Characterization of Truncated Forms of the KdpD Protein, the Sensor Kinase of the K⁺-translocating Kdp System of Escherichia coli*

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The expression of the kdpFABC operon, coding for the K⁺-translocating Kdp system, is controlled by the two regulatory proteins, KdpD and KdpE, which belong to the group of sensor kinase/response regulator systems. This study describes the construction and analysis of KdpD sensor kinases, in which different deletions in the N-terminal part of the protein were introduced. Truncated KdpD proteins, in which the membrane-spanning segments were deleted, had lost their phosphorylation capacity. Truncated KdpD proteins, in which the four membrane-spanning helices were untouched, were still phosphorylated, and the phosphoryl group could be transferred to the response regulator KdpE in vitro. Furthermore, these truncated KdpD proteins cause dephosphorylation of KdpE(P), which is comparable with that of the wild-type protein. To investigate the effect of the deletions on signal transduction in vivo the corresponding kdp genes were transferred to the chromosome. Growth studies with the mutant strains are in accord with the data obtained from the in vitro studies. Furthermore, kdp expression was investigated using a KdpA-LacZ fusion. The data obtained support the notion that the extent of kdp expression is modulated by the N-terminal part of KdpD.

The maintenance of turgor in bacterial cells is one of the most important functions served by K⁺ ions (Epstein, 1986; Epstein, 1992). Therefore, bacteria have established several types of K⁺ uptake and efflux systems as well as secondary porters and stretch-activated channels to regulate the internal K⁺ concentration in response to changes in the osmolarity of the medium (for an overview, see Bakker (1993) and Stumpe et al. (1996)). At low K⁺ concentrations (0.1 mM) the Kdp system is synthesized as an emergency system (Epstein, 1985; Laimins et al., 1978), which transports K⁺ with high affinity into the cell (Km for K⁺ uptake is about 2 mM; Rhoads et al. (1976)) and which belongs to the class of P-type ion-translocating ATPases (Siebers and Altendorf, 1993). The structural genes are organized in the kdpFABC operon (Hesse et al., 1984; Altendorf et al., 1992), and the adjacent kdpDE operon codes for two proteins, which regulate the expression of the structural genes (Polarek et al., 1992). The two regulatory proteins belong to the family of sensor kinase/response regulator systems. KdpD is a membrane-bound protein (98.7 kDa), whereas KdpE is a smaller, cytoplasmic protein (25.2 kDa) (Walderhaug et al., 1992). As described for other two component systems, the regulation of the kinase or the phosphatase activities, or both, is affected by an environmental stimulus (Parkinson and Kofoid, 1992; Parkinson, 1995; Stock et al., 1995). For KdpD, the stimulus seems to be a decrease in turgor (Epstein, 1992; Laimins et al., 1981). However, this view has been questioned by others (Asha and Gowrishankar, 1993; Csonka and Hanson, 1991). Furthermore, analysis of mutant forms of KdpD that result in constitutive expression of kdp led Sugiuira et al. (1994) to suggest that KdpD senses two signals, turgor and K⁺. The phosphorylation of the KdpD protein in vitro as well as the subsequent transfer of the phosphoryl group to the response regulator KdpE was shown (Nakashima et al., 1992; Voelkner et al., 1993).

Topological studies revealed that KdpD has extended hydrophilic domains at the C-terminal as well as at the N-terminal region connected by four membrane-spanning segments near the middle of the protein (Zimmann et al., 1995). Whereas the C-terminal domain, which contains the predicted residue His⁷³ (Voelkner et al., 1993) that becomes phosphorylated and the kinase domain that must interact with ATP and with KdpE, exhibits extended homology to other sensor kinases, the N-terminal domain does not. In order to investigate the function of the N-terminal part of KdpD, which comprises almost half of the protein mass, different truncated forms of the KdpD protein were constructed and analyzed in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were of analytical grade. [γ-³²P]ATP (3000 Ci/mmol) was purchased from Amersham Buchler. Nylon membranes (0.2 μm) and nitrocellulose membranes (0.45 μm) were obtained from Schleicher & Schuell. T4 DNA ligase, alkaline phosphatase, and restriction endonucleases were purchased from Boehringer Mannheim, Life Technologies, Inc., Biolabs, or Pharmacia Biotech Inc. Agarose was obtained from Roth. Labeling and detection of DNA was performed with the nonradioactive digoxigenin system from Boehringer Mannheim, while the T7 sequencing kit was obtained from Pharmacia. Goat anti-rabbit IgG alkaline phosphatase conjugate was purchased from Bioxin. DEAE-Sepharose CL-6B and Resource Q chromatography resin were obtained from Pharmacia. Centrifugal filter units (0.22 μm) were purchased from Millipore Corp.

