OmpR and EnvZ comprise a two-component system that regulates the porin genes ompF and ompC in response to changes in osmolarity. EnvZ is autophosphorylated by intracellular ATP on a histidine residue, and it transfers the phosphoryl group to an aspartic acid residue of OmpR. EnvZ can also dephosphorylate phospho-OmpR (OmpR-P) to control the cellular level of OmpR-P. At low osmolarity, OmpR-P levels are low because of either low EnvZ kinase or high EnvZ phosphatase activities. At high osmolarity, OmpR-P is elevated. It has been proposed that EnvZ phosphatase is the activity that is regulated by osmolarity. OmpR is a two-domain response regulator; phosphorylation of OmpR increases its affinity for DNA, and DNA binding stimulates phosphorylation. The step that is affected by DNA depends upon the phosphodonor employed. In the present work, we have used fluorescence anisotropy and phosphotransfer assays to examine OmpR interactions with EnvZ. Our results indicate that phosphorylation greatly reduces the affinity of OmpR for the kinase, whereas DNA does not affect their interaction. The results presented cast serious doubts on the role of the EnvZ phosphatase in response to signaling in vivo.

The predominant paradigm for signal transduction in prokaryotes is the two-component regulatory system (see Ref. 1 for reviews and references). The first component is a sensor kinase, often a membrane protein, which senses the appropriate environmental signal and is phosphorylated from intracellular ATP on a histidine residue. The sensor phosphokinase then transfers the phosphoryl group to an aspartic acid residue of the second component, the response regulator. Phosphorylation of the response regulator generally leads to its activation, often by increasing its affinity for DNA with a subsequent effect on transcription. In a postulated second level of regulation, it has been suggested that the sensor kinase can also stimulate dephosphorylation of the phosphorylated response regulator via a phosphatase activity, thus limiting the level of the activated regulator and resetting the system.

The two-component regulatory system that governs expression of the outer membrane porins OmpF and OmpC consists of the sensor kinase EnvZ and the response regulator OmpR. Activation of EnvZ by an unknown signal, related to the osmolality of the growth medium, leads to phosphorylation of OmpR at aspartate 55 (2–4). Phosphorylation of OmpR results in an increased affinity for the regulatory regions upstream from the ompF and ompC genes (5–8). Recent in vitro studies demonstrated that the corollary is also true, i.e. the presence of DNA increases the level of OmpR phosphorylation (9, 10). As a result of these studies, we proposed that OmpR exists as an equilibrium mixture of four distinct states (see Fig. 1): unphosphorylated OmpR (A), phosphorylated OmpR (B), unphosphorylated OmpR bound to DNA with low affinity (C), and phosphorylated OmpR bound to DNA with high affinity (D). A similar model has been proposed for the single-domain response regulator CheY, where the switch protein FltM substitutes for DNA (11, 12).

Interestingly, the reaction step that is most affected by the presence of DNA depends upon the phosphodonor employed. When phosphorylating with the small molecule phosphodonor, acetyl phosphate, DNA binding dramatically stimulates the rate of phosphorylation with little effect on the dephosphorylation rate of OmpR-P.1 Estimates of initial rates indicate that phosphorylation by acetyl phosphate is at least 25-fold faster in the presence of DNA than in its absence (i.e. C → D is much faster than A → B, Fig. 1 and Ref. 9). Furthermore, DNA binding slows dephosphorylation about 2-fold (D → C is slightly slower than B → A, Fig. 1 and Ref. 9). In contrast, when phosphorylating with the phosphokinase (EnvZ-P), the step most affected by DNA binding is the rate of EnvZ-stimulated OmpR-P dephosphorylation (i.e. D → C is much slower than B → A, Fig. 1 and Ref. 10). In either case, the overall effect of DNA is to increase the net rate of OmpR-P formation on the order of 50-fold.

