The N-terminal Input Domain of the Sensor Kinase KdpD of *Escherichia coli* Stabilizes the Interaction between the Cognate Response Regulator KdpE and the Corresponding DNA-binding Site*

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The sensor kinase/response regulator system KdpD/KdpE of *Escherichia coli* regulates expression of the *kdpFABC* operon, which encodes the high affinity K⁺ transport system KdpFABC. The membrane-bound sensor kinase KdpD consists of an N-terminal input domain (comprising a large cytoplasmic domain and four transmembrane domains) and a cytoplasmic C-terminal transmitter domain. Here we show that the cytoplasmic N-terminal domain of KdpD (KdpD/1–395) alone supports semi-constitutive *kdpFABC* expression, which becomes dependent on the extracellular K⁺ concentration under K⁺-limiting growth conditions. However, it should be noted that the non-phosphorylatable derivative KdpD/H673Q or the absence of KdpD abolishes *kdpFABC* expression completely. KdpD/1–395 mediated *kdpFABC* expression requires the corresponding response regulator KdpE with an intact phosphorylation site. Experiments with an *Escherichia coli* mutant unable to synthesize acetyl phosphate as well as transposon mutagenesis suggest that KdpE is phosphorylated in vivo by low molecular weight phosphodonors in the absence of the full-length sensor kinase. Various biochemical approaches provide first evidence that *kdpFABC* expression mediated by KdpD/1–395 is due to a stabilizing effect of this domain on the binding of KdpE–P to its corresponding DNA-binding site. Such a stabilizing effect of a sensor kinase domain on the DNA-protein interaction of the cognate response regulator has never been observed before for any other sensor kinase. It describes a new mechanism in bacterial two-component signal transduction.

The sensor kinase/response regulator system KdpD/KdpE regulates expression of the *kdpFABC* operon encoding the high affinity K⁺ transport system KdpFABC in *Escherichia coli* (1, 2). KdpD is anchored in the cytoplasmic membrane with four transmembrane domains (TM1–TM4). It also consists of two large cytoplasmic domains as follows: a C-terminal transmitter domain, which includes the typical sequence motifs of sensor kinases, and an N-terminal domain, which is part of the input domain (Fig. 1) (3, 4). Upon stimulus perception, the dimeric sensor kinase KdpD (5) undergoes autophosphorylation, and subsequently, the phosphoryl group is transferred to the cytoplasmic response regulator KdpE. Phosphorylated KdpE exhibits increased affinity for a 23-bp sequence upstream of the canonical −35 and −10 regions of the *kdpFABC* promoter and thereby triggers *kdpFABC* expression (6). The enzymatic activities of purified KdpD and KdpE were determined in vitro (7). Furthermore, KdpD also catalyzes the dephosphorylation of KdpE–P in an ATP-dependent manner (8).

Three important regions have been identified in the input domain of KdpD. (i) A cluster of positively charged amino acid residues adjacent to TM4 is important for regulation of the kinase/phosphatase ratio (9). (ii) The four transmembrane domains are important for the correct positioning of the large cytoplasmic domains to each other (10). (iii) An ATP-binding site within the N-terminal domain is important for regulation of the phosphatase activity (8, 11).

Compared with other sensor kinases, KdpD is the only known protein that has a large N-terminal domain comprising 395 amino acids (8, 11, 12). Sequence comparison of 50 KdpD proteins of different bacteria shows that the sequence of this domain is highly conserved (Fig. 2). Short versions of KdpD have been found in cyanobacteria like *Synechocystis* sp. (13), *Anabaena* sp. L-31 (14), and in *Deinococcus radiodurans* (15), which are homologous to the N-terminal domain of KdpD of *E. coli*. It is still unclear whether these short KdpD proteins function alone or whether they interact with the transmitter domain of another sensor kinase. Such an interaction is conceivable, because it has been shown that the separately produced N-terminal domain of KdpD (KdpD/1–395) is able to complement the N-terminal truncated derivative KdpD/Δ12–395 in vivo and in vitro (11). Furthermore, a chimeric *Anabaenae. coli* KdpD protein (Anacoli KdpD) comprising the N-terminal domain of *Anabaena* KdpD and the C-terminal domain plus the four transmembrane domains of *E. coli* KdpD functionally interacts with *E. coli* KdpE and activates *kdpFABC* expression in *E. coli* (14), underlining the similarity of the N-terminal domains among different bacteria and their importance for the correct function of KdpD.

