The inner membrane histidine kinase EnvZ senses osmolality via helix-coil transitions in the cytoplasm

Loo Chien Wang¹, Leslie K Morgan²,
Pahan Godakumbura², Linda J Kenney^{1,2,3,*}
and Ganesh S Anand^{1,3,*}

¹Department of Biological Sciences, National University of Singapore, Singapore, Singapore, ²Department of Microbiology and Immunology, University of Illinois-Chicago, Chicago, IL, USA and ³Mechanobiology Institute, National University of Singapore, Singapore, Singapore

Two-component systems mediate bacterial signal transduction, employing a membrane sensor kinase and a cytoplasmic response regulator (RR). Environmental sensing is typically coupled to gene regulation. Understanding how input stimuli activate kinase autophosphorylation remains obscure. The EnvZ/OmpR system regulates expression of outer membrane proteins in response to osmotic stress. To identify EnvZ conformational changes associated with osmosensing, we used HDXMS to probe the effects of osmolytes (NaCl, sucrose) on the cytoplasmic domain of EnvZ (EnvZ_C). Increasing osmolality decreased deuterium exchange localized to the four-helix bundle containing the autophosphorylation site (His²⁴³). EnvZ_C exists as an ensemble of multiple conformations and osmolytes favoured increased helicity. High osmolality increased autophosphorylation of His²⁴³, suggesting that these two events are linked. *In-vivo* analysis showed that the cytoplasmic domain of EnvZ was sufficient for osmosensing, transmembrane domains were not required. Our results challenge existing claims of robustness in EnvZ/OmpR and support a model where osmolytes promote intrahelical H-bonding enhancing helix stabilization, increasing autophosphorylation and downstream signalling. The model provides a conserved mechanism for signalling proteins that respond to diverse physical and mechanical stimuli.

The EMBO Journal (2012) **31**, 2648–2659. doi:10.1038/emboj.2012.99; Published online 27 April 2012

Subject Categories: membranes & transport; signal transduction

Keywords: amide hydrogen deuterium exchange mass spectrometry; histidine kinase; osmosensing; robustness; two-component signal transduction

*Corresponding authors. LJ Kenney, Department of Microbiology and Immunology, University of Illinois-Chicago, 835 S Wolcott Avenue, Chicago, IL 60612, USA. Tel.: +1 312 413 0576; Fax: +1 312 996 6415; E-mail: kenneyl@uic.edu or GS Anand, Department of Biological Sciences, National University of Singapore, Singapore 117543, Singapore. Tel.: +1 65 6516 7722; Fax: +1 65 6779 2486; E-mail: dbgsa@nus.edu.sg

Received: 26 September 2011; accepted: 14 March 2012; published online 27 April 2012

Introduction

The inherent capacity of prokaryotes to successfully inhabit almost all known environments can be partly ascribed to the development of versatile and sophisticated adaptive response systems that allow them to sense and relay information about their surroundings. The ability to perceive environmental stimuli, such as extracellular osmolality, is important for the growth, survival and adaptation of prokaryotes. Responses to environmental stress in bacteria are mediated by two-component signalling pathways, which consist of a histidine kinase (HK) and a cognate RR. The HK senses environmental stress and is autophosphorylated at a conserved histidine residue. The phosphoryl group is then transferred from the sensor to a conserved aspartic acid residue of the RR. The phosphorylated RR usually functions to stimulate transcription of appropriate gene targets.

The EnvZ/OmpR system is one of the best-characterized two-component systems, reciprocally regulating expression of outer membrane porins OmpF and OmpC in response to osmotic stress (van Alphen and Lugtenberg, 1977). At low osmolality, OmpF is the major porin in the outer membrane and at high osmolality, *ompF* is repressed and OmpC becomes the major porin in the outer membrane. The response to osmolality increased up to 200 mM NaCl and then was constant at higher salt concentrations. Similar effects were observed with NaCl, KCl and sucrose with little influence on the generation time. It was subsequently shown that this differential regulation required EnvZ/OmpR and the porins were regulated at the transcriptional level. Furthermore, all known functions of EnvZ required OmpR (Slauch *et al*, 1988).

The EnvZ/OmpR system regulates many genes in *Escherichia coli* (Oshima *et al*, 2002) and is essential for virulence in numerous pathogens (Dorman *et al*, 1989; Bernardini *et al*, 1990; Pickard *et al*, 1994; Vidal *et al*, 1998; Lee *et al*, 2000; Brzostek *et al*, 2003; Feng *et al*, 2003). EnvZ is an inner membrane protein with a short N-terminus in the cytoplasm (Met¹–Thr¹⁵), two transmembrane regions (Leu¹⁶–Asn⁴⁷ and Tyr¹⁶³–Ile¹⁷⁹), a periplasmic loop (Lys⁴⁸–Arg¹⁶²), and a large cytoplasmic domain (Arg¹⁸⁰–Gly⁴⁵⁰) (Forst *et al*, 1987). His²⁴³ is the site of EnvZ autophosphorylation (Roberts *et al*, 1994), while Asp⁵⁵ on OmpR is the acceptor site for phosphotransfer (Delgado *et al*, 1993). Phosphorylation of OmpR increases its affinity for the *ompF* and *ompC* promoters (Head *et al*, 1998), as well as enhancing an interaction with RNA polymerase, activating transcription (Godakumbura and Kenney, unpublished results). While the relative number of porin proteins remains constant, the OmpF:OmpC ratio is altered by the signal from EnvZ (van Alphen and Lugtenberg, 1977).

Although the EnvZ/OmpR system has been well studied, almost nothing is known as to how EnvZ senses and responds to osmotic stress. The structures of two distinct subdomains of the cytoplasmic region of EnvZ were solved separately (Tanaka

et al, 1998; Tomomori *et al*, 1999). While these structures provided insights into the topology of the individual subdomains, it is not known how the two domains interact in three-dimensional space. In all of the intact cytoplasmic domain structures of HKs that have been solved to date, the orientation of the ATP-binding domain with respect to the four-helix bundle differs in each case (Szurmant and Hoch, 2010). This has limited our understanding of the phosphotransfer and signalling mechanism. Furthermore, there are conflicting reports as to whether the periplasmic and transmembrane domains are important for signalling (Tokishita *et al*, 1991, 1992; Tabatabai and Forst, 1995). Support for the functional importance of membrane anchoring came from an engineered truncated EnvZ protein that was missing the first 38 amino acids (nearly eliminating the first transmembrane segment) and fused to eight amino acids of LacZ. As a result of the fusion, the LacZ-EnvZ chimera was not inserted into the membrane and was expressed in inclusion bodies (Igo and Silhavy, 1988). This fusion was capable of autophosphorylation and interaction with OmpR (Igo and Silhavy, 1988; Kenney, 1997), but it did not restore the normal regulation of OmpF and OmpC in response to changes in osmolality. Presumably because of this result, the ability to restore osmotic signalling of a plasmid expressing only the cytoplasmic C-terminus of EnvZ (EnvZ_c) was not reported (Park *et al*, 1998).

Amide hydrogen/deuterium exchange mass spectrometry (HDXMS) is a powerful method to probe conformational dynamics of proteins in solution (Hoofnagle *et al*, 2003). We used HDXMS to probe the conformational dynamics of EnvZ_c in the presence of low and high osmolality and discovered structural changes in EnvZ_c resulting from increased osmolality. Our results indicate that the locus for osmosensing lies within the N-terminal four-helix subdomain and dimer interface. Two amphipathic helices from each monomer, one of which contains the phosphorylatable histidine, exhibit decreased exchange in the presence of osmotic stimuli. Additional evidence from HDXMS reveals that this helical region exists as an ensemble of multiple, slowly interchanging conformations in solution, reflecting local unfolding within the helical region. Osmolytes enhance autophosphorylation and downstream signalling by shifting the equilibrium to favour a more folded (less exchangeable) conformation. This is a novel example of coupling secondary structure dynamics in proteins to downstream signalling. In addition, osmosensing was completely uncoupled from nucleotide binding. Surprisingly, EnvZ_c was capable of sensing osmolality *in vivo* without being inserted in the inner membrane and was also able to signal to OmpR. Its higher activity compared with the wild type raises doubts as to the robustness of the EnvZ/OmpR system. Our *in vitro* and *in vivo* results are consistent with the four-helix bundle as the osmosensor. These findings will likely be generalizable to other sensor proteins from both prokaryotes and eukaryotes that respond to a range of physical and mechanical stimuli.