The EnvZ kinase has the following enzymatic activities: (a) EnvZ + ATP → EnvZ-P + ADP (autophosphorylation); (b) EnvZ-P + OmpR → EnvZ + OmpR-P (phosphotransfer); and (c) EnvZ + OmpR-P → EnvZ + OmpR + P, (phosphatase). As a result, by controlling either the phosphotransfer activity or the phosphatase activity, EnvZ can modulate the level of OmpR-P in vivo. A current model for osmoregulation proposes that at high osmolarity, the level of OmpR-P increases because of a reduction in the dephosphorylation rate catalyzed by the kinase EnvZ (13). Unphosphorylated OmpR does not play a role in porin gene expression, because envZ deletion strains are effectively OmpF−OmpC− (14). Results from the EnvZ/OmpR system have been extended to other two-component regulatory systems, leading to the conclusion that phosphatase activity of the kinase EnvZ is the step regulated or altered by signal input (15–19).

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1 The abbreviations used are: OmpR-P, phosphorylated OmpR; EnvZ-P, phosphorylated EnvZ; EnvZc, cytoplasmic domain of EnvZ; HPLC, high pressure liquid chromatography.
Phosphorylation Affects OmpR/EnvZ Interactions

Phosphorylation Decreases the Affinity of OmpR for EnvZ—We wanted to determine whether or not phosphorylation of OmpR altered its interaction with EnvZ. For these experiments, OmpR was labeled with fluorescein at the amino terminus, and the labeled protein was separated from the free label. EnvZc was then added incrementally. Representative binding curves are shown in Fig. 2. For the curves shown in Fig. 2A, fluorescein-labeled OmpR was phosphorylated with phosphoramidate. In this particular experiment, the affinity for EnvZc binding to OmpRc is 589 nM (triangles). The average $K_d$ for EnvZc binding to OmpRc from 10 independent experiments is $425 \pm 127$ nM. EnvZc binds to OmpRc-P with such low affinity that its $K_d$ is beyond the detection limit of the assay (circles). We were consistently unable to detect saturable binding of EnvZc to OmpR-P. Thus, phosphorylation has a dramatic effect on OmpRc/EnvZc interactions, decreasing the affinity of OmpR for EnvZc by at least 10-fold.

It has recently been suggested that OmpRc-P/DNA and OmpRc-P-EnvZc interactions are mutually exclusive (10). We set out to examine this proposal directly, i.e. to determine whether DNA binding by OmpRc or OmpRc-P altered its affinity for the EnvZ kinase. In Fig. 2B, the dissociation constant ($K_d$) for EnvZc binding to OmpRc-P is 519 nM (filled triangles); for EnvZc binding to OmpRc in the presence of ompc DNA (C1-C2-C3) the $K_d$ is 568 nM (open triangles). The average $K_d$ for EnvZc binding to OmpRc in the presence of ompc DNA from six separate curves is $385 \pm 162$ nM. The dissociation constants are the same, within the error margin of the assay. The presence of ompc DNA did not alter the binding of EnvZc to OmpRc (Fig. 2B) or to OmpRc-P (Fig. 2A, compare closed circles with open circles). Thus, although phosphorylation has a dramatic effect on the affinity of OmpRc for EnvZc (Fig. 2A), the presence of specific DNA does not affect the ability of EnvZc-P to bind to OmpRc (Fig. 2B). This result is in conflict with the recently proposed role for DNA in the OmpR/OmpRc-EnvZ equilibrium mediated by EnvZc (10).

The Role of DNA in Phosphorylation—We have been studying the native linker in OmpR and its role in OmpRc/DNA and OmpRc/EnvZc interactions. We engineered and expressed a mutant in which the native Q linker (25) is replaced with a linker of equal length but substituted with glycine and lysine residues (hereafter referred to as GGK). The GGK mutant is able to activate expression of ompF but not ompC (OmpF+ OmpC−). GGK is also able to protect the ompF and ompC regulatory regions in a DNase I footprinting assay; a complete characterization of the mutant is described elsewhere. In the present work, we first examined the steady-state phosphorylation of

