Phosphorylation and dephosphorylation of a bacterial transcriptional activator by a transmembrane receptor

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Signal transduction in the bacterial Omp, Che, and Ntr systems involves the phosphorylation and dephosphorylation of response regulators (OmpR, CheY and CheB, NRr) that share a homologous domain. We show that in the Omp system, the transmembrane sensor EnvZ, catalyzes both the phosphorylation of OmpR and the dephosphorylation of OmpR-P. The phosphorylation reaction proceeds by a mechanism shared with the Ntr and Che kinases, NRn, and CheA. EnvZ can phosphorylate NRr and can stimulate transcription from the glnA promoter, and similarly, CheA can phosphorylate OmpR and can stimulate transcription from the ompF promoter. OmpR-P formed by either CheA or EnvZ is much more stable than CheY-P and NRr-P, but is rapidly hydrolyzed to OmpR and Pj by EnvZ in the presence of ATP, ADP, or nonhydrolyzable analogs of ATP. Because EnvZ is normally a transmembrane receptor with a periplasmic sensory domain, our results suggest that the role of EnvZ may be to control the intracellular concentration of OmpR-P in response to environmental signals.

[Key Words: Autophosphorylation; protein kinase; phosphatase; membrane receptor; signal transduction]

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Expression of the two major outer membrane porins of Escherichia coli K-12 fluctuates in response to changes in medium osmolarity. At low osmolarity, the porin encoded by ompF is expressed preferentially, whereas at high osmolarity, the porin encoded by ompC is expressed preferentially [van Alphen and Lugtenberg 1977; Kawaji et al. 1979]. This reciprocal regulation of porin expression requires the products of the ompR locus, which consists of the ompR and envZ genes [Sarma and Reeves 1977; Hall and Silhavy 1981]. OmpR is a DNA-binding protein [Norioka et al. 1986; Maeda and Mizuno 1988; Mizuno et al. 1988] that is required for transcription of both ompF and ompC. The ability of OmpR to activate transcription is controlled by the envZ product [Hall and Silhavy 1981; Garrett et al. 1983; Slauch et al. 1988]. EnvZ is an integral cytoplasmic membrane protein believed to consist of a periplasmic sensory domain and a cytoplasmic domain involved in signal transduction [Liljestrom 1986; Forst et al. 1987; Mizuno and Mizushima 1987; Igo and Silhavy 1988]. Genetic analysis indicates that EnvZ is involved both in the activation of transcription from the ompF promoter by OmpR at low osmolarity, and in the repression of this transcription and the activation of transcription from the ompC promoter by OmpR at high osmolarity [Slauch and Silhavy 1989].

The membrane location and very low cellular concentration of EnvZ have hampered efforts to obtain purified material for biochemical analysis. It has been demonstrated recently that a mutant EnvZ that lacks one of the putative membrane-spanning segments, EnvZ115, stimulates transcription from the osmotically regulated ompF and ompC promoters in vivo, but does not respond to alterations in osmolarity [Igo and Silhavy 1988]. EnvZ115 does not localize to the membrane. This protein has been purified and shown to be a kinase that catalyzes the phosphorylation of OmpR [Igo et al. 1989]. Similar results have been obtained with other truncated EnvZ proteins [Aiba et al. 1989a; Forst et al. 1989]. Highly purified EnvZ115 greatly increases the ability of OmpR to activate transcription from the ompF promoter when these proteins are allowed to interact in the presence of ATP, suggesting that EnvZ115 stimulates this transcription indirectly by phosphorylating OmpR [Igo et al. 1989].

On the basis of DNA sequence analysis, EnvZ and OmpR are members of two families of bacterial signal transduction proteins [Stock et al. 1985; Nixon et al. 1986]. Previous studies have shown that the EnvZ homologs involved in nitrogen regulation [NRr (NtrC)] and chemotaxis [CheA] use ATP to autophosphorylate at histidine residues [Hess et al. 1987; Keener and Kustu 1988; Ninfa et al. 1988; Stock et al. 1988b; Weiss and Magasanik 1988; Wylie et al. 1988]. Then the phosphoryl group is transferred from the histidine in the EnvZ homologs to one or more aspartyl side chains in the corresponding OmpR homologs, NRr (NtrC) in the nitrogen system and CheY in the chemotaxis system. Phosphorylated NRr (NRr-P) binds to an upstream en-

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hancer element and activates transcription of the glnALG operon from the nitrogen regulated glnAp2 promoter (Ninfa and Magasanik 1986, Ninfa et al. 1987, Weglenski et al. 1989) and phosphorylated (CheY-P) interacts with flagellar motor components to cause tumbling swimming behavior (Macnab 1987, Ninfa et al. 1988).

