The Hydrophilic N-terminal Domain Complements the Membrane-anchored C-terminal Domain of the Sensor Kinase KdpD of Escherichia coli*

The putative turgor sensor KdpD is characterized by a large, N-terminal domain of about 400 amino acids, which is not found in any other known sensor kinase. Comparison of 12 KdpD sequences from various microorganisms reveals that this part of the kinase is highly conserved and includes two motifs (Walker A and Walker B) that are very similar to the classical ATP-binding sites of ATP-requiring enzymes. By means of photoaffinity labeling with 8-azido-[α-32P]ATP, direct evidence was obtained for the existence of an ATP-binding site located in the N-terminal domain of KdpD. The N-terminal domain, KdpD/1–395, was overproduced and purified. Although predicted to be hydrophilic, it was found to be membrane-associated and could be solubilized either by treatment with buffer of low ionic strength or detergent. The membrane-associated form, but not the solubilized one, retained the ability to bind 8-azido-[α-32P]ATP. Previously, it was shown that the phosphatase activity of a truncated KdpD, KdpD/12–395, is deregulated in vitro (Jung, K., and Altendorf, K. (1998) J. Biol. Chem. 273, 17406–17410). Here, we demonstrated that this effect was reversed in vesicles containing both the truncated KdpD and the N-terminal domain. Furthermore, coexpression of kdpD/Δ12–395 and kdpD/1–395 restored signal transduction in vivo. These results highlight the importance of the N-terminal domain for the function of KdpD and provide evidence for an interaction of this domain and the transmitter domain of the sensor kinase.

The membrane-bound sensor kinase KdpD and the soluble response regulator KdpE comprise a sensor kinase/response regulator system of Escherichia coli, which regulates expression of the kdpFABC operon (1) encoding the high affinity transport systems TrkG, TrkH, and Kup of E. coli (2). Expression is induced under K+-limiting growth conditions (less than 2 mM), where the constitutive K+-translocating systems TrkG, TrkH, and Kup of E. coli are unable to maintain the required intracellular pool of K+. In mutants lacking these K+ transport systems, kdpFABC is already expressed in media containing less than 50 mM K+. Therefore, KdpD seems to sense the cell’s “need” for K+ to maintain turgor (11–13). Control by turgor is supported by the finding that a sudden increase in medium osmolarity, which reduces turgor, transiently turns off kdpFABC expression. This model has been challenged by the finding that under some conditions expression of kdpFABC is significantly induced when the osmolarity of the medium is increased by salt and not in case of sugar (9, 14, 15). Based on studies with KdpD proteins, in which different Arg residues were individually replaced with Gln, an electrostatic switch mechanism within the protein is proposed, which regulates the ratio of kinase to phosphatase activity (9). The concept of this is supported by the fact that KdpD kinase activity is dependent on negatively charged phospholipids (10).

The stimulus, which KdpD senses, is believed to be a decrease in turgor or an effect thereof. Expression of kdpFABC is induced under K+-limiting growth conditions (less than 2 mM), where the constitutive K+-translocating systems TrkG, TrkH, and Kup of E. coli are unable to maintain the required intracellular pool of K+. In mutants lacking these K+ transport systems, kdpFABC is already expressed in media containing less than 50 mM K+. Therefore, KdpD seems to sense the cell’s “need” for K+ to maintain turgor (11–13). Control by turgor is supported by the finding that a sudden increase in medium osmolarity, which reduces turgor, transiently turns off kdpFABC expression. This model has been challenged by the finding that under some conditions expression of kdpFABC is only significantly induced when the osmolarity of the medium is increased by salt and not in case of sugar (9, 14, 15). Based on kdpD mutants, which give rise to K+-independent kdpFABC expression but regain the ability to respond to changes in medium osmolarity independently of the solute, it is suggested that KdpD senses two stimuli: increase in osmolarity and K+ limitation (16). This presumption is supported by the differentiated response of KdpD proteins with individual substitutions of Arg residues toward K+ limitation and osmotic upshock (9).