**EXPERIMENTAL PROCEDURES**

**Protein Purification—**OmpR was expressed and purified as previously described (6, 21). GGK was constructed by ligating synthetic oligonucleotides into engineered restriction sites in OmpR, resulting in the replacement of endogenous amino acids 124–137 of the linker region with Gly-Gly-Lys-Gly-Gly-Lys-Gly-Gly-Lys-Gly-Gly-Lys-Gly-Gly. The protein was expressed as described (6) and purified on a HiTrap heparin column (Amersham Biosciences, Inc.). The construct encoding the soluble carboxyl-terminal fragment of EnvZ (EnvZc) was a mutant in which the native Q linker (25) is replaced with a linker region with Gly-Gly-Lys-Gly-Gly-Lys-Gly-Gly-Lys-Gly-Gly-Lys-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-G...
Phosphorylation Affects OmpR/EnvZ Interactions

In the presence of specific DNA, EnvZ binds OmpR with a K_d of 568 nM (Fig. 3). Unphosphorylated OmpR runs as a single peak on C4 reversed phase HPLC (Fig. 3). When DNA is included in the reaction, OmpR-P levels increase nearly 2-fold compared to DNA-free reactions (Fig. 3). GGK phosphorylates and responds to DNA binding has the opposite effect from wild type OmpR on OmpR binding to EnvZc (Fig. 3). The average apparent dissociation constant for EnvZc binding to OmpR from 10 independent experiments is 425 ± 127 nM. Closed circles indicate that OmpR-P binds EnvZc with only very low affinity. This binding is unchanged in the presence of C1-C2-C3 DNA (open circles). A saturable binding curve for EnvZc binding to OmpR-P was never obtained using this approach (n = 4). B, OmpR binds to EnvZc with a K_d of 519 nM (closed triangles). The presence of specific DNA, EnvZc still binds OmpR, with a K_d of 568 nM (open triangles). The average K_d for EnvZc binding to OmpR + C1-C2-C3 from six separate curves is 589 ± 162 nM.

GGK by the small molecule phosphodonor, acetyl phosphate (Fig. 3). Unphosphorylated OmpR runs as a single peak on C4 reversed phase HPLC (Fig. 3A). Acetyl phosphate phosphorylates OmpR at aspartate 55, and an additional peak that corresponds to OmpR-P appears (Fig. 3B and Refs. 6, 9). As reported previously, when a 2-fold molar excess of specific DNA is included in the reaction, OmpR-P levels increase nearly 2-fold (Fig. 3C and Ref. 9). The GGK mutant also elutes as a single peak on C4 reversed phase HPLC (Fig. 3D), and acetyl phosphate is similarly able to phosphorylate GGK (Fig. 3F). The addition of DNA also increases the steady-state level of phosphorylated GGK (Fig. 3F). Thus, when acetyl phosphate is used as a phosphodonor, GGK phosphorylates and responds to DNA similarly to wild type OmpR.

The Effect of DNA on GGK Phosphorylation by EnvZ—We next determined the effect of DNA on the phosphorylation of GGK by the EnvZ kinase. EnvZ is autophosphorylated upon incubation with [γ-32P]ATP (Fig. 4, A and B, lane 1). When OmpR is added to the reaction, the phosphoryl group is transferred from EnvZ-P to OmpR, and OmpR-P is formed. OmpR-P breaks down, and a residual OmpR-P decays slowly between 20 and 120 min (Fig. 4A, lanes 2-4). Similarly, incubation of GGK with EnvZ-P results in the formation of GGK-P (Fig. 4B, lanes 2-4). When OmpR is added to EnvZ-P in the presence of either ompF DNA (Fig. 4A, lanes 5-7) or ompC DNA (Fig. 4A, lanes 8-10), the level of residual OmpR-P is increased compared with no addition of DNA. However, when GGK is added to the reaction in the presence of ompF or ompC DNA, a decrease in the amount of GGK-P is observed (Fig. 4B, lanes 5-10). This behavior is in striking contrast to that observed with wild type OmpR (Fig. 4A, lanes 5-10). Even though the interaction of GGK with DNA appears to be similar to wild type OmpR in stimulating phosphorylation from acetyl phosphate (Fig. 3), the interaction of GGK-P with the kinase EnvZ is dramatically altered in the presence of DNA. The decrease in the amount of GGK-P upon incubation with EnvZ-P and DNA (Fig. 4) indicates either that the kinase is less able to phosphorylate DNA-bound GGK (C → D is slow, Fig. 1) or that EnvZ rapidly dephosphorylates DNA-bound GGK-P (D → C is fast, Fig. 1).