It has been demonstrated by use of purified components that the kinases NRn and CheA have cross specificity. CheA can catalyze the phosphorylation of NRn and thus bring about the activation of transcription from glnAp2 in vitro; NRn can catalyze the phosphorylation of CheY and act in place of CheA to cause tumbling swimming behavior [Ninfa et al. 1988]. In both of these cases, the phosphorylation occurs more slowly and requires higher protein concentrations than does the phosphorylation of the consonant response regulator protein. In this report, we demonstrate using purified components that the Omp signal transducers EnvZ and OmpR can cross-react with components of the Ntr and Che signal transduction systems: EnvZ115 can catalyze the phosphorylation of NRj and thus stimulate transcription from the glnAp2 promoter, and CheA can catalyze the phosphorylation of OmpR and stimulate the activation of transcription from the ompF promoter.

Results

Protein phosphorylation in the Omp, Che, and Ntr signal transduction systems occurs by a common mechanism

We performed two experiments that indicated that components of the Omp regulon, EnvZ and OmpR, and those of the Ntr and Che systems exhibit cross specificity. First, we demonstrated that the Omp protein kinase EnvZ115 can phosphorylate the Ntr transcription factor NRj and can stimulate the activation of transcription from the glnAp2 promoter. Second, we demonstrated that the kinase of the Che system, CheA, can phosphorylate OmpR and can stimulate the activation of transcription from the ompF promoter.

EnvZ115 can catalyze the phosphorylation of NRj and thereby activate transcription from the glnAp2 promoter

Purified EnvZ115 was autophosphorylated by incubation with [γ-32P]ATP [Fig. 1, lane 1]. Addition of NRj to this reaction mixture resulted in the phosphorylation of NRj [Fig. 1, lanes 2–6]. NRj is not phosphorylated if EnvZ115 is omitted from the reaction mixture. Although EnvZ115 can phosphorylate NRj, even at the relatively high NRj concentration used, the accumulation of NRj-P was slow and the steady-state level of EnvZ115 phosphorylation was not decreased appreciably.

EnvZ115 increased the ability of NRj to activate transcription from the glnAp2 promoter. In the absence of EnvZ115, a few transcription complexes were initiated at glnAp2 when the supercoiled template [10 nM] was incubated with excess core RNA polymerase, sigma S4, NRj, and the nucleotide triphosphates ATP, CTP, and GTP [Fig. 2, lane 2]. Addition of EnvZ115 [7.8 μM] resulted in a large increase in the amount of transcription initiated at glnAp2 [Fig. 2, lane 3]. EnvZ115 did not stimulate transcription from glnAp2 in the absence of NRj [Fig. 2, lane 4]. These results indicate that EnvZ115 acts in concert with NRj to stimulate transcription from glnAp2 and suggest that the kinases EnvZ115 and NRj act by a common mechanism.

CheA can catalyze the phosphorylation of OmpR and activate transcription from the ompF promoter

Purified CheA was autophosphorylated by incubation with [γ-32P]ATP [Fig. 3, lane 1]. Addition of OmpR to this reaction mixture resulted in the phosphorylation of OmpR. As shown, the phosphorylation of OmpR occurred in a roughly linear fashion for at least 10 min [Fig. 3, lanes 2–4, 9–11]. OmpR was not phosphorylated when CheA was not added to the reaction mixture. The phosphorylation of OmpR by CheA did not diminish noticeably the degree of phosphorylation of CheA [Fig. 3, lane 1–4, 9–11], which suggests that the rate of phosphotransfer from CheA-P to Omp is slower than the rate of CheA autophosphorylation.