Results

High osmolality alters the conformation of a helix containing the His²⁴³ phosphorylation site

Because EnvZ_c was soluble and well behaved in solution, we performed HDXMS (Hoofnagle *et al*, 2003) to determine

whether the cytoplasmic domain was sensitive to changes in osmolality. When we compared the exchange rates of different EnvZ_c peptides in low (15.5 mM Tris · HCl, pH 7.6) versus high osmolality (15.5 mM Tris · HCl, pH 7.6 with 0.5 M NaCl or 20% (w/v) sucrose), there were major differences. Three and two peptides with NaCl and sucrose, respectively, were observed to be osmosensitive and exhibited altered deuterium exchange. Two of these three osmosensitive peptides were located within the N-terminal four-helix bundle subdomain and a third spanned a loop connecting it to the ATP-binding domain. A complete summary of the relative deuterium exchange rates of 37 pepsin fragment peptides of EnvZ_c is represented as difference plots for NaCl (Figure 1A) and sucrose (Figure 1B). Supplementary Table S1 summarizes the results for 18 peptides that were analysed at varying concentrations of NaCl and sucrose following 5 min deuterium exchange. Varying the osmolality did not result in any change in deuterium exchange within the ATP-binding domain (Figure 1). The effect of osmolality on the EnvZ_c four-helix bundle was not merely a generic salt effect, since both NaCl and sucrose altered deuterium exchange at the same locations. Thus, only three peptides, all located within the N-terminal four-helix bundle subdomain, exhibited changes with osmolality. Of these three peptides, peptides I and III showed increased exchange with osmolality, reflecting increased dynamics. Peptide II (residues 238–254) showed the largest decreases in exchange with osmolality (Figure 2). This is significant, as it contains His²⁴³, the site of EnvZ_c autophosphorylation required for downstream signalling. Isotopic envelopes of the spectra at low and high osmolality reveal clear decreases in exchange at high osmolality (Figure 2A). These differences were also evident from kinetic plots of deuteration for this fragment with a clear dependence on NaCl or sucrose concentrations (Figure 2B and C). This difference was most evident from 2 to 5 min and indicated a difference of 2 deuterons exchanged in a peptide of 17 amino acids. Addition of 2 H-bonds would contribute substantially to increased helicity in this region and is consistent with a previous NMR study that noted that residues 242–248 were poorly defined and displayed increased flexibility relative to the rest of the four-helix bundle (Tomomori *et al*, 1999). We quantitated HDX rates across the protein at a range of NaCl (0 (100 mOsm/kg), 20 (110 mOsm/kg), 80 (200 mOsm/kg), 150 (330 mOsm/kg), 300 (600 mOsm/kg), 500 mM (960 mOsm/kg)) and sucrose concentrations (0% (100 mOsm/kg), 5% (200 mOsm/kg), 10% (370 mOsm/kg), 15% (570 mOsm/kg), 20% (780 mOsm/kg)). We observed decreased HDX only at the His-containing peptide and within the other helical segment within the four-helix bundle. The estimated concentration at which we observed half maximal observed deuterium exchange (after 5 min deuterium exchange) was 300 mM NaCl (600 mOsm/kg) and 15% sucrose (570 mOsm/kg) (Supplementary Table S1), that is, nearly identical osmolalities. No differences were observed for all of the remaining peptides (Figure 2; Supplementary Figure S1).

We also examined the deuterium exchange of all peptides of an EnvZ_c H243A mutant, which is incapable of autophosphorylation. The H243A mutant peptide was insensitive to NaCl, further highlighting the importance of His²⁴³ in osmosensing (Figure 2D). Although the alanine substitution would favour increased helicity of this peptide, the histidine was

Figure 1 Comparison of deuterium exchange for EnvZ_c at low and high osmolality. (A) Difference plot of the relative deuterium exchange for each pepsin digested fragment of EnvZ_c at low (100 mOsm/kg) and high osmolality (0.5 M NaCl; 960 mOsm/kg) following 2, 5, 10 and 30 min deuterium exchange (shown in solid orange, pink, blue and black lines, respectively). Each dot represents a pepsin digest fragment of EnvZ_c and residue numbers are indicated from N- to C-terminus on the x-axis. The diagram below the residue numbers indicates the corresponding domain organization of EnvZ_c. Positive and negative deuterium exchange differences denote decreases or increases in deuterium exchange at high osmolality. All of the differences in exchange indicated are not corrected for deuterium back exchange in the experiment. Changes in deuterium exchange $> \pm 0.5$ Da alone (dashed line) are considered significant for comparative deuterium exchange analysis. Three peptides that demonstrated significant changes in deuterium exchange were identified: I (residues 220–233) and II (residues 238–254) are peptides of the N-terminal four-helix bundle and III (residues 302–310) forms part of the loop connecting the four-helix bundle to the ATP-binding domain. Although 37 peptides were analysed, only 27 peptides are displayed. The overlapping peptides were not shown to avoid crowding the plot. (B) Difference plot of the average relative fractional uptake for EnvZ_c at low (100 mOsm/kg) and high osmolality (20% (w/v) sucrose; 780 mOsm/kg) after 2, 5, 10 and 30 min deuterium exchange. Details are as described in (A). Two of the same peptides were identified as in A: I (residues 220–233) and II (residues 238–254) are peptides of the N-terminal four-helix bundle.

required for osmosensing. Thus, the dimerization interface formed by the four-helix bundle subdomain, including the helical fragment containing His²⁴³, forms an osmosensitive core of EnvZ_c (Figure 2E).

As a control experiment, EnvZ_c was first pre-digested with pepsin, upon which the pH was readjusted to 7.6, and then subjected to HDXMS at low and high osmolality. No changes in deuterium exchange were observed, indicating that osmolality-dependent changes in EnvZ_c only occurred in the native state and were not a non-specific effect on deuterium exchange (Supplementary Figure S2). We also examined a control protein, the regulatory subunit RI α (91–244) of protein kinase A (PKA), for non-specific effects of NaCl and none was detected (data not shown). Furthermore, there were no effects of NaCl or sucrose on deuterium back exchange for EnvZ_c or for the regulatory subunit, RI α of PKA. We further tested the suitability of other salts (KCl, NaI) and sugars (lactose, maltose and glucose) as osmolytes. The His-containing peptide showed decreased exchange in response to all of these osmolytes in the ranges and timescales observed with NaCl and sucrose (data not shown).

The site of OmpR binding to EnvZ exhibits ensemble behaviour

All of the peptides that we examined demonstrated EX2 kinetics (Hoofnagle *et al*, 2003), that is, the rate of folding was faster than deuterium exchange, except residues 267–278 ($m/z=682.8$, $z=+2$; AESINKDIEECN) of the second helix in the four-helix bundle. It exhibited an entirely different deuterium exchange profile from the other peptides

(Figure 3A), exemplified by EX1 kinetics where exchange was faster than folding/unfolding. At low osmolality, the AESINKDIEECN peptide displayed a bimodal distribution in the amide HDX profile within the deuterium exchange timeframes analysed, consistent with this region undergoing local unfolding events (Kaltashov and Eyles, 2005). The shift in the bimodal distribution showed a direct correlation with osmolytes, where higher concentrations of NaCl (Figure 3A) and sucrose (Figure 3B) significantly increased the intensity of the lower-exchanging spectral envelope. The salt concentration dependence mirrored the effects on the His²⁴³-containing peptide in that increasing NaCl led to reduced deuterium exchange. A total of five overlapping peptide fragments covered this region and all of the peptides minimally spanning residues 269–278 were characterized by bimodal deuterium exchange profiles (data not shown). Interestingly, this bimodal distribution was much diminished in the H243A mutant, favouring the higher-exchanging Envelope II (Figure 3C). This result further highlights that the conformations of the two helices of the four-helix bundle are coupled and together, they form the osmosensing locus for EnvZ_c.