Bacterial Strains, Plasmids, and Growth Conditions—Escherichia coli strains and plasmids used are listed in Table I. All strains are derivatives of E. coli strain K12 and carry mutations in the genes coding for the other K⁺ uptake systems, TrkG, TrkH, and Kup (Bossemeyer et al., 1989; Dosch et al., 1991).

KML complex medium (1% KCl, 1% casein hydrolysate, 0.5% yeast extract) and minimal medium were prepared as described (Epstein and Davies, 1970). LB, media (1% casein hydrolysate, 0.5% yeast extract) contain a residual amount of 9.1 μM potassium, which was determined by flame photometry. To vary osmolality and the potassium concentration in these media, different concentrations of sucrose, NaCl, and/or KCl were added as indicated. Minimal media were designated K0.02 up to K115, corresponding to the millimolar concentration of potassium.

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ions added, while osmolality in this medium did not change due to the addition of corresponding amounts of sodium chloride. In general, glucose (1%) served as carbon and energy source. For the preparation of membrane vesicles, strain TKR2000 was transformed with plasmids pWP1–92, pWP2–92, pWP3–92, pWP4–92, pWP6–92, and pWP5, respectively. Purified transformants were grown in 5 liters of KML medium containing 50 μg/ml ampicillin to an absorbance at 610 nm of approximately 1.0, and cells were harvested by centrifugation at 7,000 × g. Growth experiments and incubation of cells for β-Gal activity measurements were performed in minimal and LB media, with the designated NaCl and sucrose concentrations under aerobic conditions at 37°C, and the optical density was measured at 610 nm. Samples for β-Gal activity measurements were taken as indicated in the figure legends.

Recombinant DNA Techniques—The conditions used for handling recombinant DNA and transformation of E. coli cells were as described (Ausubel et al., 1991; Sambrook et al., 1989). Treatment of DNA with restriction endonucleases, T4 DNA ligase, and calf intestinal phosphatase was performed following the protocols of the suppliers. DNA fragments were recovered from agarose gels using the Geneclean kit from Bio 101. Sequencing of DNA was performed by the dyeoxyribonucleotide chain termination method (Sanger et al., 1977) using the T7 sequencing system from Pharmacia. As template, double-stranded plasmid DNA denatured with NaOH was used as described in the protocol of the sequencing kit. The 17-mer sequencing primers hybridizing with different parts of the kdpD gene were synthesized by Dr. Holger Lill (University of Osnabrück).

