K+ Stimulates Specifically the Autokinase Activity of Purified and Reconstituted EnvZ of Escherichia coli*

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The histidine kinase/response regulator system EnvZ/OmpR of Escherichia coli regulates transcription of the genes ompF and ompC, encoding two porins of the outer membrane. Although the total amount of OmpF and OmpC remains constant, the relative levels of the two proteins fluctuate in a reciprocal manner depending on medium osmolality. The membrane-anchored sensor EnvZ somehow monitors changes in environmental osmolality. To characterize the nature of the stimulus perceived by EnvZ, this protein was overproduced, purified, and reconstituted into proteoliposomes. Autokinase activity of purified and reconstituted EnvZ was stimulated by an increase of the K+ concentration. Rb+, Na+, and NH4+ also stimulated the activity but to a smaller extent, whereas an osmotic upshift imposed by various sugars or increasing concentrations of glycerol, betaine, proline, or Tris/MES were without influence. Neither the transfer of the phosphoryl group from EnvZ–P to OmpR nor the EnvZ-mediated OmpR–P dephosphorylation were affected by one of the tested solutes. Experiments with the reconstructed signal transduction cascade including DNA fragments demonstrated a substantial increase of the amount of phosphorylated OmpR in the presence of K+ and to a lower extent in the presence of Na+, Rb+, and NH4+. Various K+ salts were tested indicating that the determined effects were K+-specific and not dependent on the anion. In a further in vitro test system, which utilizes right-side-out membrane vesicles, the K+-specific activation of EnvZ autokinase from the luminal side was confirmed. These results clearly indicate a regulation of EnvZ autokinase activity by monovalent ions, specifically K+. Whether K+ accumulation, which is one of the first responses of E. coli after an osmotic upshift, is related to the stimulation of the EnvZ autokinase activity in vivo is discussed.

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This article is dedicated to Karlheinz Altendorf (Osnabrück) on the occasion of his 60th birthday.

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In contrast to other sensor processes where ligand binding is involved (e.g., nitrate binds to the sensor kinase NarX (21)), and citrate binds to the sensor kinase CitA (22)), sensing of changes in osmolality is difficult to attribute to a specific ligand. Because EnvZ responds both to polar and nonpolar solutes, it is proposed that EnvZ is activated by changes in the cytoplasmic, periplasmic, or extracellular water activity (aw) (23). However, the picture is even more complex. It is known that glycine betaine antagonizes the osmotic repression of ompF (24) and that procaine represses ompF and induces ompC at low osmolality (25, 26).

Most of the in vitro analyses for EnvZ were done with a soluble truncated form (EnvZc), because of the lack of a method for solubilization and reconstitution of EnvZ. Here we describe the influence of various solutes on the activities of purified and reconstituted full-length EnvZ. The results obtained with two different in vitro test systems reveal a stimulation of the autokinase activity of EnvZ by monovalent cations, especially K+. 

**EXPERIMENTAL PROCEDURES**

**Materials**—[^32]P-ATP was purchased from Amersham Pharmacia Biotech. Goat anti-rabbit IgG-alkaline phosphatase conjugate was obtained from Dako. Antibodies were from Qiagen, and Bio-Beads were from Bio-Rad. Detergents were from Calbiochem. Purified E. coli lipids were purchased from Avanti Polar Lipids. Synthetic oligonucleotide primers were from Life Technologies, WH56 was used as carrier for the plasmids described and for overexpression of ompR, and outside) contained 20 mM MgCl2. Autokinase activity of EnvZ in proteoliposomes (10 mg/ml) was found to be linear within the first 2 min. To obtain sufficient amounts of phosphorylated EnvZ in all experiments, the reaction was stopped after 2 min by the addition of an equal volume of 2× concentrated SDS sample buffer (39). When the osmolality outside of the RSO-MV was varied, the vesicles were incubated in lysis buffer for 1 min with ATP and centrifuged (14,000 × g, 0.5 min), and then the pellet was resuspended in the higher osmolal buffer (50 mM Tris/HCl, pH 8.0, plus osmolytes) lacking ATP and Mg2+ after 1 min of incubation, the reaction was stopped as described above.

