaryotic histidine kinases).

Escherichia coli has 30 histidine kinases (Mizuno, 1998). Of these, the osmosensor EnvZ has been extensively investigated. EnvZ is a prototype, dimeric, transmembrane histidine kinase. The enzymatic functions of EnvZ resides in its cytosolic domain, which has the highly conserved regions for histidine kinases, the H, N, F, G_1 and G_2 boxes (Parkinson and Kofoid, 1992). EnvZ *trans*-autophosphorylates its conserved His-243 residue using ATP (Yang and Inouye, 1991), which is subsequently transferred to the conserved Asp55 residue on its cognate response regulator OmpR. EnvZ can also dephosphorylate phospho-OmpR (Igo *et al*., 1989). ATP, ADP and non-hydrolysable analogues such as AMP–PNP are positive cofactors for this reaction. Thus, EnvZ possesses dual opposing enzymatic functions, both phosphorylation (kinase) and dephosphorylation (phosphatase) of OmpR, the ratio of which determines the final levels of phospho-OmpR in the cell (for reviews on EnvZ/OmpR phosphorelay see Forst and Roberts, 1994; Pratt and Silhavy, 1995; Egger *et al*., 1997). Phosphorylated OmpR functions as a transcription factor for *ompF and ompC* genes encoding the outer membrane porin proteins OmpF and OmpC respectively (Nikaido and Vaara, 1987).

While significant progress has been made in understanding the cellular roles of many His–Asp signal transduction systems, a major drawback has been the lack of availability of structural information. The kinase domain of EnvZ (residues 223–450) consists of two domains, A (residues 223–289) and B (residues

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290–450) (Park *et al*., 1998). The 67 residue dimeric domain A contains His-243, the site of autophosphorylation and phosphotransfer to OmpR. Domain B is monomeric and binds ATP. Isolated domains A and B can autophosphorylate only when combined together in solution (Park *et al*., 1998). This approach enabled us to solve the NMR-derived solution structure of AMP–PNP-bound catalytic domain B (Tanaka *et al*., 1998). The X-ray crystal structure of a fragment containing the dimerization, ATP binding and regulatory domains of the chemotactic histidine kinase CheA from the thermophilic bacterium *Thermotoga maritima* has been determined (Bilwes *et al*., 1999). The catalytic domain of this CheA homologue was found to be very similar to that of EnvZ. Most recently, the NMR solution structure of the domain A of EnvZ has also been determined (Tomomori *et al*., 1999). However, the manner by which a substrate phosphotransfer domain and a catalytic ATP-binding domain are topologically arranged to constitute a histidine kinase active centre remains one of the most challenging open questions in this field.

Here, we present the construction and characterization of a monomeric, functional histidine kinase derived from EnvZ on the basis of the structures of domains A and B. This monomeric kinase was indeed soluble, stable and exhibited both kinase and phosphatase activities. Using this construct, we provide an important structural insight into the *trans*-autophosphorylation mechanism of EnvZ. A model is also proposed for the kinase domain of EnvZ emphasizing the orientation of the catalytic ATP-binding domain with respect to the histidine-containing substrate domain.

Results

Construction of EnvZc[AAB] fusion proteins

While we now know the structures of the individual domains A (Tomomori *et al*., 1999) and B (Tanaka *et al*., 1998) of EnvZ, we have yet to determine how they are topologically arranged with respect to each other. Attempts to obtain the structure of the entire cytoplasmic domain of EnvZ by either X-ray crystallographic or NMR spectroscopic means have so far failed. It appears that the dimeric, multimodular histidine kinases present obstacles to structural analysis. We therefore attempted to construct a monomeric EnvZ kinase, which can phosphorylate OmpR and dephosphorylate phospho-OmpR.

The EnvZ domain A homodimer consists of a four-helix bundle with twofold symmetry along the helix axis (Tomomori *et al*., 1999). Each subunit folds into a compact structure consisting of antiparallel helices connected by a turn. The four amphipathic helices pack against each other to form a hydrophobic core aligned along their long axes. We reasoned that, by fusing in tandem two domains A amino proximal to a domain B, thereby forcing the formation of an *intra*molecular four-helix bundle, dimerization could be averted without significantly altering the essential architectural features of an EnvZ dimer. Domains A and B of separate subunits in a dimer together constitute the active site for the EnvZ *trans*-autophosphorylation reaction. The proposed monomeric protein, consisting of only one such active site, would therefore give us important insights into the structural basis of the *trans*-autophosphorylation mechanism that is a distinguishing feature of EnvZ and histidine kinases in general. Therefore, we added an extra domain A (residue 223–289) to the N-terminal end of EnvZ kinase domain (residue 223–450) consisting of a single domain A and domain B, and termed it EnvZc[AAB]. Thus, EnvZc[AAB] was expected to be a monomer.