CheA increased the ability of OmpR to drive transcription from the ompF promoter [Fig. 4]. In this assay,
Phosphorylation and dephosphorylation

at high concentration in the presence of ATP greatly facilitated the OmpR-dependent stimulation of transcription by CheA. These results indicate that CheA acts through OmpR to stimulate transcription from the ompF promoter, and this suggests that the phosphorylation of OmpR and the chemotaxis proteins CheY and CheB occurs by a common mechanism.

**OmpR-P is more stable than CheY-P and NRj-P**

We directly compared the stability of OmpR-P, CheY-P, and NRj-P formed by phosphotransfer from CheA-P (Fig. 5). For this experiment, CheA was incubated in the presence of [γ-32P]ATP and then excess ATP was removed from the CheA-P by gel filtration. As shown in Figure 5, purified CheA-P was dephosphorylated rapidly by CheY with the intermediate formation of the unstable CheY-P and was similarly dephosphorylated by NRj at a slower rate, with the formation of the unstable NRj-P, as described previously [Hess et al. 1988b; Ninfa et al. 1988; Wylie et al. 1988]. CheA-P was also rapidly dephosphorylated by OmpR, but the OmpR-P formed was much more stable than NRj-P and CheY-P. Even though CheA-P was almost entirely dephosphorylated by OmpR within 10 min, a significant amount of the Omp-P that had been formed remained after 1 hr.

OmpR-P made by wild-type EnvZ was of similar stability. In the experiment shown in Figure 6, OmpR-P was formed by phosphotransfer from immobilized EnvZ-P as described in Materials and methods. The amount of the soluble OmpR-P that remained after removal of the EnvZ was examined as a function of time. As shown, much of the OmpR-P remained after a 1-hr incubation and some OmpR-P could still be detected after 4 hr [Fig. 6]. From these data, the half-time for hydrolysis appears to be ~1.5 hr, which is similar to the values obtained for NRj-P or CheY-P under denaturing conditions [Stock et al. 1988a; Weiss and Magasanik 1988].

We examined the effect of stopping the phosphorylation reaction on the persistence of OmpR-P. To stop the phosphorylation of OmpR by CheA, we added CheB to reaction mixtures in which the phosphorylation of OmpR by CheA had been allowed to proceed for 5 min [Fig. 3, lanes 5–8]. Because the rate of phosphotransfer...
from CheA to CheB is much more rapid than transfer to OmpR (Hess et al. 1988b; Stock et al. 1988b), such an addition effectively prevented the further incorporation of phosphate into OmpR by causing the rapid dephosphorylation of CheA-P. The addition of CheB had little effect, however, on the amount of OmpR-P present (Fig. 3, lanes 5–8). When the gel shown in Figure 3 was subjected to autoradiography for a longer period of time, we were able to observe a small amount of the highly unstable CheB-P in those reactions where CheB was present (data not shown). This result indicates further that under non-denaturing conditions, OmpR-P made by CheA was considerably more stable than CheB-P, CheY-P, and NR-P and suggests that under these conditions the kinase reaction is not readily reversible.

**EnvZ115 catalyzes the dephosphorylation of OmpR-P**

EnvZ controls ompF and ompC expression by communication with OmpR and it seems likely that at least part of this communication involves the regulation of the levels of OmpR-P. The stability of OmpR-P is surprising, because without additional input, this regulation would be cumbersome and the response time to environmental changes would require several generations. Indeed, phosphatase activity has been observed in reactions that contain OmpR, EnvZ, and ATP (Aiba et al. 1989a). However, it is not clear from these observations if EnvZ is required. Because we found OmpR-P to be quite stable, we examined whether purified EnvZ115 catalyzed the dephosphorylation of OmpR-P.

We incubated OmpR with CheA-P for 30 min to allow the formation of OmpR-P (Fig. 7, lanes 2–5). Then the stability of OmpR-P was examined after the addition of EnvZ115, ATP, and the combination of ATP and EnvZ115. As shown, neither EnvZ115 (Fig. 7, lanes 6–8) nor ATP (Fig. 7, lanes 9–11) when added alone had any discernible effect on the stability of OmpR-P, but the addition of both ATP and EnvZ115 resulted in the rapid dephosphorylation of OmpR-P (Fig. 7, 12–14). This result suggested that the dephosphorylation of OmpR-P by EnvZ115 required ATP. Further analysis of the dephosphorylation reaction indicated that ADP and the ATP analogs ATP-γ-S, AMP-PNP, and AMP-PCP also stimulated the dephosphorylation of OmpR-P by EnvZ115.

