Truncation of Amino Acids 12–128 Causes Deregulation of the Phosphatase Activity of the Sensor Kinase KdpD of Escherichia coli*

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The kdpFABC operon, which encodes the structural genes for the high affinity K⁺ transport complex KdpFABC, is regulated by the sensor kinase KdpD and the response regulator KdpE. KdpD is a bifunctional enzyme catalyzing the autophosphorylation by ATP and the dephosphorylation of the corresponding response regulator KdpE. Here, we demonstrate that the phosphatase activity of KdpD is dependent on ATP, whereas GTP, ITP, CTP, ADP, and GDP have no effect. The phosphatase activity requires only ATP binding, because nonhydrolyzable analogs (adenosine-5′-[(γ-thio)triphosphate and adenosine-5′-[(β,γ-imido)triphosphate) work as well. However, KdpD proteins missing amino acids 12–128 are characterized by a phosphatase activity that is independent of ATP. These proteins are still able to respond to K⁺ starvation, but an increase in osmolarity is no longer sensed. Comparison of different KdpD sequences reveals a conserved motif in this amino acid region that is very similar to a classical ATP-binding site (Walker A motif). Replacement of the conserved Gly⁵³, Lys⁸⁸, and Thr³⁹ residues in the consensus ATP-binding sequence results in a KdpD protein that causes a kdpFABC expression pattern comparable with that seen with KdpD proteins missing amino acids 12–128. However, in vitro phosphatase activity is comparable with that of wild-type KdpD. These results suggest that amino acids 12–128 of KdpD are important for its activity and that an additional ATP-binding site in the N-terminal region seems to be involved in modulation of the phosphatase activity.

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Phosphorylation and Dephosphorylation Assays—Inverted membrane vesicles containing KdpD (2 mg protein/ml) were incubated at room temperature in phosphorylation buffer, containing 50 mM Tris/HCl, pH 7.5, 10% glycerol, 0.5 mM MgCl₂, and 2 mM dithiothreitol. Phosphorylation was initiated by addition of 20 μM [γ-32P]ATP (2.38 Ci/mmol). At different times, aliquots were removed and mixed with an equal volume of double concentrated SDS sample buffer (25). After incubation for 4.5 min, an equimolar amount of KdpE was added to the KdpD-containing fractions, and the incubation was continued. Further aliquots were removed at different times and mixed with SDS sample buffer as described above.

To test dephosphorylation, purified KdpE prepared as described (17) was phosphorylated in the following manner. Wild-type KdpD (4 mg/ml) was incubated in phosphorylation buffer, except that MgCl₂ was replaced with 5 mM CaCl₂, and phosphorylation was initiated with 20 μM [γ-32P]ATP (2.38 Ci/mmol). After 5 min, purified KdpE (0.1 mg/ml) was added, and the incubation was continued for 1 min. KdpD-containing membrane vesicles were removed by centrifugation. ATP was removed by gel filtration through Sephadex G25 (preincubated in 50 mM Tris/HCl, pH 7.5, 10% glycerol, 2 mM dithiothreitol, 0.5 mM CaCl₂). Purified Kdp-E-P was used immediately. Dephosphorylation was initiated by addition of 20 mM MgCl₂, 20 μM ATP (or other nucleotides as specified), and inverted membrane vesicles containing KdpD (1 mg/ml). At different times aliquots were removed, and the reaction was stopped by addition of SDS sample buffer as described above.

All samples were immediately subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (25). Shortly before stopping SDS-PAGE an [γ-32P]ATP standard was loaded on the gels. Gels were dried, and phosphorylation of the proteins was detected by exposure of the gels to a phosphor screen. Phosphorylated proteins were quantified by image analysis using the PhosphorImager system of Molecular Dynamics.

RESULTS

Kinase and Phosphotransfer Activities and the Influence of N-terminal Truncations in KdpD—Inverted membrane vesicles containing equal amounts of either wild-type or truncated KdpD, as judged by Western blot analysis (data not shown), were tested for kinase activity. The time courses of autophosphorylation of different truncated KdpD proteins in comparison with wild-type KdpD is shown in Fig. 2. Autophosphorylation was tested to be linear within 0.5 min. Furthermore, transfer of the phosphorlyl group to KdpE was determined. The transfer of the phosphorlyl group to KdpE was very fast (within 15 s the phosphotransfer was complete). Phosphotransfer was detectable for all truncated KdpD proteins (data not shown).