Figure 1A depicts such constructs: EnvZc[AsAB] and EnvZc[AssAB]. In the former, two A domains were connected by a four-residue peptide, GGSI, and in the latter, by an eight-residue peptide, GGSGGGSI. The connecting spacer peptides (s or ss) were designed to make a flexible connection between the two A domains to minimize constraints in helix association to form an intramolecular four-helix bundle. Each construct was placed under a T7 promoter and transformed into *E. coli* BL21 cells. When induced with 1 mM IPTG, the overexpressed fusion proteins were soluble and constituted about 65–75% of total cellular proteins (Fig. 1B). After 33% ammonium sulphate fractionation, the proteins were subjected to gel filtration using a Sephacryl S-100HR column. EnvZc[AsAB] eluted in two distinct fractions: 70–80% of the protein at the position corresponding to the size of the monomer (fraction number 32–40), while the remaining protein eluted at the position corresponding to the dimer (fraction number 20–24) estimated on the basis of the elution profile of molecular weight standards (Fig. 1B). Both proteins cross-reacted to anti-EnvZ antiserum (data not shown) and migrated to the same position in a 17.5% SDS–PAGE gel (Fig. 1B and C). EnvZc[AssAB] behaved in a similar manner during purification as EnvZc[AsAB]. The SDS–PAGE gel migration patterns of the purified monomers and dimers of EnvZc[AssAB] are shown in Fig. 1C, lanes 4 and 6 respectively. The molecular weights of the monomer and the dimer of EnvZc[AsAB], as estimated by fast-protein liquid chromatography (FPLC) and by light scattering (using DynaPro Molecular Sizing Instrument), are shown in Table 1. These values are higher than their calculated molecular weights (32.8 kDa for the monomer and 65.6 kDa for the dimer), perhaps indicating that both these proteins are not perfectly globular but are more elongated.

Both the monomer and the dimer are very stable once

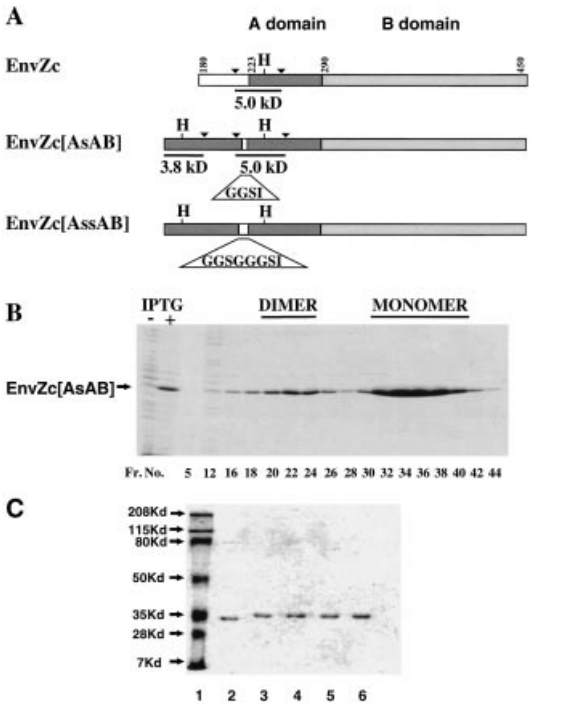

Fig. 1. Domain organization of EnvZc and EnvZc[AAB]s.
A. The domain organization of EnvZc, EnvZc[AsAB] and EnvZc[AssAB]. EnvZc consists of three regions: linker (residues 180–222), domain A (residues 223–289) and B (residues 290–450). EnvZc[AAB] proteins contain two direct repeats of domains A, which is followed by domain B. GGSI and GGSGGGSI are the amino acid spacers in EnvZc[AsAB] and EnvZc[AssAB] respectively. H represents the conserved histidine phosphorylation site. ▼ indicates the closest cutting site of V8 protease around H. The lengths of V8 digested fragments containing H are shown under the constructs.
B. Purification of EnvZc[AsAB] monomer and dimer by a Sephacryl S-100 HR gel filtration column. Each fraction was analysed on 17.5% SDS–PAGE gel. The first and second lanes represent the protein profile of BL21 cell harbouring pLQ050 in the absence and the presence of 1 mM IPTG respectively.
C. SDS–PAGE pattern of purified proteins (2 μg): EnvZc (lane 2), EnvZc[AsAB] monomer (lane 3) and dimer (lane 5), EnvZc[AssAB] monomer (lane 4) and dimer (lane 6). Lane 1, Bio-Rad broad range molecular weight marker.

formed. When the monomer fraction of EnvZc[AssAB] was concentrated to 7 mg ml^{-1} and stored at 4°C in Tris-HCl buffer (pH 8.0) containing 150 mM KCl, 1 mM dithiothreitol and 5% glycerol for 3 weeks, no dimer or other form of protein was detected by FPLC. The diluted dimer (as low as 0.02 mg ml^{-1}) did not dissociate when kept under the above conditions for 3 weeks (data not shown). The same result was obtained with EnvZc[AsAB].

Table 1. Estimation of molecular weight (MW) of EnvZc[AsAB].

	Calculated MW (kDa)	FPLC MW (kDa)	Light scattering MW (kDa)
Monomer	32.8	48	42.6
Dimer	65.6	75	84.5

Secondary structure and thermal stability

The secondary structure of the monomeric EnvZc[AssAB] was analysed by circular dichroism (CD) (Fig. 2A). The spectrum indicated that it is an α/β protein like EnvZc. Deconvolution of the spectrum was carried out using three different programs (SELCON, LINCOMB and CONTIN). The higher average helical content of EnvZc[AssAB], 63 ± 7% compared with 47 ± 7% of EnvZc, is as expected because of the α-helically rich extra domain A. The monomer forms of EnvZc[AsAB] and EnvZc[AssAB] are both very stable, as judged by their melting curves (Fig. 2B). The T_m values for EnvZc[AsAB] and EnvZc[AssAB] monomers were calculated to be 63.0°C and 63.4°C respectively.