The AESINKDIEECN peptide is a likely site for interaction with the RR OmpR (Figure 3D), based on analysis of conserved specificity determinants (Skerker *et al*, 2008), co-variance (Szurmant and Hoch, 2010) and chemical shift changes (Tomomori *et al*, 1999). At low osmolality, the peptide could be resolved as a combination of two Gaussian distributions (Figure 3E) (Gertsman *et al*, 2010). One isotopic envelope profile with higher exchange represents the population with greater disorder (coil). The

Figure 2 His²⁴³-containing peptide of EnvZ_c exhibits decreased deuterium exchange at high osmolality. (A) ESI-Q-TOF mass spectra for the peptide spanning residues 238–254 and containing the phosphorylatable His²⁴³ following 5 min of deuterium exchange at high (0.5 M NaCl (960 mOsm/kg) or 20% (w/v) sucrose (780 mOsm/kg), 15.5 mM Tris · HCl, pH 7.6) or low (15.5 mM Tris · HCl, pH 7.6) (100 mOsm/kg) osmolality. Arrowheads indicate the centroid of the mass spectral envelopes. Subtraction of the centroid of the undeuterated envelope yields the average deuterons exchanged under each condition. The lower centroids observed at high osmolality reflect decreased deuterium exchange. (B) Time course for amide HDX (1–30 min) for EnvZ_c fragment of residues 238–254 ($m/z = 646.03$, $z = +3$) at varying concentrations of NaCl and (C) sucrose. Deuterium exchange reported is corrected for back exchange as stated in Materials and methods. (D) ESI-Q-TOF mass spectra of residues 238–244 (MAGVSAD) in the H243A mutant following 5 min deuterium exchange, in the presence of 0 (100 mOsm/kg) or 0.5 M NaCl (960 mOsm/kg). (E) Structure of EnvZ_c (223–289) dimer (PDB ID: 1JOY) (Tomomori *et al*, 1999) is shown in white. Residues 238–254 (orange) show decreased deuterium exchange at high osmolality. His²⁴³ (red stick) is the site of autophosphorylation and phosphotransfer in EnvZ.

isotopic envelope profile with lower exchange represents the more helical conformation. Thus, EnvZ_c exists as an ensemble of conformations and osmolytes exert a helix stabilization effect, shifting the equilibrium to favour a more folded state. Osmolality-dependent switching between helical and coil conformations thus forms the basis for osmosensing by EnvZ_c.

EnvZ_c autophosphorylation increases with increasing osmolality

In order to correlate the decrease in exchange of the His²⁴³-containing peptide at high osmolality (Figure 2) with EnvZ signalling, we examined the effect of increasing osmolality on EnvZ_c autophosphorylation (Figure 4). Surprisingly, there

was a dramatic enhancement of autophosphorylation with increasing NaCl concentration (compare top and bottom two panels in Figure 4A; densitometric analysis is shown in Figure 4B). An explanation of this result is that increased H-bonding within the helix containing His²⁴³ as observed by decreased deuterium exchange rates (Figure 2) results in increased autophosphorylation. This higher level of EnvZ_c~P would result in higher levels of OmpR~P, and increased OmpC expression (Nara *et al*, 1986). Similar effects were observed with sucrose (Supplementary Figure S3A and B). There was remarkable agreement with the rapid phase of autophosphorylation evident in Figure 4B (0–10 min) and the peak of reduced deuteration of the His²⁴³-containing peptide (Figure 2B and C).

Figure 3 Amide HDXMS reveals local unfolding and ensemble behaviour in EnvZ_c. (A) ESI-Q-TOF mass spectra for the peptide spanning residues 267–278 following 5 min deuterium exchange. At low osmolality, the isotopic envelopes show a characteristic bimodal distribution indicative of multiple exchanging populations of peptides. This reflects a mixture of low- and high-exchanging conformations of EnvZ_c. At higher osmolality, the equilibrium is shifted to favour the low-exchanging conformation. A gradual decrease of the higher-exchanging Envelope II was observed as the NaCl concentration was increased from 0–500 mM, whereupon the lower-exchanging Envelope I increased in intensity. Concentrations of NaCl with osmolality values used are 20 mM NaCl (110 mOsm/kg), 80 mM NaCl (200 mOsm/kg), 150 mM NaCl (330 mOsm/kg), 300 mM NaCl (600 mOsm/kg) and 500 mM NaCl (960 mOsm/kg). Osmolality of 100 mOsm/kg was measured for Tris-HCl minus NaCl. (B) ESI-Q-TOF mass spectra for the peptide spanning residues 267–278 following 5 min deuterium exchange, in 0% (100 mOsm/kg) and 20% sucrose (w/v) (780 mOsm/kg). (C) ESI-Q-TOF mass spectra of residues 267–278 in the H243A mutant after 5 min deuterium exchange. Residues 267–278 no longer responded to changes in osmolality after the substitution was introduced in the His²⁴³-containing peptide of the adjacent helix, which also showed no change in deuterium exchange profile compared with wild-type (Figure 2D), suggesting that these two helices are functionally coupled. (D) Structure of an EnvZ_c (223–289) dimer (PDB ID: 1JOY) (Tomomori *et al*, 1999) is shown in white. Residues 267–278 (orange) are part of the second helix proximal to the helix containing His²⁴³ (red) and constitute a putative OmpR-binding domain based on conserved specificity determinants (Skerker *et al*, 2008) and co-evolution analysis (Szurmant and Hoch, 2010). (E) The spectral envelope of residues 267–278 following 10 min deuterium exchange (charge state: +2) was fit to the sum of two Gaussian distributions using GraphPad Prism. The overall centroid value for the spectral envelope was 684.2 Da as determined by HX-Express 2007 (Weis *et al*, 2006). Envelope II corresponded to a significantly higher exchange than Envelope I.

Nucleotide binding is uncoupled from osmosensing

The nucleotide-binding domain features a large lid-like structure, which functions to bind ATP (Tanaka *et al*, 1998). In the presence of osmolytes such as sucrose or salt, no significant differences in deuterium exchange were observed within the ATP-binding and phosphotransfer domain, that is, conditions that reduced exchange of the His²⁴³-containing peptide did not alter the ATP-binding domain (Figure 1; Supplementary Table SI). In contrast, addition of the non-hydrolyzable analogue AMP-PNP decreased deuterium exchange throughout the ATP-binding domain (Figure 5A). Specifically, residues 368–421 exhibited decreased exchange

in the presence of AMP-PNP, which directly correlated with ligand binding. There was no change in the putative OmpR-binding site upon addition of AMP-PNP (Figure 5B), that is, the bimodal distribution of the AESINKDIEECN peptide was unaffected by AMP-PNP. The effect of 0.5 M NaCl on EnvZ_c in the presence of AMP-PNP was also examined. The His²⁴³-containing peptide showed decreased exchange upon addition of 0.5 M NaCl to the same extent as observed in the absence of AMP-PNP (Supplementary Table SII). Thus, nucleotide binding was not allosterically coupled to osmosensing at the His²⁴³-containing peptide, indicating that osmosensing and ATP-binding are two distinct, discrete

Figure 4 Autophosphorylation of EnvZ_c (EnvZ_c~P) increases with increasing osmolality. (A) EnvZ_c was added at a final concentration of 4 μM and incubated for the indicated times (top) at RT. Each reaction was stopped with 3 μl of denaturing loading dye and 10 μl of the 20 μl reaction was separated by 15% SDS-PAGE. The osmolality of the reaction buffer was adjusted by addition of salt and measured by vapour point depression and is indicated in the figure. See Materials and methods for buffer conditions. (B) Densitometry of the experiment shown in (A) was performed using Molecular Dynamics ImageQuant software (v5.0). Results are plotted as a percent of the EnvZ_c~P as a function of time.

events. This result has implications for a role of the putative phosphatase activity of EnvZ as the regulated step in osmoregulation, since this enzymatic activity has been shown to be nucleotide dependent (see Discussion).