The GGK Mutant Displays Increased Turnover in the Presence of EnvZ and DNA—To distinguish between these two possibilities, we measured P_i release from GGK-P in the presence and absence of DNA and compared it to wild type OmpR (Fig. 5). If the kinase is less able to phosphorylate GGK in the presence of DNA, then P_i production should be low. If the kinase rapidly dephosphorylates DNA-bound GGK and rephosphorylation occurs, then a high level of P_i should be released. In the presence of EnvZ and ATP, wild type OmpR is phosphorylated and dephosphorylated, and the net result is P_i release at a rate of 7.1 nmol/ml/min (closed triangles). The presence of specific DNA decreases the P_i produced by wild type OmpR to a rate of 0.4 nmol/ml/min (open triangles). In contrast, the mutant GGK phosphoprotein turns over very slowly, and P_i production is low (closed circles). Because GGK is being phosphorylated (Fig. 4) under these conditions, dephosphorylation of GGK-P must be inhibited. Surprisingly, the addition of DNA greatly enhances P_i production by the mutant protein (open circles). This stimulated rate in turnover of GGK-P in the presence of DNA approaches that observed with wild type OmpR in the absence of DNA (compare closed triangles with open circles). The rate of P_i production by GGK increases from 0.3 nmol/ml/min to 4.7 nmol/ml/min upon addition of DNA. DNA binding has the opposite effect from wild type OmpR on GGK-P dephosphorylation by EnvZ. For GGK, the D → C transition is faster than the B → A transition, whereas for OmpR the D → C transition is much slower than the B → A transition (Fig. 1). Furthermore, GGK-P and EnvZ must interact when GGK-P is bound to DNA because this mutant shows an increase in the rate of EnvZ-stimulated dephosphorylation upon DNA binding (Fig. 5, open circles). Our interpretation is that the presence of the altered linker sequence prevents the B → A transition (Fig. 1) required for spontaneous dephosphorylation. When the mutant protein is bound to DNA, GGK-P can transition to an EnvZ-bound form if EnvZ is present, stimulating dephosphorylation of the protein. While this result is obtained with an OmpR mutant, it emphasizes the point that OmpR binding to DNA does not remove it from solution and prevent interaction with EnvZ, as previously proposed (10).

DISCUSSION

In the present work, we have demonstrated that phosphorylation of OmpR reduces its affinity by at least 10-fold for the kinase EnvZ. The average apparent dissociation constant for...
EnvZc binding to OmpR is 425 nM. When OmpR was phospho-
rylated using phosphoramidate (generating nearly 100%
OmpR-P), we were unable to measure a K_d for EnvZc binding
because the interaction was of such low affinity. This has
important consequences for the hypothesis that the phospha-
tase activity of EnvZ plays a regulatory role and controls
OmpR-P levels in vivo (see below).

A previous study used native gel electrophoresis to examine
the effect of DNA on the interaction of OmpR/OmpR-P and
EnvZ (10). The authors concluded that OmpR-P bound to DNA
was prevented from interacting with EnvZ, and this inability to
interact prevents EnvZ from dephosphorylating OmpR-P. We
can now interpret these results in light of our present findings.
In a solution containing a mixture of OmpR and OmpR-P,
OmpR has a higher affinity for EnvZ, and OmpR-P has a higher
affinity for DNA. The K_f for OmpR binding to an ompC binding
site, C1, is 101 nM, and the K_d for OmpR-P is 8 nM (6). Thus, in

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**Fig. 3.** Phosphorylation of OmpR and GGK by acetyl phosphate. C4 reversed phase HPLC elution profiles for OmpR (A), OmpR + acetyl phosphate (B), OmpR + acetyl phosphate + C1-C2-C3 (C), GGK (D), GGK + acetyl phosphate (E), and GGK + acetyl phosphate + C1-
C2-C3 (F).