Histidine kinase activity

The autophosphorylation activities of the monomeric and

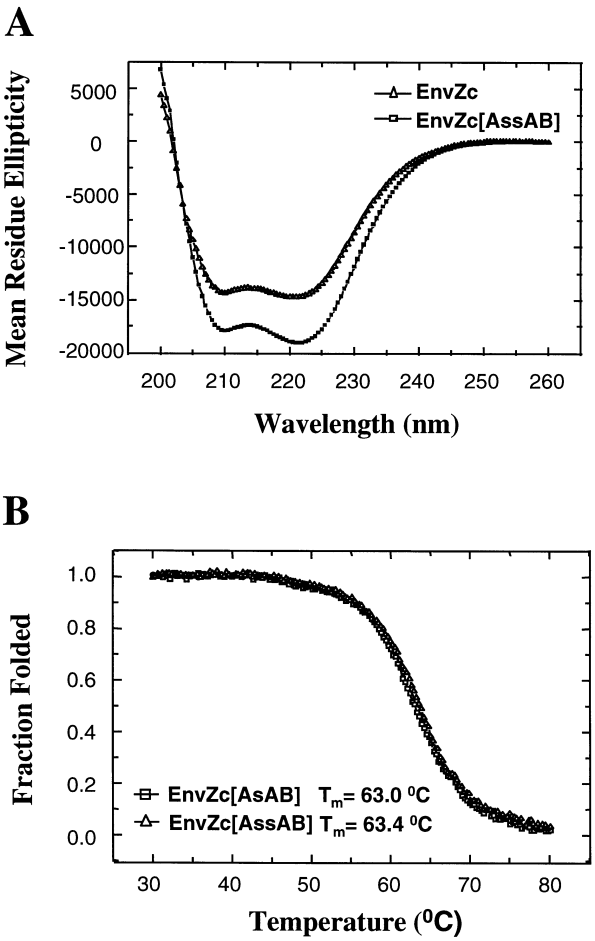

Fig. 2. CD spectroscopic analysis of EnvZc and EnvZc[AAB] proteins.
A. CD spectra of EnvZc and EnvZc[AssAB] monomer proteins were collected at 20°C from 200 to 260 nm.
B. Thermal stabilities of EnvZc[AsAB] and EnvZc[AssAB] monomer proteins were recorded at 221 nm and normalized to the fraction folded.

dimeric forms of EnvZc[AssAB] were compared with that of EnvZc in an *in vitro* biochemical assay using [γ-^{32}P]-ATP as described in *Experimental procedures* (Fig. 3). Interestingly, the initial rate of autophosphorylation of the monomeric form was identical to that of EnvZc while that of the dimeric form was approximately fourfold higher than that of EnvZc (Fig. 3B). Upon the addition of OmpR, the phosphoryl group was transferred to OmpR from both monomeric and dimeric phospho-EnvZc[AssAB] as efficiently as from phospho-EnvZc (Fig. 3C). Similar results were obtained with EnvZc[AsAB] monomer and dimer (data not shown).