The cytoplasmic domain of EnvZ is capable of osmosensing *in vivo*

In order to determine whether the transmembrane helices and periplasmic domain of EnvZ were required for osmosensing *in vivo*, we examined whether the cytoplasmic domain of EnvZ (EnvZ_c), was capable of osmosensing. Previous studies have shown that EnvZ works through OmpR to control porin gene expression (Slauch *et al*, 1988). We used a gene reporter assay measuring β-galactosidase activity of an *ompC-lacZ* fusion (Hall and Silhavy, 1979) that responds to osmotic stress (Figure 6). At low osmolality, the wild-type strain exhibited a low activity of *ompC-lacZ* that increased dramatically at high osmolality (circles). In the isogenic *envZ*-null strain, essentially no activity was observed at any osmolality (squares). When the *envZ_c*-encoding plasmid was expressed, the *ompC-lacZ* activity increased in parallel with the wild-type strain over the same osmolality range, but started at a higher level (triangles). The observation that EnvZ_c restored the normal increase in *ompC-lacZ* activity with increasing osmolality indicated that it was capable of osmosensing, even though it was no longer inserted in the inner membrane. Although there was a similar increase in activity with the wild-type EnvZ and EnvZ_c as a function of increasing osmolality, the cytoplasmic domain appeared 'leaky' in that it produced a higher background level of *ompC-lacZ* activity. The basis of the increased expression might result from the loss of a regulatory domain (e.g., the transmembrane domains or the periplasmic domain) or increased levels of EnvZ_c compared with the wild type. Previous studies that reported normal levels of OmpF and OmpC after over-expressing *envZ* in a wild-type *envZ* background suggested that the system was robust and OmpR~P levels were not dependent on EnvZ concentration (Batchelor and Goulian, 2003). The results in Figure 6 suggest that the increase in *ompC-lacZ* activity may result from loss of a regulatory domain or a lack of robustness, but in any case, osmosensing still occurs normally with the cytoplasmic domain of EnvZ alone.

Discussion

Stabilization of the four-helix bundle containing His²⁴³ at high osmolality provides the molecular basis for EnvZ signalling

Our HDXMS results emphasize that EnvZ_c is conformationally highly dynamic at low osmolality. The results are consistent with localized breathing or unfolding (Englander and Kallenbach, 1983) occurring at specific regions of EnvZ_c, particularly at the N-terminal four-helix bundle that forms the dimer interface, wherein EnvZ exists as an ensemble of fully folded and partially unfolded conformations in solution. It is interesting to note that this is the locus for autophosphorylation as well as binding and phosphotransfer to OmpR. Intrinsic dynamics were observable for residues 267–278 (AESINKDIEECN) on the basis of the bimodal distribution of isotopic envelopes, reflecting fully folded and partially unfolded states within the timeframe of our deuterium exchange experiments. While the exchange cannot be precisely measured in the peptides exhibiting bimodal distribution for deuterium exchange, the shift in distribution by osmolality provides the additional insight that this region is intrinsically dynamic. Furthermore, the effect of NaCl in stabilizing the helical segment was evident (Figure 3A). The higher-exchanging isotopic envelope (Envelope II) shows on average at least four additional deuterons exchanged and is suggestive of localized helical unfolding. The bimodal distribution was significantly shifted at high osmolality to favour the decreased exchange state and thereby a folded helical conformation of peptides. This is significant, as it suggests a basis for the intrinsic basal autophosphorylation rate and signalling activity for EnvZ_c even at low osmolality, that is, EnvZ_c exists in a dynamic equilibrium and high osmolality shifts the equilibrium to the activated state. The NaCl concentration dependence of the AESINKDIEECN peptide mirrored the effects on the His²⁴³-containing peptide. Furthermore, substitution of His²⁴³ with alanine greatly diminished the dynamics and salt stabilization of residues 267–278 (Figure 3C). Thus, the two helical segments from each monomer were dynamically coupled and further support our conclusion that the four-helix bundle subdomain forms the osmosensory locus in EnvZ_c. Taken together, our results indicate that intracellular osmolytes exert a helix stabilization effect via enhanced intrahelical

Figure 5 Effects of high osmolality and AMP-PNP binding on the ATP-binding subdomain of EnvZ_c. (A) The structure of the ATP-binding subdomain of EnvZ_c bound to AMP-PNP is shown in white (PDB ID: 1BXD) (Tanaka *et al*, 1998). The ATP-binding subdomain showed no significant change in deuterium exchange profile at high osmolality (Figure 2; Supplementary Table SI). In contrast, AMP-PNP binding greatly reduced deuterium exchange in the ATP-binding domain as expected, particularly at residues 368–421 (purple). (B) Nucleotide binding by the non-hydrolyzable ATP analogue AMP-PNP does not alter the conformational ensemble properties of EnvZ_c (residues 267–278), making it unlikely that the EnvZ phosphatase activity is regulated by osmolality or nucleotide binding (see Discussion).

H-bonding reflecting decreased deuterium exchange (Bolen and Rose, 2008). Studies with short alanine-based and poly-L-glutamate peptides have shown how ionic strength can alter helix-coil equilibria in solution, wherein osmolytes increase helicity (Scholtz *et al*, 1991; Stanley and Strey, 2008). This stable conformation enhances the autophosphorylation rate and consequently, downstream phosphotransfer and signalling.

Proposed signalling events

Our results described herein lead us to propose the following events during osmosensing: Under normal conditions (i.e.,

Figure 6 EnvZ_c is capable of osmosensing. β-Galactosidase assay of *ompC-lacZ*. MH225 is a derivative of MC4100 (Casadaban, 1976) and contains an *ompC-lacZ* fusion and is wild type (circles), *envZ*-null (squares) or *envZ*-null complemented with *envZ_c* (triangles). The strains were grown in low osmolality A medium or in A medium + various sucrose (w/v) concentrations, the osmolality is indicated on the x-axis. Measurements were made in triplicate and the experiment was repeated twice. The symbols represent the mean ± s.d. (error bars). The absence of error bars indicates that the error was less than the symbol. The activity of the Δ*envZ* strain containing the empty vector was 200–300 Miller Units compared with the *envZ*-null strain lacking the plasmid (50–150 Miller Units). Addition of a plasmid-encoding full-length *envZ* to the Δ*envZ* strain exhibited 50% of the activity of the wild-type strain.

in vivo ATP concentrations between 1–10 mM, in vast excess of ADP (Mathis and Brown, 1976), and an apparent affinity of EnvZ for ATP of 0.2 mM (Kenney, 1997), EnvZ_c exists predominantly in the ATP-bound state. An increase in external osmolality would lead to an increase in intracellular osmolality and induce conformational changes in EnvZ by enhancing helix stability through increased intramolecular H-bonding within the His²⁴³-containing peptide and OmpR-binding sites. This stable conformation facilitates increased autophosphorylation and phosphotransfer to OmpR. The lower affinity of OmpR~P compared with OmpR would promote the release of OmpR~P from EnvZ (Mattison and Kenney, 2002), enabling it to bind to the porin promoters with high affinity (Head *et al*, 1998).

Do the periplasmic and transmembrane domains of EnvZ play a role in osmosensing?

The periplasmic and/or transmembrane domains of EnvZ were previously proposed to be the putative sensor region, but results to date are not consistent with one another (Tokishita *et al*, 1991, 1992; Tabatabai and Forst, 1995). Surprisingly, although EnvZ_c has been shown to possess activities similar to its full-length counterpart (Park *et al*, 1998), it had not been reported to play a role in osmosensing. Our results show an astonishing ability of EnvZ_c to respond to osmolality *in vivo* (Figure 6). While the cellular localization was not determined, EnvZ_c protein was present in the soluble fraction and hence not bound to the inner membrane. It is possible that EnvZ_c may somehow weakly interact with the inner membrane, but our results establish that the periplasmic and transmembrane domains are not essential for osmosensing. Given that mutants have been isolated in the transmembrane regions that have effects on EnvZ activity (Tokishita and Mizuno, 1994), it would be surprising if they did not contribute somewhat to the strength of the output

response and experiments are underway to test that hypothesis. A recent study indicated that for low copy number genes in single cells, there was no correlation between transcription, translation and protein levels (Taniguchi *et al*, 2010). Perhaps, membrane anchoring of EnvZ ensures a reasonable protein copy number in cells that might otherwise be more variable, rather than playing a direct role in osmosensing. Alternatively, it may reduce a three-dimensional search for OmpR and its targets to a two-dimensional one. Our results and those of others present an emerging view of a subset of protein sensor kinases that act via cytoplasmic stimulation, rather than by transmembrane signalling (Rothenbucher *et al*, 2006; Moker *et al*, 2007).