Construction of Plasmids—Plasmid pWP1–92 with an EcoRV deletion within the kdpD gene was constructed by digestion of plasmid pPV5 with EcoRV (see Fig. 1 and Walderhaug et al. (1992)) and religation of the 6.4-kb fragment. Plasmid pWP2–92 was constructed by digestion of pPV5 with PvuII. This enzyme has two restriction sites within the kdpD gene and an additional site within the bla gene of the vector. The resulting 3.8- and 3.1-kb fragments were ligated. Construction of pWP3–92 was performed with two different digests of pPV5. From a DraI/PstI digestion, which produced several fragments due to several DraI sites in the vector part of pPV5, the PstI-DraI fragment (1.4 kb) carrying the 5' end of the kdpD gene was isolated. From a second digestion of pPV5 with EcoRV and PstI the large fragment (5.9 kb) was isolated, which comprises the 3' part of the kdpD gene at the EcoRV site at 5.9 kb up to the PstI site from the vector DNA. Both fragments were ligated with T4 DNA ligase. Plasmid pWP4–92 was obtained by closing the 0.4-kb DraI/EcoRV fragment of pWP1–92 into vector pWP1–92, which was linearized with EcoRV and treated with calf intestinal phosphatase. Plasmid pWP6–92 was constructed by introducing a PvuI restriction site at bp 5504. Therefore, a 842-bp fragment containing the StuI recognition site at bp 6062 was amplified using Vent polymerase and the following primers. Primer 1 was 5'-GCGATGTTGACAAATTTCGAGG-3' (underlined nucleotides correspond to the introduced PvuI recognition site, and the rest of the sequence is identical to bp 5505–5519 in kdpD). The second primer used is complementary to the kdpD sequence from bp 6325 to 6346. The amplified fragment was digested with StuI, and the shortened 558-bp PvuI-StuI fragment was used to exchange the PvuI-StuI fragment (bp 4350–6062) of wild-type kdpD gene in plasmid pPV5.

In each case strain TK2281 (∆kdpFABCDE) was transformed with the ligase mixtures. Plasmids were isolated from transformants and tested by restriction analysis and DNA sequencing of the junctions of the deletions, and in the case of polymerase chain reaction products, of the amplified sequence. Plasmid-bearing cells of strain TKR2000 were grown in KML medium containing 50 μg/ml ampicillin and subjected to SDS-PAGE followed by immunoblotting with anti-KdpD antiserum. Construction of plasmid pWP1lacZ was performed using synthetic linkers composed of two oligonucleotides.

1 The abbreviations used are: β-Gal, β-galactosidase; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair(s); bp, base pair(s).
from strain RH90 (Hengge-Aronis et al., 1993) into strain TK2240. Strain RH90 was kindly provided by Dr. Hengge-Aronis.

PREPARATION PROCEDURES—E. coli strain TKR2000 (λkdpFABCD E. coli strain TKR2000 (λkdpFABCD was transformed with the plasmids pWP1–92, pWP2–92, pWP3–92, pWP4–92, pWP6–92, pPV5, and pKK223–3, respectively, and cells were grown in KML media containing ampicillin (50 μg/ml).

Everted membrane vesicles were prepared from about 5 g of cells by passage through a Ribi Press fractionator, and the vesicles were washed twice in EDTA-containing buffer of low ionic strength according to Siebers and Altendorf (1988) with the following modification: Tris-HCl, pH 7.5, 10 mM MgCl₂, 10% glycerol, 2 mM dithiothreitol, 0.35 mM EDTA, 0.5 mM NaCl, and 0.005% Tween 80.

Solubilization of KdpD1–92 and KdpD3–92 from the "low speed pellet" (obtained by centrifugation at 7000 × g of the solution after the passage of cells through the Ribi press fractionator) was performed with 6 mM guanidine hydrochloride, which was then removed by stepwise passage of cells through the Ribipress fractionator. The precipitated protein was dissolved in 20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, and 0.005% Tween 80.

Protein phosphorylation and dephosphorylation experiments were performed as described by Voelkner et al. (1993). As standard phosphorylation buffer, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10% glycerol, 2 mM dithiothreitol, 0.35 mM EDTA, 0.5 mM NaCl (buffer B) was used. In addition, the same buffer lacking NaCl (buffer A) was used. In general, phosphorylation was performed for 10 min at 20°C in the presence of 0.5 μCi of [γ-32P]ATP (2.38 Ci/mmol) in phosphorylation buffer B, except that MgCl₂ was replaced with 5 mM CaCl₂. After 10 min, purified KdpE (0.1 mg/ml) was added, and the incubation was continued for 5 min. KdpD-containing membrane vesicles were removed by filtration through 0.22-μm centrifugal filter units at 5000 × g.

Dephosphorylation was initiated by the addition of 20 μM [γ-32P]ATP (2.38 Ci/mmol) in phosphorylation buffer B, except that MgCl₂ was replaced with 5 mM CaCl₂. After 10 min, purified KdpE (0.1 mg/ml) was added, and the incubation was continued for 5 min. KdpD-containing membrane vesicles were removed by filtration through 0.22-μm centrifugal filter units at 5000 × g.