Here we demonstrate that the N-terminal domain of *E. coli* KdpD alone causes semi-constitutive expression of the *kdpFABC* operon. Furthermore, the first evidence is provided that the N-terminal domain of KdpD stabilizes the binding of KdpE–P to the DNA.

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¶ The abbreviations used are: TM, transmembrane; NTA, nitrilotriacetic acid.

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"Walker A" cytoplasmic TM 1 - TM 4 G1 G2 N H F C Input Domain Transmitter Domain Linker KdpD1-395 KdpD1-395(6His) FIG. 1. Schematic presentation of the sensor kinase KdpD. The model is based on both hydropathy plot analysis and studies with lacZ/phaA fusions (3). The boxes represent the four transmembrane domains (TM1–TM4). Sequence motifs characteristic for transmitter domains (H, N, G1, F), and (G2) of bacterial kinases are indicated in the upper part. The “Walker A” motif of the regulatory ATP-binding site within the N-terminal domain is indicated by the black arrow. In the middle, the domain organization of KdpD is shown. At the bottom, the truncated form of KdpD (KdpD1–395) is shown schematically, whereby the truncation is indicated by the dotted line.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP, [35S]Labeled protein A, [α-32P]UTP, [32P]E. coli RNA polymerase, RNA guard RNase inhibitor, and RNase-free nucleotides ATP, GTP, CTP, and UTP were purchased from Amersham Biosciences. Nα-NTA resin, Nα-NTA magnetic agarose beads, the 12-tube magnet, and the Nucleotide Removal Kit were purchased from Qiagen. Goat anti-rabbit IgG (horseradish peroxidase, goat anti-rabbit IgG) horseradish peroxidase, and SuperSignal West Femto Max-12-tube magnet, and the Nucleotide Removal Kit were purchased from Pierce. Other reagents were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids—E. coli strain JM109 [recA1 endA1 gyrA96 thi-1 relA1 Δ(lac-proAB)/Δ(lac-proAB)] was used as carrier for the plasmids described. E. coli strain RK2000 [ΔkdpFABCDEΔ trkD1 traD36 proA lacZ46 thi-1 hsdR17 supE44 relA1 Δ(lac-proAB)/Δ(lac-proAB) [F proA B-lacI lacZAM15] (16)] was used as carrier for the plasmids described. E. coli strain RH001, RH003, and RH004 [ΔkdpFABCDEΔ trkD1 traD36 proA lacZ46 thi-1 hsdR17 supE44 relA1 Δ(lac-proAB)/Δ(lac-proAB) [F] (this study)] transformed with plasmid pBD or its derivatives carrying a KdpD/1 truncation is indicated by the middle within the N-terminal domain is indicated by the black arrow.

Domains (lacZ) CAE169 (this study) transformed with plasmid pBD or its derivatives carrying a KdpD/1 truncation is indicated by the middle within the N-terminal domain is indicated by the black arrow.

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eluted by increasing the imidazole concentration to 100 mM. Before use, 10His-KdpE was dialyzed against buffer E without KCl and imidazole. Alternatively, KdpE was purified following a method described before (12).