Phosphatase Activity of KdpD and the Influence of N-terminal Truncations—In addition to the kinase activity KdpD catalyzes also the dephosphorylation of phosphorylated KdpE (14). Kdp-E-P itself is very stable; within 2 h no major loss of the phosphoryl group was detected. Dephosphorylation of Kdp-E-P was initiated by the addition of inverted membrane vesicles containing KdpD, 20 mM MgCl₂, and 20 μM ATP. Within about 5 min half-life of Kdp-E-P was dephosphorylated (Fig. 3A). The rate of dephosphorylation of wild-type KdpD was comparable with that of the truncated forms. For wild-type KdpD only ATP was effective to stimulate the phosphatase activity; GTP, ITP, CTP, ADP, and GDP were without effect. Furthermore, nonhydrolyzable ATP analogs, ATP-γ-S and AMP-PNP, were as effective as ATP (data not shown). In the absence of ATP a very slow dephosphorylation was observed when wild-type KdpD and KdpD (ΔΔ28–391)

Fig. 1. Schematic presentation of wild-type and different truncated forms of the sensor kinase KdpD. The model is based on both hydrophathy plot analysis and studies with LazZ/PhoA fusions (15). The boxes represent the four transmembrane domains (TM1–TM4). Sequence motifs characteristic of transmitter domains of sensor kinases (H, N, G1, F, and G2) are indicated in the upper part. In the lower part truncated KdpD proteins are schematically presented, where lines between the stippled bars comprise deleted parts of KdpD.

Furthermore, KdpD proteins missing amino acids 12–128 are characterized by a deregulated phosphatase activity. A conserved motif that is similar to a classical ATP-binding site (Walker A motif) can be found in this region. The strong dependence of the phosphatase activity of KdpD on ATP and alteration of the proposed nucleotide binding site by site-directed mutagenesis provide first evidence that ATP-binding modulates KdpD activity.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP was purchased from Amersham Pharmacia Biotech. All nucleotides were purchased from Sigma. NAP 10 columns; restriction enzymes. The DNA fragment was isolated from agarose gels to remove ATP were obtained from Amersham Pharmacia Biotech. All nucleotides were purchased from Sigma. NAP 10 columns; restriction enzymes. The DNA fragment was isolated from agarose gels to remove ATP were obtained from Amersham Pharmacia Biotech. All nucleotides were purchased from Sigma. NAP 10 columns; restriction enzymes. The DNA fragment was isolated from agarose gels to remove ATP were obtained from Amersham Pharmacia Biotech. All nucleotides were purchased from Sigma. NAP 10 columns; restriction enzymes. The DNA fragment was isolated from agarose gels to remove ATP were obtained from Amersham Pharmacia Biotech. All nucleotides were purchased from Sigma. NAP 10 columns; restriction enzymes. The DNA fragment was isolated from agarose gels to remove ATP were obtained from Amersham Pharmacia Biotech. All nucleotides were purchased from Sigma. NAP 10 columns; restriction enzymes. The DNA fragment was isolated from agarose gels to remove ATP were obtained from Amersham Pharmacia Biotech. All nucleotides were purchased from Sigma. NAP 10 columns; restriction enzymes. The DNA fragment was isolated from agarose gels to remove ATP were obtained from Amersham Pharmacia Biotech. All nucleotides were purchased from Sigma. NAP 10 columns; restriction enzymes. The DNA fragment was isolated from agarose gels to remove ATP were obtained from Amersham Pharmacia Biotech. All nucleotides were purchased from Sigma. NAP 10 columns; restriction enzymes. The DNA fragment was isolated from agarose gels to remove AT
were tested (Fig. 3B). Interestingly, KdpD (Δ12–228) and KdpD (Δ12–395) are characterized by a phosphatase activity that is independent of the presence of ATP.