OmpR-P formed by OmpR-P phosphotransfer from wild-type immobilized EnvZ-P was purified by removal of the EnvZ as described in Materials and methods. The stability of the OmpR-P remaining after removal of the EnvZ was examined in the presence of EnvZ115 and a variety of different nucleotides. EnvZ115 caused the rapid dephosphorylation of OmpR-P when ATP, ADP, or any of several ATP analogs were present. Figure 8 shows the results of an experiment using EnvZ115 and the ATP analog AMP-PCP. Neither EnvZ115 nor AMP-PCP alone had any effect on the stability of OmpR-P (Fig. 8A, lanes 1 and 3), but the combination of EnvZ115 and AMP-PCP caused the rapid dephosphorylation of OmpR-P (Fig. 8A, lanes 2). Analysis of the OmpR-P dephosphorylation reaction by thin-layer chromatography identified the product of that reaction as P$_i$ (Fig. 8B, lane 2). These findings indicate that EnvZ115 is an OmpR-P phosphatase and that this activity is activated by adenine nucleotides. Our experiments with ATP analogs suggest that the nucleotide is not hydrolyzed in the process.

**Discussion**

*Bacterial signal transduction components cross-react with one another*

We demonstrated that the purified signal transduction components of the Omp regulon EnvZ and OmpR display cross specificity (crosstalk) with signal transduction components of the Che and Ntr signal transduction systems. This finding indicates that a common phosphotransfer mechanism is used in all three of these signal transduction systems. Previous results with the Che and Ntr systems established the basic chemistry of
autophosphorylation at a histidine residue in the kinase component followed by transfer of the phosphophoryl group to one or more aspartyl groups in the response regulators (Hess et al. 1988a; Keener and Kustu 1988; Stock et al. 1988b; Weiss and Magasanik 1988). The sensitivity of EnvZ-P to acids (Igo and Silhavy 1988) and the sensitivity of OmpR-P to hydroxylamine (unpubl.) suggest that a similar chemistry is involved in the Omp system.

**OmpR-P lacks the autophosphatase activity found in CheY-P, CheB-P, and NRj-P**

The phosphorylated response regulators of the Che and Ntr signal transduction systems are unstable in native conformation (t1/2 of hydrolysis at neutral pH ranging from 15 sec to 4 min) (Hess et al. 1988b; Keener and Kustu 1988; Weiss and Magasanik 1988; Wylie et al. 1988). When denatured with SDS or urea, the stability of the phosphophoryl group in these proteins is increased considerably (t1/2 over 1 hr), approximating the chemical stability of a typical acyl phosphate (Stock et al. 1988b; Weiss and Magasanik 1988). These observations raise the possibility that phosphorylated response regulators can possess potent autophosphatase activities. We found that OmpR-P is relatively stable with a half-life of 1–2 hr and thus lacks the potent autophosphatase activity. There is a large qualitative difference in the stability of OmpR-P and the other phosphorylated response regulators. Examination of the OmpR sequence indicates no striking differences that would explain clearly the relative stability of its phosphorylated form compared to NRj-P, CheY-P, and CheB-P.

**EnvZ is a protein kinase and a phosphoprotein phosphatase**

EnvZ is a protein kinase that, in the presence of ATP or nonhydrolyzable analogs of ATP, catalyzes the dephosphorylation of the phosphorylated regulator OmpR-P. This finding, coupled to analogous results with the Ntr system (Ninfa and Magasanik 1986; Keener and Kustu 1988), indicates that the kinase components are generally bifunctional enzymes that function both as kinases and phosphatases to regulate the levels of phosphorylated response regulators. In the Ntr system, both NRj and the auxiliary signal transduction protein P450 are required for the dephosphorylation of NRj-P. Our results with highly purified EnvZ115 indicate that this protein has phosphatase activity. Because EnvZ115 contains the highly conserved carboxyl-terminal domain and because it functions in the cytoplasm, we suspect that this conserved domain contains both kinase and phosphatase activities, and by extension, that this activity resides in NRj and will be found in other homologous bacterial information processors. CheA, which along with the closely related FrzE protein constitutes a structurally distinct subclass of the homologous information processors (for review, see Stock et al. 1989b), may be anomalous in lacking this activity. On the other hand, a similar phosphatase activity by CheA would be exceedingly hard to detect because of the great instability of the phosphorylated Che response regulators.