Phosphorylation of KdpD and KdpE was performed as described by Voelkner et al. (1993). KdpD was phosphorylated for up to 120 min in buffer A and buffer B as well as in buffer A containing 50 mM KCl in the presence of 1 μCi of [γ-32P]ATP. The reaction was stopped by the addition of SDS sample buffer.

For the dephosphorylation assay, purified KdpE was phosphorylated in the following manner. First, everted membrane vesicles containing wild-type KdpD were incubated with 20 μM [γ-32P]ATP (2.38 Ci/mmol) in phosphorylation buffer B, except that MgCl₂ was replaced with 5 mM CaCl₂. After 10 min, purified KdpE (0.1 mg/ml) was added, and the incubation was continued for 5 min. KdpD-containing membrane vesicles were removed by filtration through 0.22-μm centrifugal filter units at 5000 × g.

Dephosphorylation was initiated by the addition of 20 μM MgCl₂ and everted membrane vesicles (1 mg/ml) containing KdpD, KdpD2–92, KdpD3–92, KdpD4–92, KdpD6–92, and, in case of KdpD1–92, with the "low speed pellet." The samples were preincubated for 3 min in buffer A in the presence of 0.5 μCi of [γ-32P]2-azido-ATP. Cross-linking was achieved by irradiation of the samples with UV light at 302 nm for 2.5 min.

Analytical Procedures—Determination of protein concentrations was carried out as described (Hartree, 1972). SDS-PAGE was performed according to Laemmli (1970) using 7.5, 9, or 11% acrylamide gels. The gels were stained with Coo massie Brilliant Blue G-250 as described (Weber and Osborn, 1969) with a staining solution containing 10% trichloroacetic acid. Autoradiography of dried gels was performed with the Kodak film X-Omat AR5 for 10–16 h. The electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membranes (0.45 μm) and immunodetection of KdpD proteins with the anti-KdpD antiseraum (dilution 1:100,000) was carried out as described by Voelkner et al. (1993). The activity of β-galactosidase was determined from three different experiments and is given in Miller units calculated as described (Miller, 1972). Three units of β-galactosidase will hydrolyze 1 nmol of 2-nitrophenyl-β-D-galactopyranoside/min/mg of protein.

RESULTS

Construction of kdpD Deletion Plasmids—Five in-frame partial deletions of the kdpD gene were constructed as described under "Experimental Procedures" and diagrammed in Fig. 1.

![Diagram of Truncated Forms of the KdpD Protein](image_url)
The junctions of the deletions were confirmed by DNA sequencing. The product of each deletion construct was readily expressed (Fig. 2), reacted well with anti-KdpD antibodies, is of a size consistent with that expected, and seems stable, since no fragments reacting with the antibody were seen.

**Phosphorylation of the Truncated KdpD Proteins and Transfer of the Phosphoryl Group to KdpE**—In order to investigate the *in vitro* phosphorylation capacity of the truncated KdpD proteins, everted membrane vesicles (see “Experimental Procedures”) of strain TKR2000 carrying the KdpD wild-type or deletion plasmids were used. Phosphorylation of truncated KdpD2–92, KdpD4–92, and KdpD6–92 could clearly be detected after 10 min, when buffer B (0.5 M NaCl) was used (Fig. 3, lanes 5, 10, and 14). No significant phosphorylation occurs in the absence of NaCl (buffer A) (data not shown). The phosphorylation level of truncated KdpD proteins was comparable with that observed with intact KdpD, whereas that of KdpD4–92 and KdpD6–92 (Fig. 3) was somewhat lower. The truncated KdpD proteins, KdpD2–92, KdpD4–92, and KdpD6–92, also retained the ability to transfer the phosphoryl group to KdpE (Fig. 3). For comparison, the phosphotransfer from wild-type KdpD to KdpE is shown. No decrease in the phosphorylation intensity of truncated KdpD and wild-type KdpD could be detected if cytoplasmic fractions lacking KdpE were added (data not shown).