Influence of N-terminal Truncations in KdpD on the Regulation of kdpFABC Expression—It has been found previously that in wild-type strains kdpFABC is expressed when medium concentration of K⁺ is below 2 mM. Furthermore, an increase in medium osmolarity at constant K⁺ concentration, a maneuver that reduces turgor, caused expression of kdpFABC (7). Signal transduction mediated by truncated forms of KdpD was previously tested in E. coli deleted for other K⁺ uptake systems (TrkG and TrkH) leaving Kdp as the main K⁺ transport system. Because of the importance of the Kdp system as the major K⁺ uptake system, kdpFABC expression was never completely blocked (17). Therefore, we tested the transcriptional induction of kdpFABC with E. coli HAK006 (11) that synthesizes the constitutive K⁺ uptake systems, TrkH, TrkG, and Kup. This strain lacks the functional kdpFABC operon as well as the kdpD gene on the chromosome but contains the intact kdpE gene under the control of its own promoter. In addition, this strain harbors a kdpFABC promoter-lacZ fusion gene on the chromosome. Because the amount of regulatory proteins is very critical in signal transduction (high levels of KdpD prevent complementation of a kdpD null strain), E. coli HAK006 was transformed with plasmids pBD and its derivatives. In plasmid pBD KdpD is under the control of the arabinose promoter (22). When cells were grown in the absence of an inducer (arabinose) and in the presence of the repressor glucose, the amount of KdpD produced was sufficient to complement a kdpD null strain. The truncated proteins were tested for their response to an increase in osmolarity and K⁺ limitation in comparison with that of wild-type KdpD.

Characterization of KdpD-G37A,K38A,T39C—Sequence comparison of the first 200 amino acids of so far six known KdpD sequences of different microorganisms revealed that a conserved motif exists that is similar to a classical ATP-binding site (Walker A motif) (29) (Fig. 4). In addition, we found that KdpD proteins missing these amino acids (KdpD(12–391)) are characterized by a phosphatase activity that is independent of ATP and cause deregulation of kdpFABC expression. To test whether KdpD contains a second ATP-binding site, site-directed mutagenesis was used to inactivate this site. Therefore, codons for Gly³⁷, Lys³⁸ and Thr³⁹ were replaced by codons for Ala³⁷, Ala³⁸, and Cys³⁹ in kdpD. The resulting KdpD protein was tested for kinase and phosphatase activity. The rate of autophosphorylation was comparable with that of wild-type KdpD; after addition of KdpD the phosphorylation was increased, whereas only basal levels of β-galactosidase activity were detectable when cells were cultivated at very low K⁺ concentrations. However, β-galactosidase activity was much lower in comparison with that of wild-type KdpD.
**Phosphatase Activity of KdpD**

**TABLE I**

| KdpD construct | β-Galactosidase activity |
|----------------|-------------------------|
|                | 0.1 mM K⁺ | 1 mM K⁺ | No osmotic stress | 0.6 M NaCl |
| Wild-type KdpD | 384 ± 7 | 750 ± 6 | 13 ± 5 | 552 ± 4 |
| KdpD (Δ12–228) | 80 ± 4 | 4 ± 2 | 4 ± 2 |
| KdpD (Δ12–395) | 328 ± 5 | 3 ± 4 |
| KdpD (Δ128–391) | 3466 ± 684 | 12 ± 450 |
| KdpD-G37A,K38A,T39C | 309 ± 4 | 2 ± 4 |

G37A,K38A,T39C catalyzed the dephosphorylation of KdpE–P. In the absence of ATP the rate of dephosphorylation was very slow, and it could be stimulated in the presence of ATP. Rates were comparable with that of wild-type KdpD or KdpD(Δ128–391) (data not shown). Furthermore, transcriptional induction of $kdpFABC$ was tested using E. coli strain HAK006 transformed with plasmid pBD-G37A,K38A,T39C. Inactivation of the proposed ATP-binding site impaired $kdpFABC$ expression in response to an increase of the osmolarity of the medium (Table I). As shown before with truncated proteins missing amino acids 12–395 or 12–228, respectively, KdpD-G37A,K38A,T39C showed a modest ability to induce reporter gene expression in response to K⁺ starvation (Table I).

**DISCUSSION**

Dephosphorylation of the response regulator is well studied in various sensor kinase/response regulator systems and characterized by diverse mechanisms (16). The sensor kinase EnvZ by itself is able to enhance the rate of dephosphorylation of phospho-OmpR (31–33). In the case of NRII an auxiliary protein PII is involved (34–36). In the chemotaxis system, dephosphorylation of CheY is mediated by a separate protein, CheZ (37), and in the process of sporulation of Bacillus subtilis different phosphatases have been identified (38).