Implications for the EnvZ phosphatase activity

The EnvZ-stimulated dephosphorylation of OmpR~P (EnvZ 'phosphatase' activity) was proposed to be the regulated step in response to osmotic stress (Jin and Inouye, 1993). Nucleotide binding (but not ATP hydrolysis) stimulated the EnvZ-dependent turnover of OmpR~P (Aiba *et al*, 1989). Our HDXMS results demonstrated that conformational changes in the ATP-binding domain were independent and uncoupled from changes in the OmpR-binding site (residues 267–278; AESINKDIEECN), making it unlikely that the phosphatase activity could be regulated by osmolality and stimulated by nucleotides (Figure 5B; Supplementary Table SI). Instead, our results are consistent with an alternative view that the *in-vivo* level of EnvZ is too low for the phosphatase activity to be significant in signalling (Mattison and Kenney, 2002; Kenney, 2010) and that the affinity of EnvZ for OmpR~P is lower than its affinity for OmpR, which would drive the reaction in the wrong direction (Mattison and Kenney, 2002; King and Kenney, 2007). Furthermore, the results in Figure 4 demonstrate a direct effect of osmolytes on EnvZ_c autophosphorylation, identifying it as the osmo-stimulated activity.

A model for osmosensing: helix-coil transitions at the autophosphorylation site

Ligand binding to the external domain of the chemoreceptor generates an internal signal that modifies the intracellular kinase activity of CheA (Falke and Hazelbauer, 2001). Multiple lines of evidence suggest that the signalling mechanism requires a piston-like movement of the signalling helix towards the cytoplasm. A similar mechanism might occur in EnvZ. However, on the basis of our deuterium exchange results, we favour a stretch-relaxation model in which the stretched state undergoes a local unfolding within the helical region and the relaxed state exhibits increased folding, leading to enhanced rates of autophosphorylation (Figure 7). This model is supported by the decreased exchange that we observed within the peptide containing the autophosphorylation site, His²⁴³ (Figures 1 and 2), accompanied by an increase in autophosphorylation (Figure 4), as well as the bimodal distribution of deuterium exchange observed for the putative OmpR-binding region (residues 267–278; AESINKDIEECN; Figure 3). It is also supported by the NMR studies that noted that residues 242–248 were poorly defined and displayed structural flexibility (Tomomori *et al*, 1999). This type of stretch-relaxation has recently been measured in the p130Cas protein, a Src family kinase substrate, where substrate activation (phosphorylation) occurs upon stretch and inhibition of phosphorylation occurs upon relaxation or

Figure 7 Stretch-relaxation model for EnvZ osmosensing and signal transduction. EnvZ exists as an ensemble of conformations that are highly dynamic. This switch is suggestive of interchanging intrahelical H-bond formation and breakage. At low osmolality, a stretched conformation with weaker intrahelical H-bonding predominates in solution (left panel). High osmolality favours a more folded conformation (right panel) through increased stabilization of intrahelical H-bonds. This conformation facilitates enhanced rates of His²⁴³ autophosphorylation and phosphotransfer to OmpR.

folding (Sawada *et al*, 2006). In that system, as we observed with EnvZ_c, local unfolding of the helix is propagated as a chemical signal (phosphorylation), leading to G-protein activation. In both of these examples, EnvZ or p130Cas proteins are poised to change from a stretched (unfolded) to a relaxed (folded) state upon the appropriate signal, either osmolality or force changes.

Mechanism for osmosensing by EnvZ

On the basis of our *in-vivo* functional assays, *in-vitro* kinase measurements, and HDXMS experiments, we show that EnvZ_c is capable of osmosensing without being located in the inner membrane of the bacterial cell. This addresses an enduring question as to how osmosensory stimuli are transmitted across the membrane. The prevailing view was that the cytoplasmic domain alone was incapable of responding to extracellular osmolality in the absence of the transmembrane segments. In addition, the observation that both NaCl and sucrose signalled through EnvZ made it unlikely that stimulation was achieved via a ligand-binding event. Our results suggest that osmotic stress is transmitted passively to the cytoplasmic domain, where a stabilizing effect on key helices responsible for autophosphorylation and phosphotransfer to OmpR is exerted. This model also explains how single amino-acid substitutions located in the His²⁴³ peptide (Verhoef *et al*, 1979; Nara *et al*, 1986) can constitutively activate or inhibit autophosphorylation independently of osmotic stress through shifts in equilibria governing local folding/unfolding of the helical segments. The model at last provides a physical basis for how EnvZ can respond to diverse chemical osmolytes such as sucrose and NaCl. Molecular dynamic simulations and *in-vivo* tests of the stretch-relaxation model are currently underway in our laboratory.

Materials and methods

Materials

All chemicals were of reagent grade or higher and obtained from Sigma-Aldrich (St Louis, MO). *E. coli* strain BL21 (DE3) was purchased from Novagen (Madison, WI), tryptone and yeast extract for LB broth were from BD Biosciences (Franklin Lakes, NJ). IPTG and ampicillin were from Bio Basic Inc. (Ontario, Canada). Sodium dodecyl sulphate (ultrapure grade) was from Sinopharm Group Company Ltd. (Shanghai, China). Tetramethylethylenediamine (TEMED, electrophoresis grade) was from MP Biomedicals (Solon, Ohio), ammonium persulfate and 30% acrylamide/*bis*-acrylamide were from Bio-Rad Laboratories (Hercules, CA) and Coomassie Blue G 250 was from US Biological (Swampscott, MA). EDTA-free complete protease inhibitor cocktail tablets were purchased from Roche Diagnostics GmbH (Mannheim, Germany). TALON[®] metal affinity resin was obtained from Clontech Laboratories (Mountain View, CA) and trifluoroacetic acid (protein sequence analysis grade) was acquired from Fluka Biochemika (Buchs, Switzerland). Sucrose (ultrapure grade) and glycerol were from 1st Base (Science Park, Singapore).

Expression and purification of EnvZ_c

Plasmid pET11a-*envZ_c* expressing *E. coli envZ_c* (R¹⁸⁰ to G⁴⁵⁰) with an N-terminal hexahistidine tag was used to transform *E. coli* BL21 (DE3) competent cells for protein overexpression (~20 copies/cell). Expression and purification of EnvZ_c was carried out using procedures as described previously (Skarphol *et al*, 1997) with some modifications. The cells were grown in LB broth (10 g tryptone, 5 g yeast extract and 10 g NaCl/l) containing a final concentration of 100 µg/ml ampicillin for 1.5–2 h with vigorous aeration at 37°C. At OD₆₀₀ ~0.5, 1 mM IPTG was added (final concentration) and the culture was incubated for a further 3 h. Induced cells were harvested at 7500 g for 15 min and stored at –20°C until purification. The frozen pellet was resuspended in ice-cold lysis buffer (20 mM Tris·HCl, pH 7.6, 10 µM EDTA, 5% glycerol (v/v)) complemented with protease inhibitor cocktail and lysed by sonication (1 s pulse every 2 s for 5 min). The lysate was centrifuged at 17 600 g for 30 min at 4°C to remove non-soluble debris. The supernatant was then incubated with TALON metal affinity resin for at least 30 min at 4°C before being placed into an empty chromatography column. The resin was washed extensively with two bed volumes of lysis buffer followed by lysis buffer containing 5 and 10 mM imidazole (twice per buffer) to remove non-specific proteins. EnvZ_c was finally eluted with lysis buffer containing 250 mM imidazole. The eluate was further purified using HiLoad 16/60 Superdex[™] 75 gel filtration column with imidazole-free 15.5 mM Tris·HCl, pH 7.6 on an ÄKTA[™] FPLC system (General Electric Healthcare, Chicago, IL). Protein purity was determined from the fractions with the highest concentration using 15% denaturing SDS–PAGE.

EnvZ_c mutagenesis

The H243A mutant of EnvZ_c was generated using inverse polymerase chain reaction (PCR) method and pET11a-*envZ_c* as the template. Site-specific mutation at H²⁴³ was introduced using 5'-ATG CCG GGG GTA AGT GCC GAC TTG CGC ACG CCG-3' as the forward primer and 5'-CGG CGT GCG CAA GTC GGC ACT TAC CCC CGC CAT-3' as the reverse primer and amplification was achieved using Phusion[®] Hot Start II DNA polymerase (Thermo Fisher Scientific, Lafayette, CO). Successful point mutation was determined using DNA sequencing and the mutant-containing plasmid was transformed into *E. coli* BL21 (DE3) competent cells. Overexpression and purification of H243A EnvZ_c protein was as described above for wild-type EnvZ_c.

