The KdpF Subunit Is Part of the K⁺-translocating Kdp Complex of Escherichia coli and Is Responsible for Stabilization of the Complex in Vitro*

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K⁺ ions play a crucial role in maintaining turgor, which is vital for growth of many bacterial cells (1, 2). Therefore, Escherichia coli has 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 osmolality of the medium (for an overview see Refs. 3 and 4). At low K⁺ concentrations (<1 mM) the Kdp system is synthesized to function in a growth-limited manner (5, 6), which is not the case for high-affinity (Km, 2 μM) uptake with medium rate (Vmax, 0.15 μmol g⁻¹ min⁻¹) (7). The structural genes are organized in the kdpABC operon (8), and biochemical studies revealed that the three subunits KdpA, KdpB, and KdpC are sufficient to function as a functional transporter (Refs. 6 and 9; for reviews see Refs. 10 and 11). The largest 72-kDa KdpB subunit spans the membrane probably six times and is phosphorylated during the catalytic cycle (12, 13). KdpB is homologous to the large subunit of other P-type ATPases and shares the common key structures of this ATPase class (14, 15). The 59-kDa KdpA subunit is predicted to span the membrane 10 times and is believed to be the subunit that binds and transports K⁺ (16). The 20-kDa KdpC subunit probably traverses the membrane once close to the N terminus, leaving the C-terminal portion exposed to the cytoplasm, and is important for the assembly of the enzyme complex (17). The adjacent kdpDE operon codes for the two proteins KdpD and KdpE, which regulate the expression of the structural genes (18) and which belong to the family of sensor kinase/response regulator systems (19, 20).

An extended region was recognized between the start site of the mRNA transcript of the kdp operon and the ATG start codon of kdpA. Sequence analysis revealed that an additional open reading frame, kdpF, exists upstream of the kdpABC structural genes. Therefore, the corresponding operon should be referred to as kdpFABC. Duced from the DNA sequence, the putative KdpF protein seems to be another candidate for a diverse group of recently identified small, hydrophobic proteins associated with membrane transport complexes (for details, see “Discussion”). In bacteria, the function of these subunits has still to be elucidated.

In this report it is clearly demonstrated that kdpF is indeed expressed and that KdpF is associated with the purified complex. Although deletion of kdpF revealed that KdpF is not necessary for growth of cells on low K⁺ medium, the ATPase activity of the purified complex is impaired in the absence of KdpF. By addition of the purified KdpF or high amounts of E. coli lipids to the purified, detergent-solubilized KdpABC complex, an K⁺-stimulated ATPase activity comparable with that of the wild type enzyme could be recovered. In summary, it could be demonstrated that the KdpF protein stabilizes the enzyme complex in vitro, thereby possibly acting as a kind of lipid-like peptide for the holoenzyme.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of analytical grade. [35S]Methionine (1000 Ci/mol), [α-32P]dATP (1000 Ci/mol), and [35S]protein A (10 μCi/100 μl) were purchased from Amersham Pharmacia Biotech. CM 23-cellulose was obtained from Serva (Heidelberg, Germany). E. coli lipids were purchased from Avanti (catalog number 100600). Nitrocellulose membranes (0.45 μm) were obtained from Schleicher & Schuell. Goat anti-rabbit IgG alkaline phosphatase conjugate was purchased from Biometra (Heidelberg, Germany). Restriction endonucleases, shrimp alkaline phosphatase, T4 DNA ligase, Klenow fragment of DNA polymerase I, and dNTPs were obtained from Roche Molecular Biochemicals, Life Technologies, Inc., and New England Biolabs (Schwalbach, Germany).

Bacterial Strains, Plasmids, and Growth Conditions—E. coli strains and plasmids used are listed in Table I. All strains are derivatives of E. coli K12, and cells were grown according to Refs. 22 and 26.
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### Table I