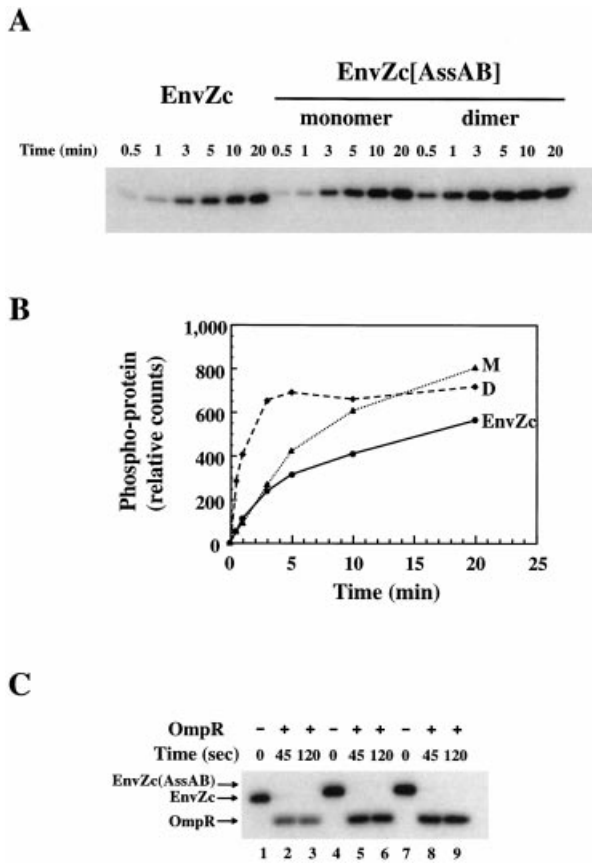

Fig. 3. Autophosphorylation and phosphotransferase activities of EnvZc and EnvZc[AssAB] dimer and monomer.
A. The autophosphorylation activities of EnvZc and EnvZc[AssAB] dimer and monomer proteins were performed as described under *Experimental procedures* and results were analysed by 17.5% SDS–PAGE gel electrophoresis. The dried gel was exposed, scanned by a phosphorimager and exposed for autoradiography.
B. Graphical representation of the phosphorimager analysis of the autophosphorylation activities of EnvZc and EnvZ[AssAB] dimer (D) and monomer (M) proteins.
C. Autoradiogram of an SDS–PAGE gel analysing the products of the phosphotransferase reaction of EnvZc and EnvZc[AssAB] dimer and monomer proteins. Each protein was autophosphorylated for 20 min using [γ-^{32} P]-ATP when OmpR was added to the reaction. Then aliquots were removed at 0, 45 and 120 s respectively. Lanes 1–3, EnvZc; lanes 4–6, EnvZc(AssAB) monomer; lanes 7–9, EnvZc(AssAB) dimer.

Identification of phosphorylated histidine residues

In 25 mM ammonium carbonate buffer (pH 7.8), V8 protease cuts specifically at the C-terminal side of Glu residues. In a V8 protease digestion of EnvZc[AsAB], the conserved His-243 residue (the autophosphorylation site) in the first domain A is expected to be in an N-terminal peptide of 3.8 kDa (from residues Met-223 to Glu-257), while the corresponding His-243 residue in the second domain A is expected to reside in a polypeptide of 5.0 kDa (residues Gln-283 in first domain A to residues Glu-257 in second domain A; see Fig. 1A). In order to identify the phosphorylation site in the EnvZc[AsAB] dimer and monomer, they were first autophosphorylated with [γ-^{32}P]-ATP (Fig. 4A, lanes 2 and 3). As a control, EnvZc was also autophosphorylated (Fig. 4A, lane 1). The phosphoproteins were subsequently digested with V8 protease and the digestion products were separated by tricine–SDS gel electrophoresis and the dried gel was exposed for autoradiography.

EnvZc yielded a radioactive band at around 5.0 kDa as expected (Fig. 4A, lane 4). The digest of the monomeric EnvZc[AsAB] showed a single band at around 3.8 kDa but not at 5.0 kDa, indicating that only the His-243 residue of the first domain A was exclusively phosphorylated. On the other hand, the dimer gave two bands after digestion: one band (expected molecular weight 5.0 kDa) migrating a little lower than that observed for EnvZc and the other migrating to a position identical to that observed for the monomeric form ($\approx$3.8 kDa). The disparity in the mobilities of these two 5.0 kDa fragments, one from EnvZc and the other from EnvZc[AsAB] dimer, may be a consequence of differences in the shapes of these peptides, arising from their different amino acid sequences.

To further confirm the above results, the His-243 residue in the first domain A of EnvZc[AssAB] was substituted with serine. The mutant protein was designated EnvZc[A(H-S)ssAB]. The monomeric and dimeric forms of the mutant protein were isolated and examined for their autophosphorylation abilities. As shown in Fig. 4B, the monomer (lane 1) was hardly phosphorylated while the same amount of the dimer was well phosphorylated with [γ-^{32}P]-ATP (lane 2). This result clearly confirms that the His-243 residue of only the first domain A can be phosphorylated in the monomer whereas the His-243 residues of both the first and second domain A can be phosphorylated in the dimer.

Note that, in the previous autokinase assay (Fig. 3), equimolar amounts (1.1 μM, based on the amount of monomer) of EnvZc, EnvZc[AssAB] monomer and dimer were used. Therefore, the total number of His-243 residues in EnvZc[AssAB] monomer is equal to that of the dimer, but twice that of EnvZc. EnvZc

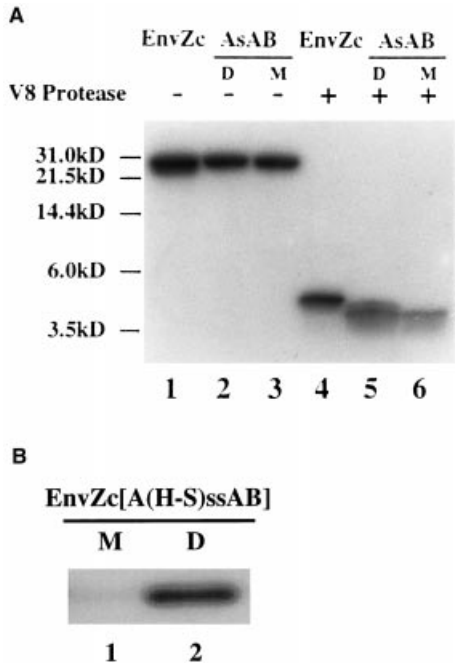

Fig. 4. Identification of phospho-histidine residues in EnvZc[AsAB] monomer and dimer.
A. Endoproteinase Glu-C (V8 Protease) digestion of phospho-EnvZc and phospho-EnvZc[AsAB] dimer and monomer proteins. EnvZc and EnvZc[AsAB] dimer and monomer proteins were autophosphorylated by [γ-^{32}P]-ATP and subjected to V8 protease digestion as described under *Experimental procedures*. The undigested phospho-proteins and their digested products were separated on a tricine–SDS–PAGE gel, which was then dried and exposed for autoradiography. Lanes 1, 2 and 3, undigested phospho-EnvZc, phospho-EnvZc[AsAB] dimer and monomer repectively. Lanes 4, 5 and 6, digested phospho-EnvZc, phospho-EnvZc[AsAB] dimer and monomer respectively.
B. Autophosphorylation of EnvZc[A(H-S)ssAB] monomer and dimer proteins was performed and the results were analysed essentially as described for EnvZc. Lane 1, monomer; lane 2, dimer.