Transposon Mutagenesis and Selection of the Mutants—For transposon mutagenesis, E. coli HAK006 was transformed with plasmids pBD3/1–395 and pNK2883. The mutagenesis was carried out in KML complex medium by incubation of the cells at room temperature for 48 h, initiating the transposition. Then the cells were plated on KML medium supplemented with 0.1% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), tetracycline (12.5 μg/ml), and chloramphenicol (34 μg/ml). The expression of the reporter gene lacZ was used as a measure for kdpFABC expression. White colonies (no kdpFABC expression) were isolated and again tested for β-galactosidase activity (28). To make sure that the mutants contain only one transposon, Pla lysates were prepared (19) and transduced in E. coli HAK006pBD3/1–395. The loss of plasmid pNK2883 was verified by a negative selection on ampicillin (100 μg/ml). Then the mutants were cured from plasmid pBD3/1–395 by negative selection on chloramphenicol.

In Vitro Transcription Experiments—In vitro transcription experiments were done similarly as described (31) in buffer TXN (40 mM Tris/Cl, pH 8.0, 50 mM KCl, 10 mM MgCl2, 10 mM dithiothreitol) in a total volume of 20 μl. To obtain phosphorylated KdpE, purified protein was incubated with 50 mM acetyl phosphate and 10 mM MgCl2 at 30 °C for 1 h. The PstI/SacI fragment from the plasmid pSM5, containing the kdpFABC promoter/operator region, was used as template. KdpE or KdpE/H9262 purified following a method described before (32) was added. After 5 min of incubation at 37 °C, the reaction was stopped after 10 min of incubation at 37 °C. The mixture was loaded on a 7 M urea, 0.1 M EDTA, 0.4% (w/v) SDS, 0.5% (w/v) bromphenol blue, 0.5% (w/v) xylene cyanol. Then samples were subjected to a 6% polyacrylamide, 7 μm urea gel in TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA). After the run, the gel was dried and exposed to a Storage Phosphor Screen. The amount of produced RNA was detected using the PhosphorImager SI (Amersham Biosciences).

Synthesis of [32P]Acetyl Phosphate and Phosphorylation of KdpE—Synthesis and determination of [32P]acetyl phosphate was carried out as described before (32). Phosphorylation of purified KdpE (0.2 mg/ml) was carried out in buffer TNaM (50 mM Tris/Cl, pH 7.5, 20 mM MgCl2, 50 mM NaCl, 0.5 M Asp) with 40 μM [32P]acetyl phosphate (6–14 μCi/mmol). The probes were incubated at 30 °C; samples were taken at indicated time points, and the reaction was stopped by addition of SDS sample buffer (29). Proteins were subjected to 12.5% SDS gels, and after the run, the gels were dried and exposed to a Storage Phosphor Screen. Phosphorylated proteins were detected and quantified using the PhosphorImager SI (Amersham Biosciences).

Electrophoretic Mobility Shift Assays—For mobility shift assays, double-stranded DNA fragments comprising the KdpE-binding site (6, 33) were used. These were obtained by annealing of two complementary oligonucleotides. The upper strand sequence (from 5′ to 3′) has the following sequence: 5′-CATTATTATACTTTTTTACACCCGCGCG-3′. After dephosphorylation of the double-stranded DNA with alkaline phosphatase (10 units), phosphorylation was carried out with polynucleotide kinase (10 units) and [γ-32P]ATP (3000 Ci/mmol) at 37 °C for 1 h. The DNA was then purified using the nucleotide removal kit. Binding assays were done with 100 nM DNA and 0–4.5 μM purified KdpE-P in TNaM buffer in a final volume of 20 μl. KdpD1/–395 (6His) (2.5 μM final concentration) in 15 μl of purification buffer (50 mM Tris/Cl, pH 7.5, 10% glycerol (v/v), 0.5 M NaCl, 10 mM β-mercaptoethanol, 0.04% n-dodecyl-β-D-maltoside), or just purification buffer was added. After incubation at 30 °C for 15 min, 3 μl of sucrose dye solution (50% (w/v) sucrose, 0.25% (w/v) bromphenol blue; 0.25% (w/v) xylene cyanol) was added, and samples were loaded onto a 5% polyacrylamide gel in TBE buffer. After the run, the gel was dried and exposed to a Storage Phosphor Screen, and the amount of KdpE-P-bound DNA was detected using the PhosphorImager SI (Amersham Biosciences).