Compared with other known sensor kinases, KdpD is the only protein that has a large, hydrophilic N-terminal domain of about 400 amino acids in length (17, 18). Sequence comparison of KdpD sequences of various bacteria reveals

* This work was supported by the Deutsche Forschungsgemeinschaft (SFB 431) and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF213466.

† Recipients of fellowships from the Deutsche Forschungsgemeinschaft: Heisenberg-Stipendium (to K. J.) and Graduiertenkolleg (to R. H.).

‡ To whom correspondence should be addressed: Universität Osnabrück, Fachbereich Biologie/Chemie, Abteilung Mikrobiologie, Barbarastr. 11, D-49069 Osnabrück, Germany. Tel.: 49-541-969-2276; Fax: 49-541-969-2870; E-mail: jung_k@biologie.uni-osnabrueck.de.

§ To whom correspondence should be addressed: Universität Osnabrück, Fachbereich Biologie/Chemie, Abteilung Mikrobiologie, Barbarastr. 11, D-49069 Osnabrück, Germany. Tel.: 49-541-969-2276; Fax: 49-541-969-2870; E-mail: jung_k@biologie.uni-osnabrueck.de.

1 The abbreviations used are: TM1–4, transmembrane domains 1 through 4; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis, LDAO, lauryldimethylamine oxide; ATPγS, adenosine 5’-(thiotriphosphate).

Ralf Heermann‡, Karlheinz Altendorf, and Kirsten Jung†‡§

From the Universität Osnabrück, Fachbereich Biologie/Chemie, Abteilung Mikrobiologie, D-49069 Osnabrück, Germany
that this domain is more conserved than other regions of KdpD. This N-terminal domain includes two sequence motifs, which are very similar to those of a typical ATP-binding site (Walker A and B motifs) (18) (Fig. 2). Sequences of short N-terminal versions of KdpD have been found in Synecocystis sp. (19) and in Anabaena sp. L-31 (GenBankTM accession number AF213466). It is suggested that such a fragment may form a functional KdpD complex with a C-terminal fragment that includes the membrane-spanning domains.

It has been shown recently that the phosphatase activity of KdpD is significantly increased in the presence of ATP or nonhydrolyzable ATP analogues, whereas other nucleotides have no effect. Truncated KdpD proteins lacking different parts of the N-terminal domain, including amino acids 12–128 are characterized by phosphatase activities independent of the presence of ATP. This finding suggests that an ATP-binding site has a regulatory role within this domain (18).

Here, we show that the N-terminal domain of E. coli KdpD can be produced separately allowing both the functional and biochemical characterization of this domain.

**EXPERIMENTAL PROCEDURES**

**Materials**—"γ-32P]ATP, NAP-5 gel filtration columns and thrombin were purchased from Amersham Pharmacia Biotech. 8-Azido-[α-32P]ATP was from ICN Biomedicals. Goat anti-(rabbit IgG)-alkaline phosphatase and anti-(mouse IgG)-alkaline phosphatase and anti-(mouse IgG)-alkaline phosphatase conjugates were purchased from Amersham Pharmacia Biotech. 8-Azido-[32P]ATP was from ICN Biomedicals. Goat anti-(rabbit IgG)-alkaline phosphatase and anti-(mouse IgG)-alkaline phosphatase conjugates were purchased from Amersham Pharmacia Biotech. All other reagents were reagent grade and obtained from commercial sources.

**Bacterial Strains and Plasmids**—E. coli strain JM 109 (recA1 endA1 gyrA96 thi hsdR17 supE44 yamD65 lacIqlacZ endpoints of histidine kinases and the position of the 6His tag are indicated in the upper part. At the bottom, the domain organization of KdpD is shown. Furthermore, the truncated forms of KdpD (KdpD/D12–395, KdpD1–395, KdpD1–395(6His)), respectively, are shown schematically, whereas the truncation is indicated by the black line.