The kinase/phosphatase activities associated with EnvZ have several important implications concerning the mechanism of porin regulation. First, our results suggest that both activities can function simultaneously so that there is the potential for constant futile cycling of OmpR phosphorylation and dephosphorylation at the expense of ATP. If this is the case, and both the kinase and phosphatase reactions are first order in EnvZ, then steady-state levels of OmpR-P should be independent of EnvZ concentration. They should depend only on the relative values of the first-order rate constants for the phosphorylation and dephosphorylation reaction. Because the level of EnvZ should directly affect only the rate of futile cycling, it makes sense that EnvZ is pro-
regulating the level of OmpR-P. We would predict that the level of OmpR-P should increase in proportion to the net concentration of OmpR present; (lane 2) 2 min after addition of OmpR; (lane 3) 10 min; (lane 4) 30 min; (lane 5) 45 min. After the 30-min time point (lane 4), aliquots from the phosphotransfer reaction mixture were removed and combined with an equal volume of a solution that contained ATP (0.4 mM) + EnvZ11S (0.5 μM) (lanes 6-8, 11, 14). Phosphorylation was assayed by removing samples after 1 min (lanes 6, 9, and 12), 3 min (lanes 7, 10, and 13), and 10 min (lanes 8, 11, and 14). Samples that contained an equal amount of CheA were then subjected to electrophoresis followed by autoradiography.

Figure 6. Time course of OmpR-P dephosphorylation. OmpR-P, purified as described in Materials and methods, was incubated with 10 mM MgCl₂ for 1 min at 37°C. Then samples were removed at the indicated times into denaturing sample buffer and subjected to electrophoresis followed by autoradiography. (Lane 1) 5 min; (lane 2) 30 min; (lane 3) 1 hr; (lane 4) 2 hr; (lane 5) 4 hr; (lane 6) 6 hr.

Produced at very low levels within the cell. On the other hand, we would predict that the level of OmpR-P should increase in proportion to the net concentration of OmpR. This could explain why an increase in the level of OmpR (from multicopy plasmid expression vectors) has such dramatic effects on porin regulation (Liljestrom et al. 1982; Gibson et al. 1987; Slauch et al. 1988).

EnvZ acts to control the expression of porin genes by regulating the level of OmpR-P.

Our results indicate that EnvZ functions to control the level of OmpR phosphorylation. Because CheA, which does not normally function to regulate transcription from ompF, can act in place of EnvZ, it is clear that OmpR phosphorylation is sufficient for transcriptional activation at ompF. EnvZ is a 450-amino-acid, transmembrane protein with a 115-residue amino-terminal periplasmic domain coupled by a 17-amino-acid membrane-spanning sequence to a 271-residue cytoplasmic domain [Forst and Inouye 1988]. Virtually the entire cytoplasmic region exhibits homology to the soluble kinase/phosphatase, NRpD, as well as to the cytoplasmic domain of numerous other membrane receptor proteins that control gene expression in bacteria (Nixon et al. 1986; Stock et al. 1988). It seems likely that the periplasmic domain of EnvZ controls the activity of the kinase/phosphatase activity of the cytoplasmic domain in response to environmental signals and that the regulation of OmpR-P is the sole function of EnvZ in the regulation of porin synthesis.