EnvZ_c phosphorylation assay

EnvZ_c phosphorylation was carried out in a buffer containing 50 mM KCl and 50 mM MgCl₂ with EnvZ_c at a final concentration of 4 µM. The reactions were initiated by the addition of 2 µCi of [γ -³²P]-ATP followed by incubation at various times at room temperature. Reactions were terminated by addition of 3 µl of denaturing solution (124 mM Tris·HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 8% (v/v) β -mercaptoethanol and 0.025% (w/v) bromophenol blue) and 10 µl of the 20 µl reaction was separated by 15% SDS–PAGE. The gel was dried for 1 h and exposed to a

phosphoimager screen and visualized on a Molecular Dynamics Storm 860 imager. The osmolality of the reaction medium was altered by adjusting the salt concentration and measured using a Wescor Vapour Pressure Osmometer. Buffer conditions were A (Tris: 12.5 mM, KCl: 12.5 mM, MgCl₂: 10 mM, NaCl: 17 mM); B (Tris: 50 mM, KCl: 50 mM, MgCl₂: 20 mM, NaCl: 17 mM); C (Tris: 50 mM, KCl: 50 mM, MgCl₂: 20 mM, NaCl: 500 mM); D (Tris: 65 mM, KCl: 50 mM, MgCl₂: 20 mM, NaCl: 850 mM). Densitometry measurements were performed using Molecular Dynamics ImageQuant Software (v5.0).

β -Galactosidase assay

The *envZ_c*-encoding plasmid was transformed into *E. coli* strains MH225 and PG189. MH225 contains a chromosomal *ompC-lacZ* fusion and PG189 is the isogenic *envZ*-null strain constructed using the λ -red-mediated gene deletion method (Datsenko and Wanner, 2000), eliminating the entire *envZ* gene. The primers used for EnvZ deletion were: 5'-ATGAGGCGATTGCGCTTCTCGCCACGAAGTGTGTAGG CTGGAGCTGCTTCG-3' and 5'-TTACCTTCTTTTGTCTGCTTCGCGG GATCCGTCGACCTG-3'. The gene deletion was confirmed using PCR and DNA sequencing. β -Galactosidase assays were performed in triplicate with at least three independent cultures as described previously to monitor the transcription of *ompC-lacZ* (Mattison *et al*, 2002). Briefly, an overnight culture of bacteria was diluted 1:100 and cells were grown to mid-exponential phase (OD₆₀₀ ~0.4) in minimal A medium at varying osmolality.

Amide HDXMS

An overview of amide HDX is described in great detail in a review (Englander and Kallenbach, 1983) and HDXMS is reviewed in Hoofnagle *et al* (2003). Briefly, protein solutions in aqueous buffer are diluted in equivalent buffers prepared with deuterium oxide (D₂O) to allow backbone amides to exchange their amide hydrogens for deuterons. A plot of the exchange with time provides insights into protein dynamics and relative solvent accessibilities in different regions of the protein. Amide exchange rates are slowed by several orders of magnitude when the pH of the reaction is between 2 and 3. Localization of exchange is carried out by complete proteolytic fragmentation of the protein after deuterium exchange under quench conditions. Pepsin proteolyzes proteins at low pH (between 2 and 3) and is therefore a protease of choice in HDXMS. Every peptide has a characteristic isotopic envelope that reflects the natural abundance of ¹³C and other higher isotopes (Hotchkiss *et al*, 2006). In addition, deuterated peptides, exhibit an extended envelope that reflects both the distribution of natural isotopes and distribution of deuterium-exchanged molecules. Differences in the centroid of the deuterium-exchanged envelope from the undeuterated envelope provide an accurate measurement of the average deuterons exchanged in the peptide (plotted as a function of deuteration time). The distribution of naturally occurring isotopes within each peptide is responsible for substantial width in the spectra. Deuterated peptides reflect an overlaying of this width with that seen upon formation of deuterated species. This prevents accurate quantitation of the number of deuterons exchanging within each peptide by direct measurement of the shift in peaks. Another consideration is the extent of back exchange. Before the deuterated samples are analysed by the mass spectrometer, a small percentage of deuterons can exchange back with the aqueous mobile phase during pepsin digestion and chromatographic separation. The deuterium back exchange constant was factored into the final measurement and is described in greater detail later in this section.

EnvZ_c was concentrated using a Vivaspın ultrafiltration centrifugal device (Sartorius Stedim Biotech GmbH, Göttingen, Germany) to at least 50 µM, as measured by Bradford assay (Bradford, 1976). Filter-sterilized Tris buffer (15.5 mM Tris·HCl, pH 7.6) was the low osmolality buffer. High osmolality solutions contained 20% (w/v) sucrose or 0.5 M NaCl. Water was removed from all three solutions using a centrifugal vacuum concentrator and replaced with 99.9% D₂O prior to the experiment. Amide HDX was carried out by incubating 2 µl of protein with 18 µl of perdeuterated buffer at 20°C for various times (1, 2, 5, 10 and 30 min), yielding a final concentration of 90% D₂O. The undeuterated sample was included as a negative control. The reaction mixture was quenched by the addition of 30 µl of pre-chilled 0.1% (v/v) TFA solution, pH 2.5. The quenched sample (50 µl) was injected into a chilled nanoACQUITY

UltraPerformance LC[®] system (Waters Corporation, Milford, MA) as previously described (Wales *et al*, 2008). In the UPLC system, the sample was trapped and digested with a 2.1 × 30 mm² Poroszyme[®] immobilized pepsin column (Applied Biosystems, Foster City, CA) supplied with 0.05% (v/v) formic acid in water at a flow rate of 100 µl/min. Peptides were eluted using an organic solvent gradient of 8–40% (v/v) acetonitrile in 0.1% (v/v) formic acid at 40 µl/min and resolved with a 1.0 × 100 mm² ACQUITY UPLC BEH C¹⁸ reversed-phase column (Waters Corporation, Milford, MA). Both the immobilized pepsin cartridge and C¹⁸ reversed-phase column were housed in a refrigerated module maintained at 2°C to minimize deuterium back exchange during analysis. Peptide signals were detected and their masses were measured using a SYNAPT[®] High Definition Mass Spectrometer[™] (HDMST[™], Waters Corporation, Manchester, UK) set to MS^E data acquisition mode, which is an unbiased, non-selective collision-induced dissociation (CID) method (Bateman *et al*, 2002; Shen *et al*, 2009).

Peptides were first identified from MS^E data of undeuterated samples using ProteinLynx Global SERVER software (PLGS v2.4) (Waters Corporation, Milford, MA) (Geromanos *et al*, 2009; Li *et al*, 2009). High-fidelity identification was achieved by searching the peptides against a database containing the EnvZ_c primary sequence cleaved by non-specific proteases with a tolerance of 10 p.p.m. deviation from the theoretical mass-to-charge (*m/z*) ratios. Continuous instrument calibration was carried out with glu-fibrinopeptide B (Glu-Fib) as a standard at a flow rate of 100 fmol/µl. Peptides identified in the undeuterated samples were used to map the deuteration profiles of experimental samples using Waters HDX Browser software (Waters Corporation, Milford, MA). Peptides with non-overlapping and favourable signal-to-noise ratio spectra were identified on the software by visual inspection, and the results with these peptides for all experimental time points were extracted and subjected to quantitative analysis using HX-Express (Version Beta) software (Weis *et al*, 2006). The software generated a centroid value for the isotopic envelope of each peptide, which reflected the average mass of the peptide. The difference in the average masses of the undeuterated and experimental peptide represented the average number of deuterons exchanged. Although the N-terminus amide could also undergo HDX, the reaction was too rapid to measure and was thus excluded from the calculation (Weis *et al*, 2006). Deuterium exchange difference plots were generated using DynamX (prerelease version) (Waters Corporation, Milford, MA). For peptides with multiple (>2) overlaps, up to two of the overlaps covering the most number of residues are displayed.