Using purified and reconstituted KdpD we have recently shown that dephosphorylation of KdpE–P is only dependent on the sensor kinase (14). In this communication we demonstrate for the first time that the rate of dephosphorylation can be significantly increased in the presence of ATP or nonhydrolyzable ATP analogs. In contrast, other nucleotides do not have an effect at all. The dephosphorylation activity of EnvZ is also stimulated by ATP, but in contrast to KdpD, also by ADP (31–33).

In comparison with other sensor kinases, KdpD contains an extended hydrophilic N-terminal domain of 400 amino acids exposed to the cytoplasm. This N-terminal domain is characterized by a high degree of homology between the so far known KdpD sequences from different organisms (Fig. 4). It is tempting to speculate that this domain, in addition to the four transmembrane helices, is involved in stimulus perception.

We found that the stimulation of phosphatase activity of truncated forms of KdpD missing amino acid 12–128 is no longer dependent on ATP. Furthermore, we demonstrated that amino acids 12–128 of the N-terminal domain of KdpD are important for the regulation of $kdpFABC$ expression. KdpD(Δ12–395) and KdpD(Δ1–228) are not able to respond to an increase in osmolarity raised by the addition of NaCl. When cells synthesizing these truncated proteins were grown under K⁺-limiting conditions, the $kdpFABC$ operon was expressed, but the level of expression was much lower in comparison with that of wild-type KdpD. In contrast, the truncation of amino acids 128–391 results in a KdpD protein that gave rise to the same expression level of the $kdpFABC$ operon as wild-type KdpD.

In addition to the strong dependence of the phosphatase activity on the presence of ATP we found that amino acids 31–39 comprise a motif that is conserved among the KdpD sequences (Fig. 4) and that is very similar to a classical ATP-binding site (Walker A motif) of many ATP-requiring enzymes (29). As indicated in Fig. 4, a Walker B motif can also be found. The most frequently published form of the Walker A motif is shown above (29). The figure was created with the PIMA Multiple Sequence Alignment program of the BCM Search Launcher (Human Genome Center, Baylor College of Medicine, Houston, TX).
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expression is reduced to the same extent as it is seen with KdpD proteins missing this putative ATP-binding site. This results are in favor of the existence of a regulatory ATP-binding site in the N-terminal domain of KdpD.

It is conceivable that the low level of kdpFABC expression is because of a decrease of the amount of KdpE–P. Because in vitro no differences were seen in the kinase activities of KdpD proteins missing this site, it is suggested that ATP-binding regulates the phosphatase activity of KdpD. Indeed, we observed a deregulation of the phosphatase activity of KdpD proteins missing this site. Therefore, an interaction of the N- and the C-terminal domain may also contribute to the regulation of the phosphatase activity. An interaction of the input and transmitter domain depending on the stimulus might influence the switch between kinase and phosphatase activity in such a way that through conformational changes the accessibility of the putative regulatory ATP-binding site may vary and thereby the phosphatase activity might be tightly regulated. Although it is known that upon an osmotic upshift intracellular ATP concentration (Walker A) either by truncation or mutagenesis diminish the phosphatase activity of KdpD. Indeed, we observed a deregulation of the phosphatase activity of KdpD proteins missing this site. Therefore, an interaction of the N-terminal domain of KdpD.

In summary, we could show here that the phosphatase activity of KdpD can be increased in the presence of ATP or ATP analogs. Furthermore, KdpD proteins missing amino acids 12–128 are characterized by a deregulated phosphatase activity in vitro. Finally, KdpD proteins missing a conserved motif in the N-terminal domain, which is similar to a classical ATP-binding site (Walker A), either by truncation or mutagenesis diminish kdpFABC expression in response to K+ limitation or osmotic upshift.

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REFERENCES

1. Polarek, J. W., Williams, G., and Epstein, W. (1992) J. Bacteriol. 174, 2145–2151

2. Altendorf, K., and Epstein, W. (1996) Bioembranes (Lee, A. G., ed) Vol. 5, pp. 403–420, JAI Press Inc., London

3. Altendorf, K., Voelkner, P., and Fuji, W. (1994) Rev. Microbiol. 145, 374–381

4. Hesse, J. E., Wizeciorz, L., Altendorf, K., Reisic, A. S., Dorus, K., and Epstein, W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4746–4750

5. Siebers, A., and Altendorf, K. (1992) Alkal Cation Transport Systems in Prokaryotes (Bakker, E. P., ed) pp. 225–252, CRC Press, Boca Raton, FL