Co-elution of KdpD/1–395 and 10His-KdpE from Ni2+–NTA-Agarose—For co-elution experiments, Ni2+–NTA magnetic agarose beads were

Fig. 2. Homology among the first 180 amino acids of KdpD proteins from 50 different bacteria. A. aciculiformis aciculiformis KdpD2 (A.a.), Anabaena sp. L-51 (A.s.), Agrobacterium tumefaciens (A.t.), Bacillus anthracis (B.a.), Burkholderia fungorum (B.fu.), Burkholderia mallei (B.m.), Campylobacter jejuni (C.j.), Caulobacter crescentus (C.c.), Clostridium acetobutylicum (C.a.), Cytophaga hutchinsonii (C.h.), D. radiodurans (D.r.), Enterococcus faecalis (E.f.), E. coli O157 (E.c.157), E. coli K12 (E.c.), Geobacter metallireducens (G.m.), Geobacter sulfurreducens (G.s.), Leptospira interrogans (L.i.), Listeria innocua (L.i.n.), Listeria monocytogenes (L.m.), Magnetospirillum magnetotacticum (M.m.), Mesorhizobium loti (M.l.), Mycobacterium avium (M.a.v.), Mycobacterium smegmatis (M.s.m.), Mycobacterium tuberculosis (M.t.b.), Myxococcus xanthus (M.x.), Nostoc punctiforme (N.p.), Nostoc sp. PCC7120 (N.s.), Pseudomonas aeruginosa (P.a.), Pseudomonas fluorescens (P.f.), Pseudomonas putida KT2440 (P.p.), Ralstonia solanacearum (R.s.), Rhodobacter sphaeroides (R.s.), Rhodopseudomonas palustris (R.p.), Rhodospirillum rubrum (R.r.), S. enterica (S.e.), Salmonella typhimurium (S.t.), Sinorhizobium meliloti (S.m.), Staphylococcus aureus (S.a.), Staphylococcus epidermidis (S.e.), Streptomyces coelicolor (S.c.), Synechocystis sp. (S.s.), Thermococcus celer (T.c.), Thiobacillus ferrooxidans (T.f.), Xanthomonas campestris (X.c.), Xanthomonas axonopodis (X.a.), Xanthomonas campestris (X.c.), and Yersinia pestis (Y.p.). Identical regions have dark shading, and regions displaying high homology have light shading. Area gaps are indicated by a (4).

The Input Domain of the Sensor Kinase KdpD

Walker A

Walker B

[51279]
used. First, magnetic beads (25 µl of suspension) were washed with 500 µl of distilled water in a 2-ml reaction tube and subsequently collected using a “12-tube magnet.” Then the beads were equilibrated with buffer E (see above), and 100 µl of cytoplasmic fraction containing 10His-KdpE was added (total protein concentration 15 mg/ml). As a control, 100 µl of cytoplasmic fraction containing 10His-OmpR (34) was added. The mixture was incubated under gentle movement at 4 °C for 30 min. In parallel, membrane vesicles containing KdpD/1–395 were solubilized with 0.2% (w/v) n-decyl-β-D-maltopyranoside by stirring on ice for 30 min and centrifuged at 4 °C at 264,000 × g in a Beckman centrifuge for 30 min. Then 1.6 ml of the supernatant (5 mg/ml total protein concentration) containing KdpD/1–395 was added to Ni²⁺-NTA-bound 10His-KdpE or 10His-OmpR, respectively, so that the final ratio of each response regulator to KdpD/1–395 was about 1:8. Samples were incubated under gentle movement at room temperature for 150 min before the magnetic beads were collected with the magnet. The beads were washed with 500 µl of buffer E containing 0.04% (w/v) n-decyl-β-D-maltopyranoside. Proteins bound to the beads were then eluted with 25 µl of buffer E containing 0.04% (w/v) n-decyl-β-D-maltopyranoside and 250 mM imidazole. Beads were removed by the magnet, and the eluted proteins were detected in an immunoblot with antibodies directed against the His tag as well as against KdpD.