In contrast, no phosphorylation could be detected with KdpD1–92 and KdpD3–92, in which the membrane-spanning segments are deleted (see Fig. 4). The truncated KdpD-containing “low speed pellet” and the solubilized proteins were also used, but phosphorylation could not be detected under all conditions tested (data not shown). Interestingly, the KdpD3–92 protein lacking the membrane-spanning segments was still associated with the membrane fraction, possibly due to hydrophobic or electrostatic interactions of N-terminal parts of KdpD with the membrane. But even the membrane-associated protein was not phosphorylated. Nakashima *et al.* (1993) have recently reported that a truncated KdpD protein, KdpD*, missing the same region within the protein as KdpD3–92, still gets phosphorylated *in vitro*. In an attempt to solve the discrepancy, we have included in our studies the plasmid pRUN-SD producing KdpD*, kindly provided by Dr. Mizuno. As can be seen from Fig. 4, the membrane-associated KdpD3–92 and the membrane-associated KdpD* were not phosphorylated in the presence of [γ-32P]ATP, even when the incubation time was extended to 120 min.

**Binding of ATP**—To test whether the ATP binding site of KdpD and its truncated forms was still intact, binding of [α-32P]2-azido-ATP was studied. Therefore, everted membrane vesicles and, in case of KdpD1–92 the “low speed pellet,” were treated as described under “Experimental Procedures.” As shown in Fig. 5, KdpD and the truncated forms were clearly labeled with [α-32P]2-azido-ATP. This finding lends support to the notion that binding of ATP to KdpD is not affected in the truncated proteins. The different intensities in background are based on various amounts of other labeled proteins in the membrane fractions and in the “low speed pellet.”

**Dephosphorylation of KdpE(P) by the Truncated KdpD Proteins**—Using the purified response regulator KdpE, the influence of wild-type KdpD and its truncated forms on the dephosphorylation of KdpE(P) was tested (Fig. 6). KdpE was phosphorylated as described under “Experimental Procedures.” KdpE(P) was not dephosphorylated by a preparation of control vesicles that lack KdpD. In contrast, KdpE(P) was dephosphorylated by the addition of wild-type KdpD. As shown in Fig. 6, dephosphorylation activity could also be observed for the truncated proteins KdpD2–92, KdpD4–92, and KdpD6–92. Whereas the activity of the KdpD2–92 protein was essentially
Phosphorylation of membrane vesicles containing KdpD, KdpD* and KdpD3–92 was performed for 10 (‘10’), 60 (‘60’), and 120 min (‘120’) in buffer A. The phosphorylated proteins were separated on 7.5% SDS-PAGE, and radioactivity was detected by autoradiography carried out for 14 h. The positions of KdpD and its truncated forms are indicated by arrows and determined from the Coomassie Blue-stained gel.

Binding of [α-32P]2-azido-ATP to KdpD and its truncated forms. Binding of [α-32P]2-azido-ATP was studied with membrane vesicles derived from the strains indicated. In the case of TKR2000/pWP1–92, the low speed pellet was used. Membrane vesicles containing KdpD (20 μg), KdpD2–92 (22 μg), KdpD3–92 (20 μg), KdpD4–92 (34 μg), KdpD6–92 (20 μg), and, in the case of KdpD1–92, the low speed pellet (24 μg) were incubated with 0.5 μCi of [α-32P]2-azido-ATP as described under “Experimental Procedures.” The proteins were separated on 9% SDS-PAGE, and radioactivity was detected by autoradiography carried out for 14 h. The positions of KdpD and its truncated forms are indicated by arrows and determined from the Coomassie Blue-stained gel.