**Preparation of Right-side-out Membrane Vesicles**—RSO-MV of E. coli strain WH56110 transformed with plasmid pR929 were prepared according to the protocol described recently (37).

**Purification of 10His-OmpR**—10His-OmpR was purified by means of Ni2+-NTA-agarose chromatography in batch. Binding of the protein (105 mg of cytosolic proteins/1 ml of Ni2+-NTA resin) was done in the presence of 10 mM imidazole in purification buffer (50 mM Tris/HCl, pH 8.0, 10% glycerol (v/v), 10 mM β-mercaptoethanol). To elute the protein, the imidazole concentration was raised to 250 mM. Subsequently, the purified protein was dialyzed against 2 liters of 50 mM Tris/HCl, pH 8.0, containing 10% glycerol (v/v), 80 mM NaCl, and 10 mM β-mercaptoethanol to remove imidazole.

**Purification and Characterization of EnvZ**—Purification of EnvZ-6His was achieved by insertion of six codons for His into the expression vector pET16b similarly as described before (32). Used for overexpression of ompR–6His was purified by means of Ni2+-NTA-agarose chromatography in batch. Binding of the protein (105 mg of cytosolic proteins/1 ml of Ni2+-NTA resin) was done in the presence of 10 mM imidazole in purification buffer (50 mM Tris/HCl, pH 8.0, 10% glycerol (v/v), 10 mM β-mercaptoethanol). To elute the protein, the imidazole concentration was raised to 250 mM. Subsequently, the purified protein was dialyzed against 2 liters of 50 mM Tris/HCl, pH 8.0, containing 10% glycerol (v/v), 80 mM NaCl, and 10 mM β-mercaptoethanol to remove imidazole.

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ous solutes as indicated. The reaction was started by the addition of 100 
µM [γ-32P]ATP (0.476 Ci/mmol) and 8 µM ADP, and samples were taken 
at the times indicated. The double-stranded C1-C2-C3 DNA fragment 
was obtained by annealing of two complementary oligonucleotides. The 
upper strand sequence (from 5’ to 3’ end) has the following composition: 
5’-GGGGTTTACATTTTGAAACATCTATAGCGATAAATGAAACATC-3’/H11032

In each case, the samples were immediately subjected to SDS-PAGE 
(39). The gels were dried, the radiolabeled proteins were detected by 
exposure of the gels to a phosphor screen, and the images were analyzed 
with a PhosphorImager system (Molecular Dynamics) using 
[32P]ATP for quantification with a PhosphorImager using [32P]ATP as stand-
ard. The molecular mass markers are shown on the left.

**RESULTS**

**Influence of the His Tag on the Expression Pattern of ompF and ompC**—To allow easy purification of EnvZ, six consecutive histidine residues were attached at the C-terminus of the protein. The functionality of this protein was comparable with the untagged EnvZ because the ompC and ompF expression pattern was comparable with that of wild-type EnvZ as tested with E. coli strains WH56 and WH57, each transformed with plasmids, pFR29 or pFR29–6His, respectively (data not shown).

**Purification of EnvZ-6His**—EnvZ-6His was purified by means of affinity chromatography. The following detergents were tested to be efficient in the solubilization of EnvZ-6His: n-octylglucoside, n-decylmaltoside, n-dodecylmaltoside, lauryldimethylamine oxide, zwitergent 3–12, and zwitergent 3–14. The autokinase activity of EnvZ-6His in these detergents was tested, and the highest activities were found with EnvZ-6His in decylmaltoside (data not shown). Therefore, for all subsequent steps of the purification, decylmaltoside was used as detergent. The highest purification results were achieved when binding of the protein to Ni2+-NTA-agarose was performed in the presence of 30 mM imidazole and 0.5 M NaCl. In a typical experiment 300 µg of EnvZ-6His was obtained from 140 mg of membrane proteins. As judged from a silver-stained gel, the purity of EnvZ-6His was greater than 95% (Fig. 1). The purified protein was either dialyzed to remove imidazole or reconstituted into E. coli phospholipids.