Analytical Procedures—Protein was assayed by a modified Lowry method (35), using bovine serum albumin as a standard.

RESULTS

Influence of KdpD/1–395 on the Expression of kdpFABC—

The story started with a control experiment in which cells producing the N-terminal domain of KdpD (KdpD/1–395) and KdpE were tested for kdpFABC expression. Surprisingly, high kdpFABC expression levels were detected at any K⁺ concentration tested. In detail, E. coli HAK006 containing a kdpFABC promoter/operator-lacZ fusion as well as a chromosomal copy of kdpE and producing KdpD/1–395 at very low amounts (nondetectable by a Western blot) was grown in minimal medium at different K⁺ concentrations. Under all conditions high β-galactosidase activities were found in the presence of KdpD/1–395 (Fig. 3A). Under K⁺-limiting conditions (<1 mol K⁺), an additional stimulation of the kdpFABC expression was observed, which was 2–3-fold higher compared with the expression produced by wild-type KdpD (Fig. 3A). High osmolality imposed by addition of NaCl or sucrose had no further stimulating effect on the constitutive expression of kdpFABC caused by KdpD/1–395 (data not shown).

To obtain further support for these unexpected results, KdpFABC production was directly measured and quantified by Western blotting (Fig. 3B). For this we used an E. coli strain (TKV2298) that contains the intact kdpFABC operon but lacks the major K⁺ uptake system Trk. It is important to note that in a Δtrk strain the onset of kdpFABC induction is shifted to higher K⁺ concentrations (<60 mM) (36, 37). Cells containing KdpD/1–395 produced higher amounts of KdpFABC complex at any K⁺ concentration than cells producing wild-type KdpD, thereby confirming the results of the transcriptional fusion experiments. Importantly, in the absence of KdpD or in the presence of KdpD with an inactivated phosphorylation site (KdpD/H673Q), no expression of kdpFABC was observed under any condition (data not shown). Furthermore, the inactivation of the regulatory ATP-binding site within the N-terminal domain by amino acid replacements (KdpD/1–395/G37A, K38A, and T38C) had only a small effect on kdpFABC expression (10–30% reduction, data not shown). It should also be mentioned that KdpD/1–395 containing a C-terminal His tag [KdpD/1–395(6His)] produced the same expression pattern as KdpD/1–395. In summary, a truncated KdpD protein lacking the transmitter domain and the four transmembrane domains caused semi-constitutive kdpFABC expression, whereas the complete loss of KdpD or the inactivation of the phosphorylation site His-673 in full-length KdpD prevented kdpFABC expression.

Influence of KdpE and the Phosphorylation of KdpE on the Semi-constitutive kdpFABC Expression Caused by KdpD/1–395—To gain further insight into this unexpected phenomenon, we investigated whether the cognate response regulator KdpE was involved in this process. To test the influence of KdpE on the kdpFABC expression caused by KdpD/1–395, plasmids pPV2/1–395 and pPV2/1–395/D52N, encoding KdpD/1–395 and KdpE, or KdpD/1–395 and KdpE/D52N, respectively, and pBD/1–395 encoding only KdpD/1–395, were transformed in E. coli strain RH003 (ΔkdpDE, chromosomal kdpFABC promoter/operator-lacZ fusion). Strains were grown in minimal media containing different concentrations of K⁺, and kdpFABC expression was determined. Importantly, in the absence of KdpE, no expression of kdpFABC was detected (data not shown). This was also the case in the presence of KdpE with an inactivated phosphorylation site Asp-52 (KdpE/D52N) (data not shown). Also, no kdpFABC expression could be detected in the alternative test system using E. coli strain TKV2298 (intact kdpFABC operon, Δtrk) transformed with the plasmids described above in the absence of KdpE or when the KdpE phosphorylation site was inactivated (data not shown). These data clearly show that the response regulator KdpE and the phosphorylation of KdpE are required for the semi-constitutive kdpFABC expression.
expression caused by KdpD/1–395. Because KdpD/1–395 is not able to phosphorylate KdpE in vitro (data not shown), there must be an alternative phosphodonor for KdpE.