The expression of both ompF and ompC requires EnvZ and, accordingly, it would appear that the phosphorylation of OmpR is required to stimulate transcription at both promoters. Indeed, it has been reported that OmpR-P binds both promoters with higher affinity than does OmpR [Aiba et al. 1989b]. Genetic studies indicate that the differential expression of ompF and ompC observed in media of different osmolarities is accomplished by two functionally distinct states: one that activates ompC and a second that both activates ompC and inhibits ompF [Slauch and Silhavy 1989]. The most likely explanation for these different states is that they represent differences in the levels of OmpR phosphorylation with the second form being more highly phosphorylated than the first. Whether this reflects simply a quantitative difference in OmpR-P concentration or a qualitative difference that results from multiple sites of phosphorylation remains to be determined.

Results obtained with purified components from the Omp, Ntr, and Che systems demonstrate considerable cross specificity. This raises intriguing questions of global control in response to environmental stimuli. The physiological significance of crosstalk in wild-type (ompR⁺ envZ⁺) cells remains to be demonstrated. However, a number of factors, in addition to media osmolarity, influence the levels of OmpF and OmpC. These include temperature, growth phase, and the composition of the growth medium [Forst and Inouye 1988; Csonka 1989]. It is possible that at least some of these factors influence porin expression by altering levels of OmpR-P through crosstalk from other histidine kinases that respond to other types of environmental signals.
Figure 8. OmpR-P made by EnvZ can be dephosphorylated by EnvZl15, releasing Pj. OmpR-P formed by phosphotransfer from EnvZ and purified as described in Materials and methods was incubated for 10 min at 37°C and then aliquots were removed and combined with an equal volume of solution containing 0.5 μM EnvZl15 [lane 1]; 0.5 μM EnvZl15 and 0.4 mM AMP-PCP [lane 3]; or buffer alone [lane 4]. Samples that contained an equal amount of OmpR were removed after 10 min into SDS (final concentration 0.2%) and subjected to electrophoresis [A] or thin-layer chromatography [B] followed by autoradiography. The position of OmpR is indicated in A. (The band at the bottom of lane 2 in A is Pj, which runs at the dye front.) The positions of Pj (lane 5) and ATP (lane 6) are indicated in B.

Functional analogies between EnvZ and other signal transducers

The Omp signal transduction system differs from the Ntr and Che systems in that both sensing and signaling activities are consolidated into a single protein, EnvZ. This protein has the transmembrane topology and sensing functions analogous to those found in the chemotaxis receptors, the protein kinase activity analogous to that found in NRn and CheA, and the phosphoprotein phosphatase activity analogous to that found in CheZ and the combination of NRn and Pn.

EnvZ is similar in many respects to eukaryotic regulatory proteins such as the epidermal growth factor [EGF] receptor. Both are composed of an intracellular kinase domain separated by membrane-spanning hydrophobic sequences from an amino-terminal sensory region that controls kinase activity, and the kinase domains of both are autophosphorylated. In response to the appropriate environmental stimulus, both receptors send a signal by phosphorylation that results in altered patterns of gene expression. Moreover, dominant structural gene mutations are known in each case (envZ473 and etrB) that cause wide ranging changes in gene expression (Hall and Silhavy 1981; Yarden and Ullrich 1988). There are, of course, important differences; most notably, the EGF receptor is a tyrosine kinase and EnvZ is a histidine kinase. It is intriguing that antibodies directed against phosphotyrosine generally cross-react with phosphohistidine, as if the two modifications share some structural similarity (Frackelton et al. 1983).] Also, phosphohistidine appears to participate in the phosphotransfer reaction directly, whereas phosphotyrosine does not. Nonetheless, detailed understanding of signal transduction in bacteria may provide insights to the function of analogous regulatory processes in vertebrate species.