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**Fig. 4.** EnvZ15 phosphorylation of OmpR and GGK. A, lane 1 shows EnvZ after autophosphorylation with [γ-32P]ATP. Lanes 2–4 show OmpR-P produced 5, 20, and 60 min after the addition of OmpR to
the reaction. Lanes 5–7 are identical to lanes 2–4, but the reaction
contains F1-F2-F3 DNA. Lanes 8–10 are identical to lanes 2–4, but the
reaction contains C1-C2-C3 DNA. B, as for A, except the assay was
performed with GGK.

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**Fig. 5.** ATPase assay with OmpR and GGK. Closed triangles show
the P_i released after incubation of OmpR with EnvZ and ATP. Open
triangles indicate that P_i release is slowed when DNA is included in the
reaction. Closed circles indicate that GGK does not stimulate the ATPase
activity of EnvZ, and open circles show that upon addition of DNA, GGK
greatly stimulates P_i production. Reactions were performed in triplicate
and repeated three times. The symbol represents the mean, and error bars
indicate the standard deviation of three data points obtained at each time
point. The data shown are a representative experiment. The slopes re-
ported in the text are the average of the nine data points collected at each
time point from three individual experiments.
Phosphorylation Affects OmpR/EnvZ Interactions

In Vivo Relevance—The fundamental question arising from this work is whether or not there is an in vivo role for the stimulation of OmpR-P breakdown by EnvZ that is observed in vitro. The results presented here suggest that the affinity of EnvZ for OmpR-P is too low to play a role in vivo in dephosphorylating OmpR-P. They are most consistent with a model in which the autophosphorylation of EnvZ or the phosphotransfer to OmpR (or both) increases with increasing osmolarity. This view is further supported by a recent study that reported elevated EnvZ autokinase activity at high potassium concentrations, with no change in the phosphotransfer or phosphatase activities (30). Spontaneous dephosphorylation of OmpR-P would then lead to OmpR-P decay. Measurements of OmpR-P turnover have reported a half-life of OmpR-P of 1–2 h, i.e. too long for spontaneous dephosphorylation to account for signal shut off (3). In other studies, about 60% of the OmpR-P was dephosphorylated within the first 10 min. Given our present findings, the kinetics of OmpR-P dephosphorylation bear careful reexamination. It is also important to emphasize that our studies are performed with an EnvZ construct that lacks the periplasmic and transmembrane domains, which could alter EnvZ/OmpR and EnvZ/OmpR-P interactions.

It has been suggested that the phosphatase activity of EnvZ is regulated in response to osmolarity, i.e. at high osmolarity the kinase activity remains constant, and the phosphatase activity decreases, increasing the cellular level of OmpR-P (13). This hypothesis is based on the results of experiments with a chimeric kinase, Taz, in which the periplasmic domain of the aspartate receptor has been fused to the carboxyl terminus of EnvZ (31). This chimeric construct only responds to high concentrations of aspartate (1–5 mM) compared with the natural aspartate receptor, which binds aspartate with a $K_d$ of 1.2 $\mu$M (32), and its effect on ompF expression has not been reported. The behavior of Taz therefore seems unlikely to accurately represent EnvZ signaling in vivo. In vitro demonstration of the phosphatase activity is beyond dispute (3, 10, 33), and mutations in EnvZ have been isolated that influence it (34–37). However, these substitutions also alter EnvZ autophosphorylation and phosphotransfer activities, and so their in vivo actions should be interpreted with caution.

The results presented here demonstrate that OmpR-P has a significantly lower affinity for the EnvZ kinase than does OmpR and suggest that alternate mechanisms must be proposed if the phosphatase activity of EnvZ controls OmpR-P levels in vivo. For example, it is possible that in order for EnvZ to bind OmpR-P, the protein must undergo a conformational change. This might be a result of interaction with RNA polymerase. However, such models that postulate extra roles for sensor kinases, beyond the environmental sensing step in vivo, involve additional assumptions and await further experimental investigation. Our present work emphasizes the need to separately characterize the partial biochemical reactions of the components in the signaling pathway in order to rationalize the mechanistic basis of the observed phenotypes.

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