Alternative Phosphodonors for KdpE in the Presence of KdpD/1–395—There are at least three different possibilities for an alternative phosphodonor for KdpE in the absence of full-length KdpD. (i) KdpE could be phosphorylated by a low molecular weight phosphodonor like acetyl phosphate. (ii) Another sensor kinase could phosphorylate KdpE (cross-talk). (iii) KdpD/1–395 could interact with the transmitter domain of another sensor kinase. The latter possibility is supported by the fact that KdpD, separated into two independent peptides (the N-terminal domain of KdpD and the C-terminal domain including the transmitter domain), supports the idea that KdpE is phosphorylated in part by another sensor kinase. The question still remained regarding how the N-terminal domain of KdpD causes semi-constitutive kdpFABC expression. In addition, no differences were observed in the alternative test system E. coli liposomes without KdpD/1–395(6His). As a control, the experiment was performed in the presence E. coli liposomes without KdpD/1–395(6His). The presence of liposomes alone already had a stimulating effect on the in vitro transcription of kdpFA, which seems to be unspecific and has to be regarded as a positive effect of the phospholipid bilayer on the transcription apparatus as described earlier (39). Presumably, because of the high background no stimulation of transcription could be detected in the case of KdpE–P compared with KdpE when liposomes were present. In summary, these results clearly show that KdpD/1–395 has a stimulating effect on the transcription of kdpFABC in vitro.

Influence of KdpD/1–395 on the Stimulation of kdpFABC Transcription—There appear to be two different possibilities regarding how KdpD/1–395 might stimulate kdpFABC transcription: (i) KdpD/1–395 facilitates the phosphorylation of KdpE by acetyl phosphate or (ii) KdpD/1–395 stabilizes the binding of KdpE–P to the DNA. To check the first possibility, the time-dependent phosphorylation of purified KdpE by [32P]acetyl phosphate was determined in vitro in the absence and presence of purified and reconstituted KdpD/1–395(6His). As shown in Fig. 6, KdpD/1–395(6His) did not stimulate the phosphorylation of KdpE. In addition, no differences were observed regardless of whether KdpD/1–395(6His) in proteoliposomes or in solution was tested (data not shown). Therefore, these results argue against the first hypothesis.

To test the second hypothesis, we applied electrophoretic mobility shift assays. For this a double-stranded DNA fragment comprising the KdpE-binding site (6, 33) was used and incubated with increasing amounts of phosphorylated KdpE. Reconstituted KdpD/1–395(6His) could not be applied in this experiment, because the proteoliposomes caused precipitation of the samples in the pockets of the native gel. For this reason, KdpD/1–395(6His) in detergent was used in this experiment and buffer containing detergent as a control. The detergent was
necessary because KdpD/1–395(6His) precipitates in buffer without detergent. As shown in Fig. 7, the amount of shifted DNA is significantly increased in the presence of soluble KdpD/1–395(6His) compared with the control. However, an additional band corresponding to the KdpD/1–207-KdpE-DNA complex was missing indicating that there is only a weak interaction between KdpD/1–395 and KdpE/H11011P. Based on these results it is proposed that the N-terminal domain of KdpD (KdpD/1–395) stabilizes the binding of KdpE/H11011P to its corresponding DNA-binding site.