**Reconstitution of EnvZ-6His into Proteoliposomes**—Reconstitution was carried out using the detergent-mediated method as described (35). E. coli phospholipids were solubilized with detergent and mixed with purified EnvZ-6His, and the detergents were removed by BioBeads. Detergent-dependent solubilization of liposomes can be followed by turbidity measurements, whereby three characteristic stages can be distinguished: onset, partial, and total solubilization. Rigaud et al. (35) found that the nature of the detergent used and the stage of solubilization of the liposomes influence the activity of the reconstituted protein. The following detergents were used for the solubilization of liposomes: Triton X-100, n-octylglucoside, n-decylmaltoside, and n-dodecylmaltoside. Purified EnvZ-6His was added at different solubilization stages. The highest activities of EnvZ-6His autokinase activity were determined when partially solubilized liposomes with Triton X-100 as detergent were used (data not shown). The efficiency of EnvZ-6His reconstitution was determined to be 53%.

**Activities of Purified EnvZ-6His in Proteoliposomes and 10His-OmpR**—In the presence of [γ-32P]ATP reconstituted EnvZ was rapidly autophosphorylated. The autokinase activity of the reconstituted protein was found to be linear for 2 min (Fig. 2A). Although autokinase activity of the solubilized protein was also detectable, activities of the reconstituted protein were about four times higher (data not shown). To test phosphotransfer and phosphatase activities, the following experimental approach was chosen. EnvZ-6His was phosphorylated under standard conditions for 5 min. Subsequently, proteoliposomes were collected by ultracentrifugation, and the activities of phosphotransfer and phosphatase were determined. The results are shown in Fig. 2B. The phosphotransfer activity of EnvZ-6His was found to be about 10 times higher than the phosphatase activity.

**FIG. 1. SDS-PAGE analysis of samples from different steps of the purification of EnvZ-6His. Lane 1, everted membrane vesicles (20 µg of protein); lane 2, decylmaltoside-solubilized proteins (20 µg of protein); lane 3, purified EnvZ-6His after elution from the Ni2+-NTA-agarose with imidazole (3.2 µg of protein). The proteins were stained with silver.**
This discrepancy is on one hand due to the incomplete collection of proteoliposomes during ultracentrifugation and on the other hand due to smaller amounts of protein loaded onto the gels. Transfer of the phosphoryl group was detectable; however, the rate was slow, and the transfer was not completed within 5 min. The same setup was used to test EnvZ phosphatase activity. Thus, after 5.5 min ADP as a cofactor was added, and further samples were taken. As shown in Fig. 2B, after the addition of ADP the amounts of phosphorylated EnvZ and OmpR were rapidly declining, and the half-life of OmpR—P was determined to be 3 min. Thus, purified EnvZ-6His and 10His-OmpR catalyzed all known enzymatic activities.

The Influence of Various Solutes on the Autokinase Activity of EnvZ-6His—EnvZ autokinase activity was tested in the presence of various solutes. It was found that this activity of EnvZ-6His was significantly stimulated in the presence of monovalent ions in a concentration-dependent manner (Fig. 3). The highest activities were detectable when KCl was added. NaCl, RbCl (Fig. 3), and NH₄Cl (data not presented) had also stimulatory effects but to a lower extent. In contrast, no stimulation of the autokinase activity was found in the presence of Tris/ MES, sucrose, or trehalose, although these compounds were tested at equal osmolalities. Glycine betaine (Fig. 3) or proline (data not shown), which are accumulated in cells exposed to an osmotic upshift under certain conditions, did not influence EnvZ autokinase activity. For clarity, the results shown are representative for the whole concentration range tested for each compound, which was 25, 50, 100, 200, 300, and 500 mM in case of salts. In the case of the uncharged compounds, higher concentrations were used to achieve comparable osmolalities. For KCl and the other salts, a concentration-dependent stimulation was observed that reached the maximum at a concentration of 300 mM. Because monovalent ions, especially K⁺, might be necessary for normal functioning of EnvZ autokinase activity, we also tested the influence of increasing concentrations of sucrose or trehalose in the presence of 50 mM KCl. Although, as expected, under these conditions higher amounts of EnvZ—P were detectable, the presence of sucrose or trehalose did not further increase activity.