**Oligonucleotide-directed Site-specific Mutagenesis**—Introduction of a thrombin cleavage site following amino acid 395 in KdpD (KdpD/Th) was achieved by individual amino acid substitutions. Constructs KdpD/1–395 and KdpD1–395(6His) were obtained by insertion of three stop codons or six codons for His and three stop codons, respectively, after the triplet corresponding to amino acid 395. All site-specific mutations were directed by synthetic mutagenic oligonucleotide primers using the polymerase chain reaction (PCR) overlap extension method (24). PCR products were purified in agarose gels and digested with appropriate restriction enzymes. DNA fragments were isolated from the gel and ligated to similarly treated pPV5(6His) (7) or pPV5-3 resulting in plasmids pPV5-3/1–395 and pPV5-3/1–395(6His), respectively. Plasmids pPV5-3/1–395/G37A, K38A, T39A and pPV5-3/1–395/G37A, K38A, T39A(6His) were obtained by restriction of plasmids pPV5-3/1–395 and pPV5-3/1–395(6His) with SacI and Stul, and the resulting fragments were ligated with a similarly treated vector, pPV5-1/G37A, K38A, T39C.

**Preparation of Intact Membrane Vesicles**—E. coli strain TKR2000 transformed with plasmids pPV5-3, pBD, or pBD3 carrying different kdpD mutations was grown aerobically at 37°C in KML complex medium (1% tryptone, 0.5% yeast extract, and 1% KCl) supplemented with ampicillin (100 μg/ml) or with chloramphenicol (34 μg/ml) and ampicillin (100 μg/ml). Overexpression of genes under control of the arabinose promoter was achieved by addition of 0.2% arabinose to the medium. Cells were harvested at an absorbance of 600 nm of ~1.0. Inverted membrane vesicles were prepared as described previously (7) with the exception that vesicles containing the N-terminal domain were not washed with buffer of low ionic strength. Vesicles were resuspended in 50 mM Tris-HCl, pH 7.5, containing 10% (v/v) glycerol, frozen in liquid nitrogen, and stored until use at −80°C.

**Purification of KdpD/1–395(6His) and KdpD/Th(6His)**—Solubilization and purification of KdpD/1–395(6His) and KdpD/Th(6His) were carried out as described for wild-type KdpD (7). Briefly, after solubilization with 2% (w/v) lauryldimethylamine oxide (LDAO) and centrifugation at 100,000 × g for 30 min, the protein was bound batchwise to the resin (equilibrated with column buffer (50 mM Tris-HCl, pH 7.5; 10% (v/v) glycerol; 0.5 mM NaCl; 10 mM β-mercaptoethanol; 0.04% (w/v) doceyl maltoside) by incubation of the solubilized protein with Ni2+-NTA resin for 30 min at 4°C. Alternatively, the N-terminal domain, KdpD1–395(6His), was purified in the absence of detergent. For this purpose, membrane vesicles were washed four times with buffer of low ionic strength (1 mM Tris-HCl, pH 7.5; 3 mM EDTA). After centrifugation at 100,000 × g for 30 min, KdpD1–395(6His) was found in the soluble fraction. 49 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, and 0.5 mM NaCl were added before the protein was loaded on a Ni2+-NTA column, which was equilibrated with column buffer without detergent. The protein-resin complex was then packed into a column, and unbound protein was removed by washing with column buffer. Bound His-tagged KdpD derivatives were eluted by increasing the imidazole concentration up to 100 mM.