autophosphorylation reaches its maximum around 20 min and $\approx$60% of His-243 is phosphorylated at this point as measured by filter-binding assay (data not shown). In the autokinase experiment described above, the total amount of phosphorylated protein was estimated by phosphor-imager analysis and found to be 0.66 μM, 0.94 μM and 0.84 μM for EnvZc, EnvZc[AssAB] monomer and dimer respectively. Because the total amount of His-243 residues in EnvZc[AssAB] monomer or dimer in the reaction is 2.2 μM, the above result indicates that 43% of the His-243 residues in the monomer and 38% of those in the dimer were phosphorylated. Because only the first His-243 residue can be phosphorylated in the monomer (from the V8 protease digestion and H243S mutation analysis), the final phosphorylation level of this residue was calculated to be 85%. Why only a fraction of the histidine residues within EnvZ proteins can be phosphorylated is presently not understood.

Phosphatase activity

Next we studied the phospho-OmpR phosphatase activity of EnvZc[AAB]. Using purified, ^{32}P-labelled phospho-OmpR as substrate, the phosphatase activity was measured by the percentage of phospho-OmpR remaining in the reaction mixture as a function of reaction time. The half-life of phospho-OmpR in the presence of EnvZc and EnvZc[AssAB] dimer was approximately 3 and 6 min respectively (Fig. 5), indicating that the phosphatase activity of the dimeric EnvZc[AssAB] is similar to that of EnvZc. However, for the monomer, the phosphatase activity was much lower: the half-life of phospho-OmpR was increased by eightfold to about 24 min (Fig. 5). Similar results were obtained with EnvZc[AsAB] monomer and dimer.

Binding interaction of His$_{10}$–OmpR with EnvZc and with EnvZc[AssAB]

The phosphoryl transfer and phosphatase activities of EnvZ require it to interact with its cognate response regulator, OmpR. The level of phospho-OmpR in the cell in turn regulates the transcription of the porin genes *ompF and ompC*. Complex formation between the kinase domain of EnvZ with OmpR was demonstrated using Ni-histidine tag affinity chromatography (Park *et al*., 1998). NMR titration experiments show that domain A (N^{15}-labelled) of EnvZ and OmpR(N) (N-terminal OmpR, residues 1–134) interact (Tomomori *et al*., 1999).

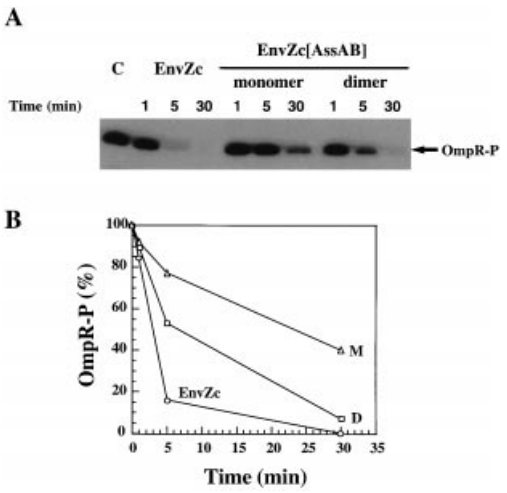

Fig. 5. Phosphatase activities of EnvZc and EnvZc[AssAB].
A. Phospho-OmpR phosphatase assay of EnvZc, EnvZc[AsAB] monomer and dimer. The control (C) represents the same amount of phospho-OmpR from the reaction mixture without any EnvZc or EnvZc[AAB]s.
B. The phospho-OmpR amount was quantitated by densitometer and represented graphically. The phospho-OmpR amount in the control lane was taken to be 100%.

Extending such domain interaction studies, we compared the affinity between OmpR and EnvZc to that between OmpR and EnvZc[AssAB] by using Ni-NTA resin-bound His_{10}–OmpR for binding analyses. From the densitometric scans of the eluted EnvZc[AssAB] and EnvZc (Fig. 6, lanes 9 and 10), it was found that the amount of EnvZc[AssAB] specifically bound to His_{10}–OmpR was 33% of the amount of His_{10}–OmpR-bound EnvZc [for this estimation the non-specific binding of EnvZc to the Ni-NTA resin (lane 7) was subtracted], indicating its decreased affinity for OmpR.

Discussion

EnvZc[AAB] monomer resembles EnvZc

In this report, we have provided biophysical and biochemical evidence that the hybrid protein EnvZc[AAB] exists predominantly as a stable monomer which displays all the enzymatic activities of EnvZc, including autokinase, OmpR kinase and phospho-OmpR phosphatase activities. Most importantly, it shares the same principle of the *trans*-autophosphorylation mechanism as EnvZc, which has been previously demonstrated genetically and biochemically (Yang and Inouye, 1991).