Identical to that of wild-type KdpD, dephosphorylation of KdpD(P) by KdpD and its truncated forms is unaffected by the chromosomal-encoded kdp deletions (data not shown). Since the latter strains were not able to grow on minimal medium containing less than 15 mM K+, it is worth mentioning that growth of all strains on minimal medium containing 115 mM K+ is unaffected by the chromosomal-encoded kdp deletions introduced. To determine the potassium dependence of kdp expression, the cells were grown on minimal media with different K+ concentrations. Cells were grown up to an absorbance at 610 nm of 0.5 and subjected to β-Gal assay. The results are shown in Fig. 7. Expression of kdp was observed in strain TK2240 (wild type) at concentrations lower than 60 mM K+. Expression of kdp increased continuously by lowering the potassium concentration in the medium, whereas the osmolarity was held constant by the addition of corresponding amounts of sodium chloride. In the case of strains TK22292 and TK22692, significant expression of kdp occurred only at K+ concentrations lower than 10 mM. The critical concentration of K+ for expression in TK22492 was at about 15 mM K+.

To study the effects of the truncated, chromosome-encoded KdpD in more detail, strains TK22192, TK22492, TK22392, TK22492, TK22692, and TK2240 (wild type) were able to grow on minimal medium containing 20 μM K+ (see below). However, strains TK22192 and TK22392 exhibit no growth on media containing less than 15 mM K+. It is worth mentioning that growth of all strains on minimal medium containing 115 mM K+ is unaffected by the chromosomal-encoded kdp deletions (data not shown).

In Vivo Effects of Chromosome-encoded, Truncated KdpD—Regulatory proteins are only present in low amounts in bacterial cells. To avoid artifacts caused by overexpression, it was therefore necessary to transfer the truncated kdpD genes from the plasmid to the chromosome (see “Experimental Procedures”). Furthermore, we had realized previously that the extent of kdp expression is affected by the presence of other K+ uptake systems. Therefore, the strains used in this study are deleted for the other K+ uptake systems. The strains used in this study are deleted for the other K+ uptake systems, leaving only Kdp for K+ transport. The resulting strains were designated TK22192, TK22292, TK22392, TK22492, and TK22692, and the successful replacement of wild-type kdpD gene by the corresponding kdpD deletions was confirmed by Southern hybridization (data not shown).

The strains constructed were tested for their ability to grow on minimal medium containing different K+ concentrations, since the Kdp system can be turned on by reducing the K+ concentration of the medium. Strains TKW22292, TKW22492, TKW22692, and TK2240 (wild type) were able to grow on minimal medium containing 20 μM K+ (see below). However, strains TKW22192 and TKW22392 exhibit no growth on media containing less than 15 mM K+.
Therefore, the influence of NaCl or sucrose on the expression of the kdp gene was used to turn on β-Gal activity upon addition of NaCl or sucrose, reaching a maximum of 2000 units. In the case of strain TKW22292 and TKW22692, the β-Gal activity in wild-type and mutant strains in response to different K+ concentrations was measured as described under “Experimental Procedures.” Strains TKW22192, TKW22292, TKW22492, TKW22692, TKV2208, and TK2240 were transformed with plasmid pWPA1lacZ. The cells were grown in minimal media containing the indicated potassium concentrations. Samples for β-Gal activity measurements were taken at an absorbance at 610 nm of 0.5. To test expression of strains TKW22192, TKW22292, and TKW2208 on media with potassium concentrations below 0.1 mM, cells were grown on K20 up to an absorbance at 610 nm of 0.5, washed in the media with lower potassium concentrations, and finally incubated for 30–120 min in the corresponding media. β-Gal activity was determined as described under “Experimental Procedures.” K0.1 to K60 indicate K+ concentrations in mM.

Cells were grown on minimal media containing 20 μM KCl. At points where the potassium concentration gets growth-limiting, 40 μM KCl were added. Samples for β-Gal activity measurements were taken at different time points of these growth experiments, and the results are shown in Fig. 8. The growth behavior of the strains was comparable with that of the wild-type. The β-Gal activity, on the other hand, showed remarkable differences. In the case of strain TKW22292 and TKW22692, the β-Gal activities were between 1000 and 1500 units at each time point of the induction protocol. In comparison with that, the kdp wild-type strain TK2240 showed an increase of β-Gal activity when the potassium concentration became growth-limiting (Fig. 8, points B/C). A much more dramatic increase in β-Gal activity (up to 6000 units) was observed when the potassium concentration in the medium became growth-limiting for the second time (Fig. 8, points D/E). A similar significant increase in β-Gal activity could only be detected in the case of TKW2292. During growth on K+-limiting media, cells eventually entered stationary phase. Since rpoS encodes a stationary phase regulator (σs), it was of interest to test the effect of rpoS mutations on kdp expression. Interestingly, strain TKW2295, in which rpoS is inactivated, also fails to show this increase of β-Gal activity.