The non-hydrolyzable ATP analogue, AMP-PNP, was used to determine the effect of ATP binding to EnvZ_c. MgCl₂ (final concentration: 2 mM) and AMP-PNP (final concentration: 0.2 mM) were added to the sample and allowed to react on ice for at least 30 min before proceeding with the amide HDX experiment detailed above. A total of 38 peptides were obtained and analysed, which spanned ~80% of the primary sequence of EnvZ_c (Supplementary Figure S4). The deuterium back-exchange loss during the experiment was determined by incubating apo-EnvZ_c with perdeuterated Tris buffer for 24 h at RT and was 32.7% ± 1.0%. Apo-EnvZ_c was also incubated with perdeuterated Tris buffers containing 20% (w/v) sucrose or 0.5 M NaCl for 24 h at RT to determine if these osmolytes affect deuterium back exchange and no significant changes were found. All deuterium-exchange values reported were corrected for back exchange by multiplying the raw centroid values by 1.49 (Anand *et al*, 2010).

References

- Aiba H, Mizuno T, Mizushima S (1989) Transfer of phosphoryl group between two regulatory proteins involved in osmoregulatory expression of the *ompF* and *ompC* genes in *Escherichia coli*. *J Biol Chem* **264**: 8563–8567
- Anand GS, Krishnamurthy S, Bishnoi T, Kornev A, Taylor SS, Johnson DA (2010) Cyclic AMP- and (Rp)-cAMPS-induced conformational changes in a complex of the catalytic and regulatory (RI α) subunits of cyclic AMP-dependent protein kinase. *Mol Cell Proteomics* **9**: 2225–2237
- Batchelor E, Goulian M (2003) Robustness and the cycle of phosphorylation and dephosphorylation in a two-component

Estimation of centroid values for calculation of average deuterium exchange for fragment peptides showing bimodal distribution of amide exchange

The peptide spanning residues 267–278 (AESINKDIEECN) exhibited two distinct spectral peaks characteristic of EX1 deuterium exchange kinetics (Figure 3A). This bimodal distribution is indicative of local unfolding of this particular peptide detectable within the timescale of the experiment. The maximum deuterons incorporated for this peptide fragment can be estimated by calculating the centroid values for each modelled spectral peak (Gertsman *et al*, 2010). To obtain the centroid for each of the two resolvable isotopic envelopes, the spectrum was fit to the sums of two Gaussian distributions using GraphPad Prism (v5.01) (San Diego, CA) and shown in Figure 3E. The centroid values for envelope I was estimated to be significantly lower than envelope II (>4 deuterons).

Amide HDXMS data analysis and display

Deuterium exchange in EnvZ_c under varying osmolyte or AMP-PNP concentrations were calculated using HX-Express (Version Beta) (Weis *et al*, 2006) and Waters HDX Browser (Waters Corporation, Milford, MA). Results were summarized via mirror and difference plots, which allow for a protein-wide overview of changes that occur at the peptide level in a protein of interest (Houde *et al*, 2011). Differences in deuterium exchange were plotted using DynamX (prerelease version) (Waters Corporation, Milford, MA). This plot displays the difference in deuterium exchange for all pepsin digest fragment peptides at all time points of deuterium exchange, listed sequentially from N- to C-terminus (residue numbers and their corresponding domains are shown on the x-axis) under alternate conditions of interest. In our study, these were used to summarize the effects of osmolality (NaCl and sucrose) and nucleotide on EnvZ_c. The maximum theoretical exchangeable amides exclude the N-terminus amino group and any proline residue (Hoofnagle *et al*, 2003). Differences in deuterium exchange >0.5 Da were considered significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

pET11a-envZ_c was a kind gift from Dr Masayori Inouye, University of Medicine and Dentistry of New Jersey. This study was supported by the Mechanobiology Institute, National University of Singapore, a grant from the VA 1101BX000372 and NIH GM-058746 to LJK and a grant from Waters Corporation to GSA. We thank Michael P Sheetz, Pascal Hersen, GV Shivashankar and Srinath Krishnamurthy for discussions and critical reading of the manuscript, and Srinath Krishnamurthy for technical support. Chun Xi Wong, Mechanobiology Institute, NUS provided Figure 7.

Author contributions: Anand and Kenney wrote the manuscript, conceived and analysed the data. Morgan, Godakumbura and Wang performed and analysed the data. Wang also wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

- regulatory system. *Proc Natl Acad Sci USA* **100**: 691–696
- Bateman RH, Carruthers R, Hoyes JB, Jones C, Langridge JI, Millar A, Vissers JP (2002) A novel precursor ion discovery method on a hybrid quadrupole orthogonal acceleration time-of-flight (Q-TOF) mass spectrometer for studying protein phosphorylation. *J Am Soc Mass Spectrom* **13**: 792–803
- Bernardini ML, Fontaine A, Sansonetti PJ (1990) The two-component regulatory system *ompR-envZ* controls the virulence of *Shigella flexneri*. *J Bacteriol* **172**: 6274–6281