6. Epstein, W. (1986) FEMS Microbiol. Rev. 39, 73–78

7. Laimins, L. A., Rhoads, D. B., and Epstein, W. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 456–468

8. Epstein, W. (1992) Acta Physiol. Scand. 146, 193–199

9. Csonka, L. N., and Hanson, A. D. (1991) Annu. Rev. Microbiol. 45, 569–606

10. Asha, H., and Gowrishankar, J. (1993) J. Bacteriol. 175, 4528–4537

11. Sugiuira, A., Hirokawa, K., Nakashima, K., and Mizuno, T. (1994) Mol. Microbiol. 14, 929–938

12. Walderhaug, M. O., Polarek, J. W., Voelkner, P., Daniel, J. M., Hesse, J. E., Altendorf, K., and Epstein, W. (1992) J. Bacteriol. 174, 2152–2159

13. Voelkner, P., Puppe, W., and Altendorf, K. (1993) Eur. J. Biochem. 217, 1019–1026

14. Jung, K., Tjaden, B., and Altendorf, K. (1997) J. Biol. Chem. 272, 10847–10852

15. Zimmann, P., Puppe, W., and Altendorf, K. (1995) J. Biol. Chem. 270, 28292–28298

16. Stock, J. B., Surette, M. G., Levit, M., and Park, P. (1995) in Two-Component Signal Transduction (Hoch, J. A., and Silhavy, T. J., eds) pp. 25–51, American Society for Microbiology, Washington, D.C.

17. Puppe, W., Zimmann, P., Jung, K., Lucassen, M., and Altendorf, K. (1996) J. Biol. Chem. 271, 25027–25034

18. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119

19. Kollmann, R., and Altendorf, K. (1993) Biochim. Biophys. Acta 1143, 62–66

20. Nakashima, K., Sugiuira, A., Kanamurau, K., and Mizuno, T. (1993) Mol. Microbiol. 7, 109–116

21. Jung, K., Heermann, R., Meyer, M., and Altendorf, K. (1998) Biochim. Biophys. Acta, in press

22. Guzman, L.-M., Belin, D., Carson, M. J., and Beckwith, J. (1995) J. Bacteriol. 177, 4121–4130

23. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467

24. Hattori, M., and Sakaki, Y. (1986) Anal. Biochem. 152, 232–238

25. Laemmli, U. K. (1970) Nature 227, 680–685

26. Epstein, W. (1970) J. Bacteriol. 101, 836–843

27. Miller, J. H. (1992) A Short Course in Bacterial Genetics, pp. 72–74, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

28. Peterson, G. L. (1977) Anal. Biochem. 83, 346–356

29. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) EMBO J. 1, 945–951

30. Beier, D., Deppiesche, H., and Gross, R. (1998) Mol. Gen. Genet. 252, 169–176

31. Ige, M. N., Ninfa, A. J., Stock, J. B., and Silhavy, T. J. (1989) Gene 77, 1725–1734

32. Aiba, H., Nakasuai, F., Mizushima, S., and Mizuno, T. (1989) J. Biol. Chem. 264, 14090–14094

33. Aiba, H., Mizuno, T., and Mizushima, S. J. (1989) J. Biol. Chem. 264, 8563–8567

34. Keener, J., and Kustu, S. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4976–4980

35. Ninfa, A. J., and Magasanik, B. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5909–5913

36. Kamberov, E. S., Atkinson, M. R., Chandran, P., and Ninfa, A. J. (1994) J. Biol. Chem. 269, 28294–28299

37. Blat, Y., and Eisenbach, M. (1994) Biochemistry 33, 902–906

38. Perego, M., Hanstein, C., Welsh, K. M., Djavakhishvili, T., Glaser, P., and Hoch, J. A. (1994) Cell 79, 1047–1055

39. Traut, T. W. (1994) Eur. J. Biochem. 222, 9–19

40. Saraste, M. Sibbald, P. R., and Wittinghofer, A. (1990) Trends Biochem. Sci. 15, 430–434

41. Huang, W., Lindqvist, Y., Schneider, G., Gibson, K. J., Flint, D., and Lorimer, G. (1994) Structure 2, 407–414

42. Ohwada, T., and Sagisaka, S. (1987) Arch. Biochem. Biophys. 259, 157–163

43. Charles, T. C., and Nester, E. W. (1993) J. Bacteriol. 175, 6614–6625