The Influence of Various Solutes on the Phosphotransfer and OmpR—P Phosphatase Activity—To test the influence of various solutes on the further activities of EnvZ-6His the experimental approach described above and in Fig. 2B was used. Centrifugation of proteoliposomes containing phosphorylated EnvZ allowed easy changes of buffers. Activities were tested in the presence of the following solutes: KCl, K⁺ glutamate, NaCl, RbCl, Tris/MES, glycine betaine, sucrose, and trehalose (each 0.3 m). None of them affected the transfer of the phosphoryl group to OmpR nor the dephosphorylation of OmpR—P significantly (data not shown). The results obtained were identical to those shown in Fig. 2B.

Reconstruction of the Whole Signal Transduction Cascade in Vitro—Because in whole cells a stepwise addition of components of the signal transduction cascade does not exist, our next aim was to establish the whole signal transduction cascade in vitro. Recent experiments of Inouye and co-workers (9) demonstrated a dramatic shift toward the phosphorylated form of OmpR in the presence of DNA comprising OmpR binding sites. Thus, purified EnvZ-6His in proteoliposomes and 10His-OmpR in a ratio of 1:4 were mixed with DNA comprising the C1-C2-C3 binding sites of OmpR (9), and the reaction was started by the addition of a mixture of ADP and ATP (ratio of 1:12.5) and [γ-³²P] ATP. Samples were taken after 1 and 30 min. The autoradiograph is shown in Fig. 4A. As shown before, the amount of phosphorylated OmpR increased in the presence of DNA by a factor of about 6, whereas the amount of phosphorylated EnvZ remained at a basal level in each case. This experiment was performed at a KCl concentration of 50 mM. In the next experiment the time-dependent signal transduction in the presence or absence of KCl was tested (Fig. 4, B and C). Under both conditions phosphorylated EnvZ was detectable, however, at a very low level. The amount of phosphorylated OmpR increased over time. In the presence of 50 mM KCl the initial rate was 17 times higher than in the absence of KCl. Whereas in the presence of KCl half-maximal phosphorylation of OmpR was already reached after 5 min, in the absence of KCl the amount of phosphorylated OmpR was steadily rising within the tested time range.

The Influence of Different Solutes on the EnvZ/OmpR Signal Transduction Cascade in Vitro—The next experiments were undertaken to test the influence of various solutes on the EnvZ/OmpR signal transduction cascade. To calculate initial rates, the reaction was stopped after 2.5 min. As already shown before (Fig. 4), under all conditions phosphorylated EnvZ was detectable at a basal level (Fig. 5). The presence of NaCl, KCl, RbCl, K⁺ glutamate, and NH₄Cl significantly increased the amount of phosphorylated OmpR, whereby the highest values were reached in the following order KCl > K⁺ glutamate > RbCl > NH₄Cl > NaCl (Fig. 5). Maximal stimulation was observed in the presence of KCl. The stimulatory effect of these salts was found to be concentration-dependent, and maximal values were detected at a concentration of 100 mM. The values for K⁺ glutamate were lower compared with KCl. Because the presence of K⁺ glutamate affected the resolution of proteins in
The Effect of Various Solutes on the Autokinase Activity of EnvZ in RSO-MV