- Bolen DW, Rose GD (2008) Structure and energetics of the hydrogen-bonded backbone in protein folding. *Annu Rev Biochem* **77**: 339–362
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254
- Brzostek K, Raczowska A, Zasada A (2003) The osmotic regulator OmpR is involved in the response of *Yersinia enterocolitica* O:9 to environmental stresses and survival within macrophages. *FEMS Microbiol Lett* **228**: 265–271
- Casadaban MJ (1976) Transposition and fusion of the lac genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J Mol Biol* **104**: 541–555
- Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97**: 6640–6645
- Delgado J, Forst S, Harlocker S, Inouye M (1993) Identification of a phosphorylation site and functional analysis of conserved aspartic acid residues of OmpR, a transcriptional activator for ompF and ompC in *Escherichia coli*. *Mol Microbiol* **10**: 1037–1047
- Dorman CJ, Chatfield S, Higgins CF, Hayward C, Dougan G (1989) Characterization of porin and ompR mutants of a virulent strain of *Salmonella typhimurium*: ompR mutants are attenuated in vivo. *Infect Immun* **57**: 2136–2140
- Englander SW, Kallenbach NR (1983) Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Q Rev Biophys* **16**: 521–655
- Falke JJ, Hazelbauer GL (2001) Transmembrane signaling in bacterial chemoreceptors. *Trends Biochem Sci* **26**: 257–265
- Feng X, Oropeza R, Kenney LJ (2003) Dual regulation by phospho-OmpR of ssrA/B gene expression in *Salmonella* pathogenicity island 2. *Mol Microbiol* **48**: 1131–1143
- Forst S, Comeau D, Norioka S, Inouye M (1987) Localization and membrane topology of EnvZ, a protein involved in osmoregulation of OmpF and OmpC in *Escherichia coli*. *J Biol Chem* **262**: 16433–16438
- Geromanos SJ, Vissers JP, Silva JC, Dorschel CA, Li GZ, Gorenstein MV, Bateman RH, Langridge JI (2009) The detection, correlation, and comparison of peptide precursor and product ions from data independent LC-MS with data dependant LC-MS/MS. *Proteomics* **9**: 1683–1695
- Gertsman I, Fu CY, Huang R, Komives EA, Johnson JE (2010) Critical salt bridges guide capsid assembly, stability, and maturation behavior in bacteriophage HK97. *Mol Cell Proteomics* **9**: 1752–1763
- Hall MN, Silhavy TJ (1979) Transcriptional regulation of *Escherichia coli* K-12 major outer membrane protein 1b. *J Bacteriol* **140**: 342–350
- Head CG, Tardy A, Kenney LJ (1998) Relative binding affinities of OmpR and OmpR-phosphate at the ompF and ompC regulatory sites. *J Mol Biol* **281**: 857–870
- Hoofnagle AN, Resing KA, Ahn NG (2003) Protein analysis by hydrogen exchange mass spectrometry. *Annu Rev Biophys Biomol Struct* **32**: 1–25
- Hotchkiss M, Anand GS, Komives EA, TenEyck L (2006) Automated extraction of backbone deuteration levels from amide H/2H mass spectrometry experiments. *Prot Sci* **15**(3): 583–601
- Houde D, Berkowitz SA, Engen JR (2011) The utility of hydrogen/deuterium exchange mass spectrometry in biopharmaceutical comparability studies. *J Pharm Sci* **100**: 2071–2086
- Igo MM, Silhavy TJ (1988) EnvZ, a transmembrane environmental sensor of *Escherichia coli* K-12, is phosphorylated in vitro. *J Bacteriol* **170**: 5971–5973
- Jin T, Inouye M (1993) Ligand binding to the receptor domain regulates the ratio of kinase to phosphatase activities of the signaling domain of the hybrid *Escherichia coli* transmembrane receptor, Taz1. *J Mol Biol* **232**: 484–492
- Kaltashov IA, Eyles SJ (2005) *Mass Spectrometry in Biophysics: Conformation and Dynamics of Biomolecules*. Hoboken, NJ: John Wiley and Sons, Inc.
- Kenney LJ (1997) Kinase activity of EnvZ, an osmoregulatory signal transducing protein of *Escherichia coli*. *Arch Biochem Biophys* **346**: 303–311
- Kenney LJ (2010) How important is the phosphatase activity of sensor kinases? *Curr Opin Microbiol* **13**: 168–176
- King ST, Kenney LJ (2007) Application of fluorescence resonance energy transfer to examine EnvZ/OmpR interactions. *Methods Enzymol* **422**: 352–360
- Lee AK, Detweiler CS, Falkow S (2000) OmpR regulates the two-component system SsrA-ssrB in *Salmonella* pathogenicity island 2. *J Bacteriol* **182**: 771–781
- Li GZ, Vissers JP, Silva JC, Golick D, Gorenstein MV, Geromanos SJ (2009) Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. *Proteomics* **9**: 1696–1719
- Mandell JG, Falick AM, Komives EA (1998) Identification of protein-protein interfaces by decreased amide proton solvent accessibility. *Proc Natl Acad Sci USA* **95**: 14705–14710
- Mathis RR, Brown OR (1976) ATP concentration in *Escherichia coli* during oxygen toxicity. *Biochim Biophys Acta* **440**: 723–732
- Mattison K, Kenney LJ (2002) Phosphorylation alters the interaction of the response regulator OmpR with its sensor kinase EnvZ. *J Biol Chem* **277**: 11143–11148
- Mattison K, Oropeza R, Byers N, Kenney LJ (2002) A phosphorylation site mutant of OmpR reveals different binding conformations at ompF and ompC. *J Mol Biol* **315**: 497–511
- Moker N, Reihlen P, Kramer R, Morbach S (2007) Osmosensing properties of the histidine protein kinase MtrB from *Corynebacterium glutamicum*. *J Biol Chem* **282**: 27666–27677
- Nara F, Matsuyama S, Mizuno T, Mizushima S (1986) Molecular analysis of mutant ompR genes exhibiting different phenotypes as to osmoregulation of the ompF and ompC genes of *Escherichia coli*. *Mol Gen Genet* **202**: 194–199
- Oshima T, Aiba H, Masuda Y, Kanaya S, Sugiura M, Wanner BL, Mori H, Mizuno T (2002) Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. *Mol Microbiol* **46**: 281–291
- Park H, Saha SK, Inouye M (1998) Two-domain reconstitution of a functional protein histidine kinase. *Proc Natl Acad Sci USA* **95**: 6728–6732
- Pickard D, Li J, Roberts M, Maskell D, Hone D, Levine M, Dougan G, Chatfield S (1994) Characterization of defined ompR mutants of *Salmonella typhi*: ompR is involved in the regulation of Vi polysaccharide expression. *Infect Immun* **62**: 3984–3993
- Roberts DL, Bennett DW, Forst SA (1994) Identification of the site of phosphorylation on the osmosensor, EnvZ, of *Escherichia coli*. *J Biol Chem* **269**: 8728–8733
- Rothenbucher MC, Facey SJ, Kiefer D, Kossmann M, Kuhn A (2006) The cytoplasmic C-terminal domain of the *Escherichia coli* KdpD protein functions as a K⁺ sensor. *J Bacteriol* **188**: 1950–1958
- Sawada Y, Tamada M, Dubin-Thaler BJ, Cherniavskaya O, Sakai R, Tanaka S, Sheetz MP (2006) Force sensing by mechanical extension of the Src family kinase substrate p130Cas. *Cell* **127**: 1015–1026
- Scholtz JM, York EJ, Stewart JM, Baldwin RL (1991) A neutral, water-soluble, alpha-helical peptide: the effect of ionic strength on the helix-coil equilibrium. *J Am Chem Soc* **113**: 5102–5104
- Shen Z, Li P, Ni RJ, Ritchie M, Yang CP, Liu GF, Ma W, Liu GJ, Ma L, Li SJ, Wei ZG, Wang HX, Wang BC (2009) Label-free quantitative proteomics analysis of etiolated maize seedling leaves during greening. *Mol Cell Proteomics* **8**: 2443–2460
- Skarphol K, Waukau J, Forst SA (1997) Role of His243 in the phosphatase activity of EnvZ in *Escherichia coli*. *J Bacteriol* **179**: 1413–1416
- Skerker JM, Perchuk BS, Siryaporn A, Lubin EA, Ashenberg O, Goulian M, Laub MT (2008) Rewiring the specificity of two-component signal transduction systems. *Cell* **133**: 1043–1054
- Slauch JM, Garrett S, Jackson DE, Silhavy TJ (1988) EnvZ functions through OmpR to control porin gene expression in *Escherichia coli* K-12. *J Bacteriol* **170**: 439–441
- Stanley CB, Strey HH (2008) Osmotically induced helix-coil transition in poly(glutamic acid). *Biophys J* **94**: 4427–4434
- Szurmant H, Hoch JA (2010) Interaction fidelity in two-component signaling. *Curr Opin Microbiol* **13**: 190–197
- Tabatabai N, Forst S (1995) Molecular analysis of the two-component genes, ompR and envZ, in the symbiotic bacterium *Xenorhabdus nematophilus*. *Mol Microbiol* **17**: 643–652
- Tanaka T, Saha SK, Tomomori C, Ishima R, Liu D, Tong KI, Park H, Dutta R, Qin L, Swindells MB, Yamazaki T, Ono AM, Kainosho M,

- Inouye M, Ikura M (1998) NMR structure of the histidine kinase domain of the E. coli osmosensor EnvZ. *Nature* **396**: 88–92
- Taniguchi Y, Choi PJ, Li GW, Chen H, Babu M, Hearn J, Emili A, Xie XS (2010) Quantifying E. coli proteome and transcriptome with single-molecule sensitivity in single cells. *Science* **329**: 533–538
- Tokishita S, Kojima A, Aiba H, Mizuno T (1991) Transmembrane signal transduction and osmoregulation in Escherichia coli. Functional importance of the periplasmic domain of the membrane-located protein kinase, EnvZ. *J Biol Chem* **266**: 6780–6785
- Tokishita S, Kojima A, Mizuno T (1992) Transmembrane signal transduction and osmoregulation in Escherichia coli: functional importance of the transmembrane regions of membrane-located protein kinase, EnvZ. *J Biochem* **111**: 707–713
- Tokishita S, Mizuno T (1994) Transmembrane signal transduction by the Escherichia coli osmotic sensor, EnvZ: intermolecular complementation of transmembrane signalling. *Mol Microbiol* **13**: 435–444
- Tomomori C, Tanaka T, Dutta R, Park H, Saha SK, Zhu Y, Ishima R, Liu D, Tong KI, Kurokawa H, Qian H, Inouye M, Ikura M (1999) Solution structure of the homodimeric core domain of Escherichia coli histidine kinase EnvZ. *Nat Struct Biol* **6**: 729–734
- van Alphen W, Lugtenberg B (1977) Influence of osmolarity of the growth medium on the outer membrane protein pattern of Escherichia coli. *J Bacteriol* **131**: 623–630
- Verhoef C, Lugtenberg B, van Boxtel R, de Graaff P, Verheij H (1979) Genetics and biochemistry of the peptidoglycan-associated proteins b and c of Escherichia coli K12. *Mol Gen Genet* **169**: 137–146
- Vidal O, Longin R, Prigent-Combaret C, Dorel C, Hooreman M, Lejeune P (1998) Isolation of an Escherichia coli K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new ompR allele that increases curli expression. *J Bacteriol* **180**: 2442–2449
- Wales TE, Fadgen KE, Gerhardt GC, Engen JR (2008) High-speed and high-resolution UPLC separation at zero degrees Celsius. *Anal Chem* **80**: 6815–6820
- Weis DD, Engen JR, Kass IJ (2006) Semi-automated data processing of hydrogen exchange mass spectra using HX-Express. *J Am Soc Mass Spectrom* **17**: 1700–1703