In contrast to its autokinase and OmpR kinase activities, the phospho-OmpR phosphatase activity and OmpR-binding affinity of the monomeric EnvZc[AAB] were reduced in comparison to those of EnvZc. Although very little is known about the mechanism of EnvZc phosphatase activity at present, both domains A and B are considered to contribute to OmpR binding and phosphatase activity (M. Inouye, unpublished data). Also, it is known that the phosphatase activity is highly sensitive to a large number of mutations in the EnvZ molecule (Hsing *et al*., 1998). Therefore, the reduced phosphatase activity of EnvZc[AAB] monomer and its lower binding affinity to OmpR may be due to the following possibilities: (i) the four-helix bundle, presumed to be formed by two covalently linked domains A in the monomeric EnvZc[AssAB], may not be identical to the four-helix bundle formed by two independent domains A; and (ii) the interaction between domain B and the monomeric four-helix bundle in EnvZc[AssAB] may be slightly distorted compared with that between domain B and the dimeric four-helix bundle in EnvZc.

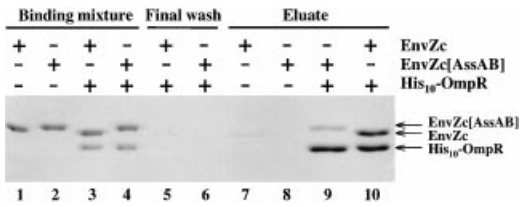

Fig. 6. Binding interaction of His_{10}–OmpR with EnvZc and with EnvZc[AssAB] by Ni-10xhistidine tag affinity chromatography. The binding interactions of His_{10}–OmpR with EnvZc and with EnvZc[AssAB] monomer were performed as described under *Experimental procedures*. The results were analysed using SDS–PAGE. Lanes 1–4, initial binding mixtures; lanes 5 and 6, filtrate from the third wash of the protein-bound Ni-NTA resins; lanes 7–10, eluates from Ni-NTA resins.

Molecular models

We propose molecular models of EnvZc[AAB] monomer and dimer and EnvZc homodimer based on the above results. The addition of an extra domain A to the EnvZ kinase domain created a stable monomer ($T_m = 63.4°C$) with a 16% increase in helical content over that of EnvZc, consistent with the notion that an intramolecular four-helix bundle is formed in the monomer. Because only the His-243 residue of the first (amino proximal) domain A can be phosphorylated in the monomeric hybrid construct EnvZc[AsAB] (Fig. 4A, lane 6), this first domain A but not the second domain A is oriented towards the catalytic domain B (Fig. 7A). In this arrangement, the ATP-binding pocket of domain B is constrained to face helix I of the first domain A, thus allowing only the His-243 residue of the first domain A to be phosphorylated (Fig. 7A II) but not the His-243 residue of the second domain A, excluding the possibility of the arrangement shown in Fig. 7A I. This is consistent with the previous genetic and biochemical data supporting *trans*-autophosphorylation of EnvZ (Yang and Inouye, 1991).

In contrast to the monomer, in the case of the dimeric EnvZc[AAB], two contiguous four-helix bundles must be formed by inter- and intrahelical interactions between the four domains A as depicted in Fig. 7B in order to maintain the similar hydrophobic core as demonstrated in the A domain homodimer (Tomomori *et al*., 1999). The spacer peptides (s or ss) connecting these two four-helix bundles are flexible enough to allow the two bundles to move freely with respect to each other. This arrangement would allow the conserved His-243 residues on both domains A of one EnvZc[AAB] molecule to be accessed by domain B of the other molecule in the dimer for phosphorylation. This would also account for the fact that a substitution of the His-243 residue to serine on the first domain A did not abolish autophosphorylation in the dimer (Fig. 4).

Thus the present study provides further insights into the structural basis for the *trans*-autophosphorylation reaction of EnvZ and the topological arrangement of domain A with respect to domain B. The association of two domains A of two individual subunits results in the formation of the four-helix bundle in an EnvZc homodimer (Tomomori *et al*., 1999). Therefore, the first domain A in the hybrid monomeric EnvZc[AssAB] corresponds to domain A