It has been found previously (Laimins et al., 1981) that a sudden increase in medium osmolality, a maneuver that reduces turgor, was able to turn on kdp expression transiently. Therefore, the influence of NaCl or sucrose on the expression of kdp was tested. Cells were grown in LB0 media containing the different solutes up to an absorbance at 610 nm of 0.5. To achieve reasonable growth even under conditions of high osmolality, we found it necessary to use LB0 medium for this set of experiments. As shown in Fig. 9, β-Gal activity of TK2240 (wild type) rises upon addition of NaCl or sucrose, reaching a maximum of about 600–700 units. At 0.4 mM NaCl, growth of the cells is already affected, which is indicated by a decrease in kdp expression. In comparison with wild type, expression of kdp in the presence of NaCl is reduced to different levels in strains TKW22492, TKW22692, and TKW22292. No expression at all was detectable in case of strain TKW22192 and TKW22292 (data not shown). No significant increase of kdp expression in dependence of sucrose on LB0 media could be detected in all the deletion strains tested (Fig. 9). The absence of σs in strain TKW2295 rpoS::Tn10 kdp- has no influence on the sucrose-induced expression of kdp. Finally, it is interesting to note that in wild-type and mutant strains the level of kdp expression is reduced when, in the presence of NaCl, the K+ concentration in the medium is increased (data not shown).

**DISCUSSION**

The predicted overall topology of the KdpD protein as shown in Fig. 1 is based on sequence analysis (Walderhaug et al., 1992), on studies with kdpD-lacZ and kdpD-phoA fusions, and on protease treatment of spheroplasts in the presence and absence of Triton X-100 (Zimmann et al., 1995). The four membrane-spanning regions and the extended N-terminal cytoplas-
mic domain of KdpD exhibit no significant homology to other sensor kinases. To obtain information about the function of the N-terminal domain and the four membrane-spanning helices, five deletions comprising these regions were constructed (see Fig. 1).

KdpD2–92, KdpD4–92, and KdpD6–92 lacking the N-terminal amino acids Arg12–Asp228, Ile128–Phe391, and Arg12–Trp395, respectively, but leaving the four membrane-spanning helices untouched, still exhibited phosphorylation capacity and phototransfer activity in vitro. Also, the KdpE(P) dephosphorylation capacity of these three truncated forms was not affected. In contrast, no phosphorylation could be detected in the case of KdpD3–92, in which the membrane-spanning segments comprising Lys892–Asp930 were lacking, and in KdpD1–92 in which a stretch of amino acids from Ile128–Asp136 was deleted.

However, the binding of ATP to all five truncated forms of the protein was not affected, in comparison with wild-type KdpD, as indicated by the binding of 2-azido-ATP. Therefore, the membrane-spanning segments of KdpD seem to play an important role in the phosphorylation capacity of the protein.

Our results of the in vitro phosphorylation of the membrane-associated KdpD3–92 protein are in contrast with the results obtained for KdpD* (Nakashima et al., 1993), for which phosphorylation was observed after 120 min of incubation in the phosphorylation buffer. It is important to note that, in comparison with KdpD3–92, the same restriction sites have even been used to generate an identical deletion in kdpD, leading to the truncated form KdpD*. When both proteins were subjected to the same preparation protocol, no phosphorylation could be detected. It is therefore conceivable that variations in the preparation and in the renaturation as well as the further purification of KdpD* by fast protein liquid chromatography, which may eliminate further regulatory factors, are probably responsible for this discrepancy. Nevertheless, it should be emphasized again that both KdpD* and KdpD3–92 are not functional in vivo.