Materials and methods

Purified proteins and nucleotides

Previously described preparations of OmpR (Igo et al. 1989), NRn (Ninfa et al. 1989), sigma 54 (Ninfa et al. 1989), and core RNA polymerase (Ninfa et al. 1989) were used. NRn2302 was purified as described by Hunt and Magasanik (1985) through the hydroxylapatite step, followed by gel filtration on BioRad A 0.5 M (Bio-Rad) and by chromatography on DEAE-Sephacel. Insoluble EnvZ115 was prepared by sucrose gradient centrifugation of aggregated EnvZ115, and then EnvZ115 was solubilized with deoxycholate [DOC] as described previously (Igo et al. 1989). Freshly prepared DOC-solubilized EnvZ115 was used for all experiments except the glnAp2 transcription assay; in that experiment, insoluble EnvZ115 was used, because DOC was somewhat inhibitory to the transcription assay. CheA (Stock et al. 1988a), CheY (Stock et al. 1989a), and CheB (Simms et al. 1985) were purified essentially as described previously and were provided by E. Ninfa and A. Stock. Sigma 70 was kindly provided by Carol Gross. Each of these proteins was at least 90% pure. In the phosphatase assays (experiments shown in Figs. 7 and 8), ATP, ADP, and the ATP analogs ATP-γ-S, AMP-PNP, and AMP-PCP were obtained from Boehringer–Mannheim.
Igo et al.

**The phosphorylation of NRj by EnvZ115**

EnvZ115 (0.5 μM) and 0.4 mM [γ-32P]ATP were incubated in transcription buffer [final concentrations: 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl2, 0.1 mM EDTA, 1.0 mM dithiothreitol (DTT)]. After 5 min, an aliquot was removed into denaturing sample buffer [124 mM Tris-HCl (pH 6.8), 4% SDS, 8% vol/vol 2-mercaptoethanol, 20% vol/vol glycerol], to the remainder of the reaction mixture NRj was added [final concentration 4.5 μM] and the incubation was continued at 37°C. Samples were removed into denaturing sample buffer at the indicated times. Samples containing an equal amount of EnvZ115 then were subjected to electrophoresis on an SDS–7.5% acrylamide gel and to autoradiography.

**Stimulation of glnAp2 transcription by EnvZ115**

This assay was performed as described previously [Ninfa and Magasanik 1986, Ninfa et al. 1989]. All reaction mixtures contained the template [pTH8, 10 nM], core RNA polymerase [0.1 μM], sigma 54 (0.1 μM), and the nucleotides ATP, CTP, and GTP [0.4 mM each]. NRj [90 nM], NRj [360 nM], and insoluble EnvZ115 [7.8 μM] were present where indicated. The transcription assay mixtures were incubated for 15 min, after which heparin and labeled UTP were added, and the incubation was continued for an additional 10 min to produce full-length transcripts from initiated ternary complexes [Ninfa et al. 1989]. The transcripts were recovered, subjected to electrophoresis on denaturing urea–acrylamide gels, and detected by autoradiography as described previously [Ninfa et al. 1989].

**The phosphorylation of OmpR by CheA**

The experiment shown in Figure 3 was performed as follows: CheA [final concentration, 5 μM] was incubated with 0.4 mM [γ-32P]ATP. After 5 min, a small sample was removed and added to denaturing sample buffer, to the remainder of the sample, a mixture of OmpR and concentrated buffer was added such that the buffer remained constant. Thus, in the reaction, OmpR was present at 20 μM, CheA was present at 4.4 μM, and ATP was present at 0.35 mM. Samples were removed into denaturing sample buffer after 1, 5, and 10 min, after which the sample was split into two halves. CheA was added to a final concentration of 5 μM to one-half of the reaction mixture (this addition resulted in a 7% dilution of all other components in the reaction mixture) and samples were removed after 1, 2, 5, and 10 min into denaturing sample buffer. To the other half of the reaction mixture, H2O was added to effect a 7% dilution and samples were removed after 1.5, 2.5, and 5.5 min into denaturing sample buffer. Samples that contained an equal amount of CheA were then subjected to electrophoresis on an SDS–10% acrylamide protein gel and to autoradiography.

**Stimulation of ompF transcription by CheA**

This assay was performed as described previously [Igo et al. 1989] with the modification described below. All reactions contained template [pIP23, 10 nM], core RNA polymerase [0.1 μM], and sigma 70 [0.1 μM]. In the experiment shown in Figure 4A, all components were added directly to transcription reaction mixtures without prior preincubation as described previously [Igo et al. 1989]. For the experiment shown in Figure 4B, OmpR (17 μM) was preincubated with ATP (0.4 mM) or with ATP (0.4 mM) and CheA (18 μM) in the same buffer used in the transcription assay. After 20 min at 37°C, various dilutions of these preincubation mixtures were added to transcription reactions, which then were incubated further for 20 min at 37°C. Then a mixture that contained heparin and labeled UTP was added to allow production of full-length transcripts from the initiated ternary complexes. The transcripts were recovered, subjected to electrophoresis, and detected by autoradiography.