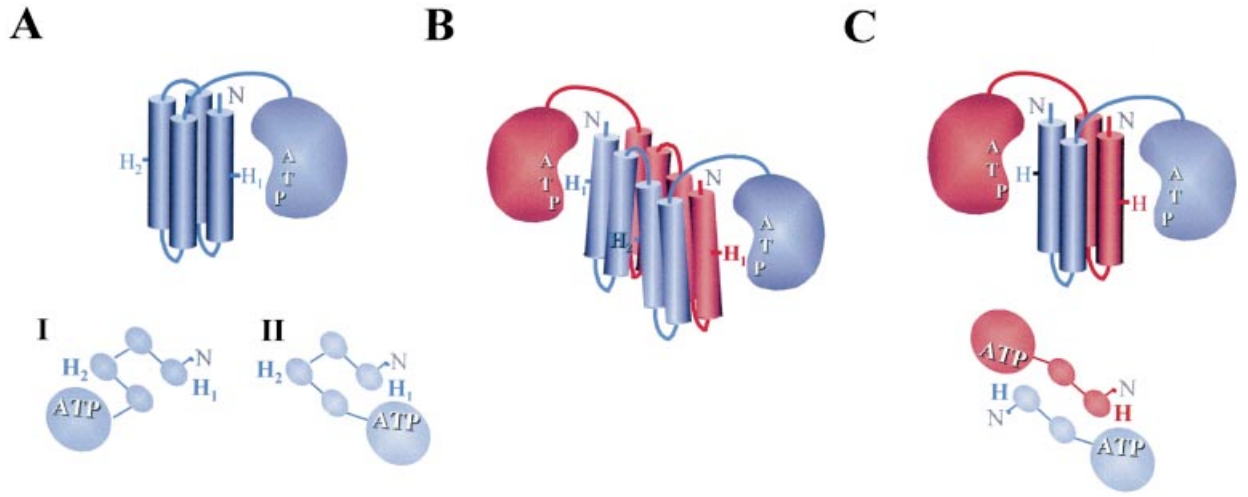

Fig. 7. Molecular models of EnvZc[AAB] monomer, dimer and EnvZc homodimer.
A. Molecular models of EnvZc[AAB] monomer.The monomer of EnvZc[AAB] was modelled as follows: the four-helix bundle that is normally formed by two domains A from two EnvZc molecules in an EnvZc homodimer is proposed to be formed *intra*molecularly in an EnvZc[AAB] monomer. The top panel presents the side view and the bottom presents top views of two possible orientations (I and II) of domain B with respect to the four-helix bundle.
B. Model of EnvZc[AAB] dimer.The dimer of EnvZc[AAB] was modeled as follows: two four-helix bundles are formed by both intramolecular and intermolecular interactions. Thus a four-helix bundle is formed by helices 1, 2 (or 1′, 2′) from one molecule and helices 3′, 4′ (or 3, 4) from the other molecule. The flexible linker peptides connect these two four-helix bundles.
C. Molecular model of the homodimeric EnvZ kinase domainThe ATP-binding site of domain B of one subunit is placed in close proximity to the His-243 residue on helix I of domain A of the other subunit in a homodimer. The top presents the side view and the bottom presents the top view.

from the partner subunit in an EnvZc homodimer as shown in Fig. 7. The two domains B thus flank either side of the bundle with the ATP-binding pocket of one monomer facing His-243 on helix I of the other monomer. The results obtained with EnvZc[AAB] strengthen the possibility for this topological arrangement. The proposed model for the homodimeric EnvZ kinase domain (Fig. 7C) has implications for the structures of other histidine kinases.

Further structural studies of the EnvZc[AAB] monomer are currently under way in our laboratory that may be useful in determining how domains A and B are topologically related. The determination of the precise topology between the histidine phosphotransferring domain A and the catalytic, ATP-binding domain B will be an important step in our understanding of the regulatory mechanisms of histidine kinase function.

Experimental procedures

Plasmids

The plasmid pET11a-EnvZc (Park and Inouye, 1997) was used to express the cytoplasmic domain of EnvZ (residues 180–450). By using primer 5′TACATATGGCGGCTGGTGT-T3′ and 5′GTATTAATGGATCCGCCGCGCAGGTAGTCGA TAAACT3′, a fragment containing the domain A region (Met-223–Arg-289 of EnvZ) was PCR amplified from pET11a-EnvZc. This fragment was subsequently cloned in T/A vector (Invitrogen), sequenced, amplified by miniprep and finally released by *Nde*I and *Ase*I double digestion. The pET11a-EnvZc plasmid was digested by *Nde*I, releasing the linker region of EnvZ. The remaining part of the plasmid was ligated with the above fragment. Because the fragment could be ligated into the vector in two directions, sequencing of the insertion was performed to confirm the correct direction after picking up candidate colonies. The resulting plasmid, pLQ050, expresses EnvZc[AsAB] with four extra amino acid residues (GGSI) between the two domains A under the regulation of the T7 promoter.

To construct EnvZc[AssAB], which contains a longer spacer (GGSGGGSI), two oligos (5′GATCCACCGCCT3′ and 5′GATCAGGCGGTG3′) were synthesized and annealed to each other to create a small linker. This linker was inserted into pLQ050 which had been partially digested by *Bam*HI. PLQ050 has two *Bam*HI sites, one located on the pET11a backbone, the other located in the spacer sequence between two A regions. The resulting plasmid, pLQ051, was sequenced to confirm the correct direction and insertion site of the small linker sequence.

To construct plasmid expressing His_{10}–OmpR, plasmid pT7OmpR was first cut by *Nde*I and *Bam*HI. The released fragment, containing the OmpR coding sequence starting from *Nde*I site, was ligated with *Nde*I–*Bam*HI digested pET16b (Novagen).

Purification of EnvZc, EnvZc[AAB]s and His_{10}–OmpR

E. coli BL21-DE3 was used for the expression and purification of all the above proteins. The cells harbouring different

plasmids were cultured to mid-log phase when 1 mM IPTG was added. After another 3 h incubation, cells were harvested and broken by French Press. EnvZc was purified by ammonium sulphate fractionation and anion-exchange chromatography (DE52) as previously reported (Forst *et al*., 1989). Purification of EnvZc[AAB]s was similar to that of EnvZc except that the DE52 column chromatography step was omitted. His_{10}–OmpR was purified using a Ni-NTA resin column and subsequent Sephacryl S-100HR column chromatography.