**Biochemical Evidence for the Interaction between KdpD/1–395 and KdpE—**To obtain direct evidence for an interaction between the N-terminal domain of KdpD (KdpD/1–395) and KdpE, a co-elution experiment with 10His-KdpE and KdpD/1–395 from Ni²⁺-NTA agarose magnetic beads was performed. Briefly, 10His-KdpE was first bound to the beads and then solubilized KdpD/1–395 was added in a final ratio of 1:8 (KdpE:KdpD/1–395) (see “Experimental Procedures” for details). As a control, 10His-OmpR, the response regulator of the EnvZ/OmpR system of E. coli (34), was bound to the magnetic beads and incubated in the same manner with KdpD/1–395 as described above. Then the non-bound proteins were first washed off, and bound proteins were eluted from the beads and detected in an immunoblot with antibodies directed against the His tag as well as against KdpD. As shown in Fig. 8, the non-tagged KdpD/1–395 co-eluted with 10His-KdpE from the column (lane 1), but no co-elution of KdpD/1–395 was observed with the control protein 10His-OmpR (lane 2), demonstrating the specific interaction between the N-terminal domain of KdpD and the response regulator KdpE.

**DISCUSSION**

KdpD differs from other sensor kinases by its very large 395-amino acid-comprising, N-terminal domain, which is part of the input domain (3, 11). Here we describe that in the absence of the C-terminal transmitter domain and the four transmembrane domains, the N-terminal domain of KdpD causes semi-constitutive kdpFABC expression in vivo. This observation is very astonishing, because in a kdpD null mutant or in cells producing KdpD with an inactivated phosphorylation site (KdpD/H673Q), no expression of kdpFABC was observed. However, we found that this unusual signaling requires the response regulator KdpE with an intact phosphorylation site. As expected and experimentally confirmed in this study, purified 10His-KdpD/1–395 was not able to phosphorylate KdpE. This led to the conclusion that KdpE was phosphorylated in a different way in vivo. It has been shown before that KdpE can be phosphorylated by acetyl phosphate in vitro (38). Furthermore, other response regulators can be phosphorylated by small molecules like imidazole phosphate, acetyl phosphate, carbamoyl phosphate, or phosphoramidate, whereby high concentrations...
of these substances must be present \textit{in vitro} (40–42). To test whether KdpE is phosphorylated by acetyl phosphate \textit{in vivo}, we used \textit{E. coli} strains that are unable to synthesize acetyl phosphate. In these mutants, KdpD/1–395–mediated kdpFABC expression was decreased up to 50%. This suggests that KdpE is in part phosphorylated by acetyl phosphate under these circumstances. Similar results were obtained for the response regulators OmpR of \textit{E. coli} and PhoP of \textit{Salmonella enterica} that are phosphorylated by acetyl phosphate in the absence of their cognate sensor kinases (43, 44). Moreover, for the response regulator RssB of \textit{E. coli}, no cognate sensor kinase is known, and it is thought that the phosphorylation of RssB is exclusively regulated by the intracellular concentration of acetyl phosphate (45). Alternatively, KdpE could be phosphorylated by another sensor kinase, the so-called "cross-talk," or KdpD/1–395 forms a hybrid kinase with the transmitter domain of another sensor kinase, which then phosphorylates KdpE. These are obviously very often discussed theories that are difficult to prove. However, by using transposon mutagenesis no additional gene was detected. Therefore, we conclude that KdpE is phosphorylated by acetyl phosphate in the absence of an intact KdpD. However, the results clearly indicate that acetyl phosphate is not the only phosphoryl donor. It is quite conceivable that KdpE is phosphorylated by other phosphoryl donors like carbamoyl phosphate or phosphoramidate.

The question still remained how KdpD/1–395 causes semi-constitutive kdpFABC expression. KdpD/1–395 did not affect the phosphorylation of KdpE by acetyl phosphate \textit{in vitro}. However, there must be a specific function of KdpD/1–395 because no kdpFABC expression was detectable in the absence of KdpD. We could clearly show that the transcription of \textit{kdpFABC} was enhanced \textit{in vitro} in the presence of reconstituted KdpD/1–395. By performing such experiments, care has to be taken because transcription is enhanced \textit{per se} in a lipidd environment (39). Because the amount of \textit{kdpFA} transcripts was highest in the presence of reconstituted KdpD/1–395, we conclude that the enhanced \textit{kdpFABC} transcription \textit{in vitro} is probably due to two effects: (i) the presence of KdpD/1–395, and (ii) the presence of a lipidd environment presented by the phospholipid vesicles.