**Photoaffinity Labeling with 8-Azido-[α-32P]ATP**—Purified KdpD/Th(6His) was reconstituted into E. coli phospholipids essentially as described (7). Photoaffinity Labeling with 8-Azido-[α-32P]ATP—KdpD/Th(6His) was purified and reconstituted into proteoliposomes as described above. Proteoliposomes (10 μg of protein) in buffer (50 mM Tris-HCl, pH 7.5; 10% (v/v) glycerol; 10 mM MgCl2; and 2 mM dithiothreitol) were incubated with 5 μCi of 8-azido-[α-32P]ATP (20 Ci/mmol) for 5 min at room temperature. Cross-linking was achieved by irradiating with UV light.
N-terminal Domain of the Sensor Kinase KdpD

RESULTS

Overproduction, Localization, and Purification of KdpD/1–395(6His)—A short version of KdpD comprising the N-terminal domain (amino acids 1–395) followed by six His residues (KdpD/1–395(6His)) was generated by insertion of six codons for His and three stop codons at the appropriate site in kdpD. This gene was overexpressed in E. coli strain TKR2000, which was transformed with plasmid pPV5-3/1–395(6His) (data not shown). Localization studies indicated that KdpD/1–395(6His) was membrane-associated (Fig. 3, lane 2); none of the protein was found in the cytoplasm (Fig. 3, lane 3). KdpD/1–395(6His) could either be completely solubilized by detergent (data not shown) or partially detached from the membrane under low ionic conditions (Fig. 3, lanes 4 and 5) (see also “Experimental Procedures”). Protein obtained by both methods was used for purification. As illustrated in Fig. 4, KdpD/1–395(6His) binds either in the presence or absence of detergent specifically to Ni-NTA agarose and can be eluted from the resin by increasing the imidazole concentration to 100 mM. Because under low ionic buffer conditions only membrane-associated proteins are released, purification of KdpD/1–395(6His) reaches about 95% homogeneity, whereas in the presence of detergent two major contaminating proteins are detectable (Fig. 4).

Detection of ATP Binding to the N-terminal Domain by PhosphorImaging—The N-terminal domain is highly conserved among all thus far known KdpD sequences of various microorganisms. Moreover, it contains a highly conserved region, which is very similar to a classical ATP-binding site (Walker A and Walker B motifs) (Fig. 2). Direct biochemical evidence for binding of ATP to this site was obtained by phosphorimaging labeling with 8-azido-α-32P]ATP. The purified and reconstituted full-length sensor kinase, carrying a thrombin cleavage site at amino acid position 395 (KdpD/Th(6His)) to separate the ATP-binding sites of the N-terminal and C-terminal domain, was first labeled with 8-azido-α-32P]ATP and then treated with thrombin. The protein fragments were loaded on an SDS gel, and the corresponding autoradiography was performed (see Fig. 5). Besides the labeled and uncleaved KdpD, two labeled fragments were detectable; however, their size did not correspond to the expected fragment size after thrombin cleavage. Determination of the N-terminal sequences of these fragments confirmed that the large (−29 kDa) fragment repre-
The importance of the N-terminal domain can complement the truncated KdpD in vitro, plasmids pBD6-92, encoding KdpD/12–395, and pBD3/1–395(6His), encoding the N-terminal domain of KdpD, were cotransformed in E. coli strain TKR2000. Cells were cultivated, the two kdpD derivatives were overexpressed, and KdpD phosphatase activity was determined after preparation of inverted membrane vesicles. As shown in Fig. 7B, inverted membrane vesicles containing both the truncated KdpD as well as the N-terminal domain are characterized by a KdpE–P phosphatase activity, which shows a restored dependence on ATP. These data further support the assumption of a direct interaction of the N-terminal domain (KdpD/1–395) with the rest of the protein and stress the importance of the ATP-binding site located in this region.