In this context it is worth mentioning that in other sensor kinases, e.g. FixL and EnvZ, the membrane-spanning segments are not essential for autophosphorylation in vitro. In the case of the soluble EnvZ* (Roberts et al., 1994), comprising amino acid residues from Ser215 to Gly450, the autophosphorylation capacity is indistinguishable from that of the wild type.

So far, these findings lend support to the notion that in KdpD the membrane-spanning segments are important for the correct conformation of the protein ready to receive the proper stimulus (changes in turgor) possibly via membrane stretching and/or via conformational changes. However, it is also conceivable that a possible dimerization of KdpD may be influenced by changes within the membrane-spanning segments and that this dimerization may be important for phosphorylation as shown for the nitrogen regulator NR11, in which the autophosphorylation reaction occurs within a dimer by a trans, inter-subunit mechanism (Ninfa et al., 1993).

It has been mentioned before that the expression of kdp is turned on by reducing the K+ concentration in the medium. That the N-terminal region of KdpD affects this process, therefore playing a role in modulating signal transduction, can be taken from the expression level of kdp as monitored by the β-Gal activity measurements. In strains carrying different deletions within the N-terminal region, the onset of kdp expression in dependence of the K+ concentration in the medium is, in comparison with the wild-type, shifted toward lower concentrations (approximately 15 mM or even lower; see Fig. 7). The low expression of kdp, as tested with polyclonal antibodies, is also reflected by the reduced growth rate of the deletion strains in the corresponding media.

If the K+ concentration in the medium is lowered even further (20–40 μM), a dramatic increase in the expression of kdp in the wild-type strain TK2240 can be observed (see Fig. 8). This increase in expression is somewhat lower in TK22492, in which amino acids 128–391 of KdpD are deleted, but it could not be detected at all if amino acids 12–228 (TK22292) or 12–395 (TK22692) are missing. It is interesting to note that the growth behavior of these mutant strains under these conditions is indistinguishable from that of the wild-type, whereas the level of kdp expression is significantly different.

The ability of KdpD to respond to high osmolarity, a maneuver that reduces turgor, is also impaired in those mutant strains. Depending on the strain tested, the induction of kdp expression is decreased in the case of NaCl and almost not detectable in the case of sucrose (see Fig. 9). Raising the K+ concentration in the medium from 9.1 mM to about 20–30 mM, kdp expression is reduced in the wild-type and mutant strains. These findings support the notion that KdpD senses two signals, changes in turgor and K+ (Sugiura et al., 1994). Whether KdpD senses K+ directly or indirectly, via K+ uptake followed by changes in turgor, is difficult to dissect.

In this context it should be noted that no increase in kdp expression under low K+ concentration (20 μM) could be detected when a kdp wild-type strain was used in which the alternative σ factor (σ*) encoded by the rpoS gene was inactivated by Tn10 insertion (Hengge-Aronis et al., 1993), whereas this mutation had no effect on the observed induction by sucrose. These data indicate that elevated kdp expression is dependent on the putative alternative σ factor (σ*) (as seen if K+ gets growth-limiting), while in general the σ70 factor is discussed as being responsible for kdp transcription (Altendorf et al., 1994). Whether σ* affects kdp expression in a more direct or indirect manner has still to be established.

We have demonstrated that signal transduction via the KdpD system is dependent on the membrane-spanning segments of KdpD. Furthermore, we propose that signal transduction is modulated by the N-terminal part of KdpD. The postulated modulation may take place at the level of dephosphorylation of KdpE via a phosphatase activity of KdpD, as described for other sensor kinases like NR11 (Ninfa and Magasanik, 1986), EnvZ (Igo et al., 1989), or FixL (Lois et al., 1993). So far, we have been unable to detect significant changes in the phosphatase activity of KdpD or its truncated forms in vitro. However, it is conceivable that in the way the in vivo experiments were conducted it has been difficult to mimic the extreme in vivo conditions.
conditions, under which we were able to detect the different expression levels of kdp.

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