There are at least two possibilities regarding how KdpD/1–395 affects \textit{kdpFABC} transcription: (i) the stabilization of phosphorylated KdpE, and (ii) the stabilization of the binding of KdpE–P to its corresponding binding site upstream of the \textit{kdpFABC}–promoter/operator region. In the presence or absence of KdpD/1–395 nearly the same phosphorylation kinetics of KdpE by acetyl phosphate \textit{in vitro} were observed, which argues against the first hypothesis. The mobility shift experiments provide evidence for the second hypothesis; a much higher amount of DNA-bound KdpE–P could be observed in the presence of KdpD/1–395 compared with the control experiment. In comparison to earlier results (18), the affinity of KdpE–P to the DNA was relatively low in our experiments. This might be due to the presence of detergent, which was necessary to keep KdpD/1–395 in solution. These conditions might also weaken the interaction between KdpD/1–395 and KdpE, and therefore a signal for a ternary complex consisting of KdpE–P, DNA, and KdpD/1–395 is missing. Finally, electrophoretic mobility shift assay experiments in the presence of KdpD/1–395 in proteoliposomes resulted in the formation of aggregates in the gel pockets and therefore led to misleading results. Despite these experimental difficulties, a stabilizing effect of KdpD/1–395 on the KdpE–P–DNA interaction was clearly detectable.

This is the first example for a stabilization of the interaction between a response regulator and its corresponding DNA-binding site by a domain of the cognate sensor kinase. Furthermore, it seems likely that a lipidd environment also promotes transcription efficiency, an effect that was already described earlier (39). Coincidentally, the separately produced N-terminal domain of KdpD results in a membrane-attached polypeptide, which can be released from the membrane by detergent or by washing with buffer of low ionic strength (11). Moreover, the analysis of truncated KdpD derivatives lacking different parts of the transmembrane spanning domains indicated that a certain distance between the N- and C-terminal cytoplasmic domains is required to allow signal transduction. In addition, it has been demonstrated that the N- and C-terminal domains interact with each other (10, 11). Based on these results a model was established according to which the N- and C-terminal domains move toward each other under inducing (phosphorylating) conditions. It is conceivable that the movement of the cytosolic domains also includes conformational changes leading to the liberation of structures within the N-terminal domain which then promote the stabilization between KdpE–P and the DNA (Fig. 9). This would explain why cells producing the non-phosphorylatable KdpD/H673Q prevent or cells producing the N-terminal truncated KdpD/Δ12–395 (8) diminish \textit{kdpFABC} expression. The direct interaction between the N-termi-
The Input Domain of the Sensor Kinase KdpD

The domain of KdpD and KdpE has been shown by co-elution experiments. According to the literature a quite similar interaction has been shown for the sensor kinase UhpB and the response regulator UhpA. UhpB participates not only in the phosphorylation control of UhpA—P but also binds UhpA specifically. Therefore expression of the target gene uhpT is tightly regulated (31, 46).

Despite the stabilizing effect of the N-terminal domain of KdpD on the KdpE−P-DNA interaction, the question remains why kdpFABC expression was found to be dependent on the extracellular K⁺ concentration under K⁺ limiting growth conditions. These effects are difficult to explain right now. It seems likely that other proteins channel additional information to KdpE under extreme K⁺ limiting conditions. In this regard it is interesting to note that H-NS (hns) and thioreredox reductase (trxB) somehow affect kdpFABC expression in E. coli (47). Furthermore, a very recent report (48) proposes the interaction of the input domain of KdpD and lipoproteins, namely LprJ and LprF, in of the input domain of KdpD and lipoproteins, namely LprJ and LprF, in

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