To ensure that the determined effects are related to the cation and not to the chloride anion, the influence of the following K+ salts was tested: KNO$_3$, K$_2$SO$_4$, and KBr. These salts increased the level of phosphorylated EnvZ to the same extent as KCl (data not shown). The addition of KCl (50 mM) varied. At the indicated times, samples were taken, separated by SDS-PAGE, and analyzed as phosphorimages. A comprehensive study that describes ATP accessibility was described earlier (37). In the first experiment the influence of NaCl and KCl on the autokinase activity of EnvZ was tested under isoosmolar conditions. In the presence of KCl at concentrations of 300 mM and higher, a significant increase of the autokinase activity of EnvZ was observed (Fig. 6). In contrast, no stimulatory effect was observed by increasing the concentration of NaCl. In the next experiments the buffer concentration inside of the vesicles was held constant, and the osmolality was raised outside. To obtain reasonable amounts of phosphorylated EnvZ and to mimic more physiological conditions, the buffer inside of the vesicles contained 300 mM KCl. For each test, RSO-MV were loaded with radiolabeled ATP, collected by centrifugation and resuspended in buffer of increasing osmolality. The influence of the ionic solutes NaCl and KCl as well as the nonionic solutes sucrose, glucose, and sorbitol was tested. However, none of these compounds influenced EnvZ autokinase activity significantly (data not shown). Thus, a rise of the osmolality outside of the vesicles did not affect EnvZ autokinase activity.

**DISCUSSION**

Here we described a procedure for the purification and reconstruction of the whole signal transduction cascade in vitro. As shown before (9), the amount of phosphorylated OmpR was drastically increased in the presence of DNA fragments comprising the OmpR-binding site. Our studies have shown that the addition of increasing concentrations of KCl raised tremendously the initial rate of OmpR phosphorylation as well as the steady state accumulation of OmpR$^-P$. An activation of the signal transduction cascade, as detected by the increased accumulation of OmpR$^-P$, was also achieved in the presence of other monovalent cations such as Na$^+$, K$^+$, or NH$_4^+$, although to a lower extent compared with KCl. Because increasing concentrations of a Tris/MES buffer did not stimulate the signal transduction cascade, it is unlikely that changes of the ionic strength influence EnvZ activity. The data also imply that the stimulation is not due to an increase of the osmolality, because sucrose or trehalose did not show any effect. Osmotic unphosphated cells are able to take up substantial amounts of proline or glycine betaine, but none of these compounds exhibited a stimulatory effect on EnvZ autokinase activity. Recently, it was shown that chloride ions are important for the osmoregulation of the halophilic bacterium *Halobacillus halophilus* (44). To ascertain that the determined effects are due to the cation and not to the chloride anion, the influence of the following K$^+$ salts was tested: KNO$_3$, K$_2$SO$_4$, and KBr. These salts increased the level of phosphorylated EnvZ to the same extent as KCl (data not shown).

**FIG. 4.** The EnvZ/OmpR signal transduction cascade in vitro. A, influence of DNA on the EnvZ/OmpR signal transduction cascade. EnvZ (1 µM) and OmpR (4 µM) were mixed in the presence of buffer containing 50 mM KCl and 5 µM C1-C2-C3 DNA fragment when indicated. The reaction was started by the addition of 100 µM $[^{32}P]$ATP and 8 µM ADP. At the indicated times samples were taken, separated by SDS-PAGE, and analyzed as phosphorimages. B and C, time course of the EnvZ/OmpR signal transduction cascade and the influence of KCl. The experimental conditions were those described in A, except that in each experiment the C1-C2-C3 DNA fragment was present, but the addition of KCl (50 mM) varied. At the indicated times, samples were taken, separated by SDS-PAGE, and analyzed as phosphorimages (B), and quantified with a PhosphorImager using $[^{32}P]$ATP as standard (C).
not to the anion, we tested the influence of different salts. We found that the presence of KNO₃, K₂SO₄, or KBr had the same stimulatory effects as KCl. Thus, K⁺ and related monovalent ions specifically enhance the production of phosphorylated OmpR. Earlier, Mizuno and co-workers (25) characterized EnvZ in membrane vesicles, and they found also an increased accumulation of phosphorylated OmpR depending on an increase of K⁺, Na⁺, or Li⁺. They attributed this effect to a decrease of the EnvZ phosphatase activity; however, they did not measure phosphatase activity directly. Our studies of the single enzymatic activities of EnvZ revealed that K⁺ and other monovalent cations specifically stimulate the autokinase activity, whereas the rates of phosphotransfer and phosphatase activities of EnvZ are unchanged. Therefore, we conclude that accumulation of phosphorylated OmpR in the presence of K⁺ is due to an increased autokinase activity of EnvZ.