Circular dichroism spectroscopy

CD spectra were recorded on an Aviv Model 62DS spectropolarimeter with a Hewlett-Packard Peltier thermoelectric temperature controller. CD spectra were recorded in 1 mm path length cells at 20°C from 200 to 260 nm at 0.5 nm intervals with a 5 s recording time at each point. For melting curves, the ellipticity was monitored at 221 nm and the sample temperature was raised from 0°C to 80°C in increments of 0.3°C over a time span of 10 h.

Autokinase and phosphotransferase assays

EnvZc, EnvZc[AssAB] dimer and monomer proteins (1.1 μM each) were incubated in 20 mM Tris-HCl buffer (pH 8.0) containing 50 μM KCl, 5 mM $CaCl_2$ and 5% glycerol with 50 mM [γ-^{32}P]-ATP (1 Ci mmol^{-1}) at room temperature. Aliquots were removed from the reaction mixture at 0.5, 1, 3, 5, 10 and 20 min and the reaction was stopped with 5× SDS loading dye. At 20 min, OmpR was added to the reaction mixture at a final concentration of 2.8 μM. Aliquots were removed and reactions stopped using 5× SDS loading dye at 0, 45 and 120 s. The phospho-proteins were separated on a 17.5% SDS–PAGE gel. The dried gel was exposed and scanned by a phosphorimager for quantitative comparison. It was then exposed for autoradiography.

Endoproteinase Glu-C (V8 protease) digestion

EnvZc and EnvZc[AsAB] dimer and monomer proteins (4 μg each) were autophosphorylated by [γ-^{32}P]-ATP as described above. After 15 min incubation at room temperature, an equal aliquot from each reaction mixture was removed and the reaction was stopped with tricine–SDS sample buffer (50 mM Tris-HCl pH 6.8 containing 4% SDS, 12% glycerol, 2% β-mercaptoethanol and 0.01% Serva Blue G250 dye). The remaining phospho-protein was subjected to V8 protease digestion. The phosphorylated proteins were incubated for 3 h at 25°C in the digestion buffer of 25 mM ammonium carbonate (pH 7.8), containing 0.01% SDS and V8 protease (Boehringer), whose ratio to phosphorylated protein was 1:20. The reaction was stopped with tricine–SDS sample buffer. The undigested phospho-proteins and the digested products were separated on a tricine–SDS–PAGE gel (Schagger and von Jagow, 1987) using a Bio-Rad Mini Protean II gel-electrophoresis system. The separating gel composition was 16.5% T and 6% C, the spacer gel was 10% T and 3% C, whereas the stacking gel was 4% T and 3% C. The gel was run at 200 V until the serva blue dye reached the bottom of the gel. The wet gel was exposed for autoradiography.

Phosphatase assay

Phospho-OmpR was prepared as previously reported (Park *et al*., 1998). Purified ^{32}P-labelled phospho-OmpR in total amount of 3.2 μM OmpR was incubated with 3.2 μM various EnvZ proteins at 22°C in the presence of 1 mM ADP and 5 mM Ca^{2+}. Aliquots were removed from the reaction mixture at indicated time points, mixed with 5× SDS loading dye and separated on 17.5% SDS–PAGE gel. After drying, the gel was exposed for autoradiography.

Binding assay on Ni-Nitrilotriacetic Acid (NTA) Resin

The binding experiments between His_{10}–OmpR with EnvZc and with EnvZc[AssAB], respectively, were carried out as follows. Purified His_{10}–OmpR (5 μg) was incubated with purified EnvZc (10 μg) or EnvZc[AssAB] monomer (10 μg) in 50 μl binding buffer A, containing 20 mM Tris (pH 8.0), 20 mM β-mercaptoethanol and 5% glycerol. EnvZc or EnvZc[AssAB] was mixed in binding buffer A without His_{10}–OmpR to measure non-specific binding of these proteins to the Ni-NTA resin. Five microlitres of initial binding mixture was removed and mixed with 5× SDS–PAGE loading dye. Then the binding mixtures were incubated for 1 h at room temperature. Ni-NTA resin (Ni-NTA agarose from Qiagen; 5 μl of resin per 5 μl of buffer A) was added to the binding mixture and the mixture incubated at 4°C for 45 min with gentle shaking. The binding mixture was then transferred to an ultrafree-MC filtration tube (UFC30HV00; Millipore) and filtered (centrifugation at 14 000 r.p.m. for 1 min). The resulting protein-bound Ni-NTA resin was washed three times, within 3 min, at 4°C, with buffer A containing 350 mM NaCl to avoid dissociation of the complex. The Ni-NTA resin was then treated with 10 μl of buffer A containing 100 mM EDTA for 10 min at room temperature and then filtered by centrifugation at 14 000 r.p.m. for 2 min. This elution step was repeated with 5 μl of elution buffer and the eluates combined. The eluates were mixed with 5× SDS dye and applied to 17.5% SDS–PAGE. The gel was stained with Coomassie brilliant blue and the amounts of proteins were estimated using a Bio-Rad Model GS-670 Imaging Densitometer.

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