**Phosphotransfer from CheA-P to OmpR, NRj, and CheY**

CheA-P was purified by gel filtration of autophosphorylated CheA as described previously [Wylie et al. 1988]. The final preparations were about 1 μM CheA-P in 0.1 mM KP buffer [pH 7.0]. For the phosphotransfer experiments shown in Figure 5, MgCl2 (10 mM) was added to autophosphorylated CheA and the mixture was incubated for 1 min at 37°C. Additional proteins were added. Samples were removed at the indicated times into denaturing sample buffer and subjected to electrophoresis on SDS–acylamide gels followed by autoradiography. In the experiment shown in Figure 5A, CheA-P and CheY were each present at 0.2 μM and the samples were separated on an SDS–15% acrylamide minigel [Bio-Rad Laboratories mini protein II]. In the experiment shown in Figure 5B, CheA-P was present at 0.5 μM, NRj was present at 5 μM, and the samples were separated on a standard-length SDS–10% acrylamide gel. In the experiment shown in Figure 5C, CheA-P was present at 0.5 μM, OmpR was present at 42 μM, and the samples were separated on a standard-length SDS–12% acrylamide gel. The experiments shown in Figure 5, A–C were performed using the same CheA-P preparation. In the experiment shown in Figure 7, a second CheA-P preparation was used.

**Phosphotransfer from immobilized EnvZ115 to OmpR**

Wild-type EnvZ was precipitated with antisera and autophosphorylated by incubation with [γ-32P]ATP as described previously [Igo et al. 1989], except the ATP concentration was 0.1 mM. Then autophosphorylated EnvZ in the immune complex was washed repeatedly with STE [10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 1 mM EDTA]; a total of 20 ml of STE buffer was used in this washing step. The autophosphorylated EnvZ in the immune complex, largely free of ATP, was washed two times in 1 ml of TMK buffer [10 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 50 mM KCl] and resuspended in TMK buffer. OmpR [13.3 μM] was added, and the reaction mixture was incubated at 37°C for 15 min, after which the immune complex that contained EnvZ was removed by centrifugation. The supernatant solution, which contained OmpR, then was incubated at 37°C. Samples were removed at the indicated times into denaturing sample buffer and subjected to electrophoresis followed by autoradiography.

**Phosphatase assay**

OmpR-P was generated by phosphotransfer from gel filtration purified CheA-P for 30 min as shown in Figure 7, lanes 1–5. Aliquots of this reaction mixture were mixed with an equal volume of a solution that contained either EnvZ115, nucleotide, EnvZ115 plus nucleotide, or H2O. As a result of these additions, the OmpR concentration was 20.5 μM, the CheA concentration was 0.25 μM, the buffer concentration was 0.25 mM KP, [pH 7.0], the MgCl2 concentration was 5 mM, and where indicated, the EnvZ115 concentration was ~0.5 μM, and the nucleotide concentration was 0.4 mM. Samples were removed into denaturing sample buffer at the indicated times after the addition and subjected to electrophoresis followed by autoradiography.

To use OmpR-P that was made by phosphotransfer from wild-type EnvZ as the substrate in phosphatase assays, we pre-
pared an immune complex that contained wild-type EnvZ, auto-
tophosphorylated this protein, washed out the ATP, and trans-
ferred the phosphoryl group to OmpR as described above. After removing the immobilized EnvZ by centrifugation, the super-
natant solution that contained OmpR-P was subjected to gel
filtration on a small G-50 column equilibrated in TMK buffer.
This gel filtration step took ~10 min. The OmpR-P obtained
then was incubated with EnvZ115 [0.5 μm], ATP or analogs [0.4
mm], or the combination of EnvZ115 and ATP or analogs. After
a 10-min incubation at 37°C, samples that contained an equal
amount of OmpR were removed into SDS (final concentration
0.2%). Aliquots (10 μl) of these denatured samples then were
subjected to electrophoresis followed by autoradiography and 1
D.

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