The K⁺-specific effect on EnvZ autokinase activity was confirmed when we used another in vitro test system, which is based on RSO-MV. When the K⁺ concentration in the lumen was increased, autokinase activity of EnvZ was stimulated. This effect was not observed in the presence of NaCl or when outside of the vesicles the concentration of KCl, NaCl, sucrose, glucose, or sorbitol was increased. Thus, domains of EnvZ exposed to the luminal side, which are the cytoplasmic domains, are sensitive toward K⁺.

It is known that expression of both ompF and ompC requires phosphorylated OmpR; preferential expression of one of these genes depends on the amount of phosphorylated OmpR (4). Here we demonstrated that the amount of phosphorylated OmpR–P is substantially increased by stimulating the autokinase activity of EnvZ with increasing concentrations of K⁺ ions. Uptake of K⁺ appears to be the earliest response of E. coli after an osmotic upshift (45). Therefore, it is suggested that the increased K⁺ concentration in osmotic stressed cells raises the autokinase activity of EnvZ, thereby increasing the amount of phosphorylated OmpR–P and leading to ompC expression. Earlier, Epstein and co-workers (46) had shown the interdependence of K⁺ and glutamate accumulation in cells exposed to an osmotic upshift. In accord with a stimulation of EnvZ autokinase activity by K⁺ is the finding that ompC is not induced when the osmotic upshift is done in the presence of betaine, a condition that reduces the rate and the extent of K⁺ accumulation (24). Regulation of one of the enzymatic activities of EnvZ is in agreement with the numerous EnvZ derivatives with altered enzymatic properties that lead to altered ompF/ompC expression patterns. Furthermore, the sensitivity of the cytoplasmic domains toward K⁺ fits well with EnvZ mutants with altered sensing properties, which have single amino acid replacements within the linker or the X domain, which are both cytoplasmic (14, 15). Despite these agreements, the correlation between the accumulation of K⁺ and the stimulation of EnvZ autokinase in vivo still has to be shown. In addition, in vitro studies indicated that maximal amounts of phosphorylated OmpR were already detected at physiological K⁺ concentrations. Therefore, an increase of the intracellular K⁺ concentration seems to be one primary stimulus perceived by EnvZ but not the only one. Integration host factor and DNA bending have already been shown to be involved in the transcriptional regulation of ompF (47–49).

The obtained results are clearly distinct from the activation mechanism of the osmosensor and transporter ProP of E. coli. Transport activity of purified ProP in proteoliposomes (50) or RSO-MV (42) was significantly increased upon an osmotic upshift imposed by NaCl or sucrose. In addition, the results obtained for EnvZ are different to KdpD, a sensor kinase, which responds to K⁺ limitation or an osmotic upshift imposed by salts. In the RSO-MV test system, KdpD autokinase activity was activated by an increase of the NaCl concentration and inhibited by K⁺ ions in the lumen of the vesicles (37). When the salt concentration outside of the vesicles was raised, a small but significant stimulation of autokinase activity of KdpD was observed, whereas no effects were seen for EnvZ under these conditions.

In summary, EnvZ catalyzes several reactions: its autophosphorylation, the transfer of the phosphoryl group to OmpR, and the dephosphorylation of OmpR–P. Our results reveal that the
autokinase activity of EnvZ is stimulated by monovalent cations, specifically K+ ions. The logical consequence of these results is the search for EnvZ mutants that lost the sensitivity toward K+.

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