**DISCUSSION**

The stimulus that KdpD senses to activate signal transduction, which results in induction of kdpFABC expression, is believed to be a decrease in turgor or some effect thereof, reflecting the role of K⁺ as an important cytoplasmic solute (12). However, there is an ongoing debate as to whether KdpD is a turgor sensor or not (13–16). Despite this, the nature of the primary stimulus for KdpD is still unknown. Although KdpD shares high similarity to transmitter domains of other histidine kinases, it is unusual in regard of the large input domain. This input (sensor) domain comprises an extended N-terminal cytoplasmic region, four transmembrane domains, and part of the C-terminal domain (Fig. 1). Three regions within the input domain have been identified to be important for the activity of KdpD: (i) A cluster of positively charged Arg residues close to transmembrane domain four (TM4) is involved in the shift...
between kinase and phosphatase activities of KdpD (9). (ii) The four transmembrane domains seem to be important for the correct positioning of N- and C-terminal domains to each other as well as for perception and/or signal transduction (7, 17, 22). (iii) Amino acids 12–128, including a slightly modified Walker A site, seem to be important for the regulation of the phosphatase activity of KdpD (18). Here, we describe the overexpression and purification of the N-terminal domain of KdpD as well as its functional importance.

Although the Walker A motif found in the N-terminal domain of KdpD is slightly different from a classical motif (an additional amino acid is found between the first and second Gly), photoaffinity labeling by 8-azido-[$\alpha$-$\gamma$P]ATP shown here provides direct evidence that the N-terminal domain binds ATP. This has been shown for the full length sensor kinase and for the separately produced domain. The other known example for the same modification of the Walker A motif is found in the dethiobiotin synthetase (28), for which ATP binding was shown. ATP binding was detected only for the membrane-
Although other amino acid replacements within KdpD alter the growth conditions\(^{3}\) a sudden increase of the intracellular ATP main is unproved. However, the direct link between the increased intracellular ATP concentration and binding of ATP to the N-terminal domain is unproved.

In summary, the results presented here indicate that the N-terminal domain is essential for modulating the phosphatase activity of KdpD and thus proper signal transduction. The data provide direct evidence for the existence of an ATP-binding site within this domain. The role of this binding site remains unclear. KdpD activity can be restored \textit{in vitro} as well as \textit{in vitro} from two separately produced domains providing indirect evidence for the interaction between the N-terminal domain and the rest of the protein. Finally, the purification protocol for the N-terminal domain presented here provides the prerequisite to characterize this interaction in more detail.

Acknowledgments—We thank Dr. Roland Schmid for N-terminal determinations of KdpD proteolysis fragments and Mechthild Krabusch for technical assistance. We also thank Dr. Wolf Epstein, University of Chicago, for critical reading of the manuscript.

REFERENCES

1. Walderhaug, M. O., Pularek, J. W., Voelkner, P., Daniel, J. M., Hesse, J. E., Altendorf, K., and Epstein, W. (1996) \textit{J. Bacteriol.} 174, 2152–2159
2. Altendorf, K., and Epstein, W. (1992) \textit{Immunol. Today} 13, 177–178
3. Malli, R., and Epstein, W. (1998) \textit{J. Bacteriol.} 179, 498–502
4. Voelkner, P., Puppe, W., and Altendorf, K. (1993) \textit{Eur. J. Biochem.} 217, 1019–1026
5. Nakashima, K., Sugii, A., Momoi, H., and Mizuno, T. (1992) \textit{Mol. Microbiol.} 6, 1777–1784
6. Sugiura, A., Nakashima, K., Tanaka, A., and Mizuno, T. (1992) \textit{Mol. Microbiol.} 6, 1769–1776
7. Jung, K., Tjaden, B., and Altendorf, K. (1997) \textit{J. Biol. Chem.} 272, 10478–10852
8. Heeremann, R., Altendorf, K., and Jung, K. (1998) \textit{Biochim. Biophys. Acta} 1413, 114–124
9. Jung, K., and Altendorf, K. (1998) \textit{J. Biol. Chem.} 273, 26415–26420
10. Stallkamp, I., Dowhan, W., Altendorf, K., and Jung, K. (1999) \textit{Arch. Microbiol.} 172, 289–302
11. Launins, L. A., Rhoads, D. B., and Epstein, W. (1981) \textit{Proc. Natl. Acad. Sci. U. S. A.} 78, 464–468
12. Epstein, W. (1992) \textit{Acta Physiol. Scand. Suppl. 607}, 193–199
13. Malli, R., and Epstein, W. (1998) \textit{J. Bacteriol.} 180, 5102–5108
14. Sutherland, L., Cairney, J., Elmore, M. J., Booth, I. R., and Higgins, C. F. (1986) \textit{J. Biol. Chem.} 261, 805–814
15. Asha, H., and Gowrishankar, J. (1993) \textit{J. Biol. Chem.} 278, 4528–4537
16. Sugiura, A., Hirokawa, K., Nakashima, K., and Mizuno, T. (1994) \textit{Mol. Microbiol.} 14, 929–938
17. Puppe, W., Zimmann, P., Jung, K., Lucassen, M., and Altendorf, K. (1996) \textit{J. Biol. Chem.} 271, 25027–25034
18. Jung, K., and Altendorf, K. (1998) \textit{J. Biol. Chem.} 273, 17406–17410
19. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Assamizu, E., Nakamura, Y., Miyagawa, N., Hiroawa, M., Sugii, S., Satsudo, T., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. (1996) \textit{DNA Res.} 3, 109–116
20. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) \textit{Gene} 33, 103–119
21. Kollmann, R., and Altendorf, K. (1993) \textit{Biochim. Biophys. Acta} 1143, 62–66
22. Jung, K., Heeremann, R., Meyer, M., and Altendorf, K. (1998) \textit{Biochim. Biophys. Acta} 1372, 311–329
23. Guzman, M. L., Belin, D., Carson, M. J., and Beckwith, J. (1995) \textit{J. Bacteriol.} 177, 4121–4130
24. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) \textit{Gene} 77, 51–59
25. Epstein, W., and Davies, M. (1970) \textit{J. Bacteriol.} 101, 836–843
26. Laemmli, U. K. (1970) \textit{Nature} 227, 680–685
27. Peterson, G. L. (1977) \textit{Anal. Biochem.} 83, 346–356
28. Huang, W., Jia, J., Gibson, K. J., Taylor, W. S., Rendina, A. R., Schneider, G., and Lindqvist, Y. (1995) \textit{Biochemistry} 34, 10985–10995
29. Shyng, S. L., and Nichols, C. G. (1998) \textit{Science} 281, 1138–1141
30. Ohwada, T., and Sagisaka, S. (1987) \textit{Arch. Biochem. Biophys.} 259, 157–163
31. Walker, J. E, Saraste, M., Runswick, M. J., and Gay, N. J. (1982) \textit{EMBO J.} 1, 945–951

\(^{3}\) K. Jung, unpublished observation.

Associated N-terminal domain, but not for the solubilized peptide. Although the N-terminal domain is mainly composed of hydrophilic amino acid residues, there is a stretch of 17 hydrophobic amino acid residues around the Walker A site (3). From the differences found in the ability to bind axido-ATP, it is concluded that only the membrane-associated domain retains the proper folding to accommodate the nucleotide. It has been shown for the ATP-sensitive channel (K\textsubscript{ATP} channel), which is blocked by intracellular ATP, that binding affinity changes significantly depending on the membrane phospholipid phosphatidylinositol-4,5-bis-phosphate (29). Because negatively charged phospholipids influence the kinase activity of KdpD (10), it is conceivable that alterations in the attachment of this domain to the lipid bilayer influences the binding of the ATP.
The Hydrophilic N-terminal Domain Complements the Membrane-anchored C-terminal Domain of the Sensor Kinase KdpD of *Escherichia coli*

Ralf Heermann, Karlheinz Altendorf and Kirsten Jung

*J. Biol. Chem.* 2000, 275:17080-17085.
doi: 10.1074/jbc.M000093200 originally published online March 21, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000093200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 13 of which can be accessed free at http://www.jbc.org/content/275/22/17080.full.html#ref-list-1