| Strain/plasmid | Relevant markers | Source/reference |
|---------------|------------------|------------------|
| TKW2305       | ΔkdpFABC ΔatpA thr lacZ nagA trkA405 thrD1 | lab collection   |
| TK2205        | ΔkdpFABC thrA lacZ nagA trkA405 thrD1 | this study |
| TK228505      | ΔkdpFABC thrA lacZ nagA trkA405 thrDK1 polA | this study |
| TKT2285-001   | TKW228505 with integrated plasmid pTM001 | this study |
| TKT2285-201   | TKW228505 with integrated plasmid pTM001 | this study |
| TKT2285-301   | TKW228505 with integrated plasmid pTM001 | this study |
| TKT2285-401   | TKW228505 with integrated plasmid pTM001 | this study |
| TKT2285-501   | TKW228505 with integrated plasmid pTM001 | this study |
| DK6           | F' ΔatpB-C str tetr hsdR miniA miniB purE pdcX his ile met | 23 |
| pJLA503       | 3°-promoter Amp' | 24 |
| pJL101        | pJLA303 hdpFA' | this study |
| pJL302        | pBR322/Amp'/ECOR fragment of the tet gene Amp' | lab collection |
| pJID          | pDkFABC/pJd + 4.9-kilobase fragment with kdpFABC (constitutive expression) | lab collection |
| pSR4          | kdpFABC/under control of the kdp promoter Amp' | 25 |
| pTM001        | pSR4/Amp'/SacI fragment downstream of kdpC | this study |
| pTM201        | pTM001 kdpF (275-97) | this study |
| pTM301        | pTM001 kdpF (292C) | this study |
| pTM401        | pTM001 kdpF (G29A) | this study |
| pTM501        | pTM001 kdpF (35-59) | this study |
| pTM502        | pJIDkdp/Amp'/SacI fragment downstream of kdpC | this study |
| pTM902        | pMTM001 kdpF (292C) | this study |
| pTM100        | pMTM002 kdpF (G29A) | this study |

**Construction of Plasmids and Strains—** The methods used for handling recombinant DNA and for transformation of *E. coli* cells were as described (27). Treatment of DNA with restriction endonucleases, T4 DNA ligase, shrimp alkaline phosphatase, and isolation of plasmids was performed following the protocols of the suppliers.

Plasmid pTM1 was generated by digestion of vector pJLA503 (24) with SalI treatment, followed by double digestion with Klenow DNA polymerase and shrimp alkaline phosphatase, and ligation of the *SalI* fragment from plasmid pTM002 (see below) containing the *kdpF* gene. The resulting plasmid was digested with EcoRI (to remove sequences from pTM002) and religated to yield pTM1.

All other vectors used in this study are derivatives of plasmids pSR4 (25) and pJIDkdp (kindly provided by Dr. W. Epstein, University of Chicago). In both plasmids an *EcoRI* deletion was introduced to inactivate the posterior of two *EcoRI* restriction sites enclosing the *kdpFABC* genes. Each of the resulting plasmids pTM001 and pTM002 gave rise to a series of mutant vectors, designated pTMX01 and pTMX02 (Fig. 1). The difference between vectors of the series pTMX01 and pTMX02 resides in the regulatory region upstream of the *kdpFABC* operon, where the *EcoRI* vector region resembles that of the wild type sequence including 472 bp upstream of the *kdpA* start codon. The expression of the *kdp* genes is under control of the regulatory proteins KdpD and KdpE, which are chromosomally encoded. In vectors of the series pTMX02 the *kdpFABC* genes are constitutively expressed under control of a promoter, which is still part of the truncated tetra-cycline resistance gene of the vector. With the latter series of vectors it was possible to express the mutated *kdp* genes in minicells.

Transfer of *in vitro* constructed, plasmid-encoded *kdpF* mutations to the chromosome was carried out using the *polA* technique based on the method described by Gutterson and Koshland (28). For this purpose strain TKW228505 was used. Only carbunculin-resistant clones growing well on K10 agar plates were used for further investigations.

Site-directed Mutagenesis—To introduce point mutations in the two stage PCR technique was used (29). The outside primers kdp11 (5'-CTCCATAAAGTCTGTTTTATTACTGG-3') and kdp22 (5'-AGATTAAGGCTACAGTGACCTCAGG-3') corresponding to the 3' parts (underlined) of the following sequences in *kdp*: bp 62–77 (kdp21) and bp 32–16 (kdp22). The 5' part of the primers contains a HindIII restriction site and in addition 7 nucleotides to ensure the cleavability of the PCR products by HindIII. With these primers a 24-bp deletion was produced at the 5' end of *kdpF* and at the same time a new HindIII restriction site suitable for identification of relevant clones was generated. After the first stage of PCR, the resulting fragments were tailor-cut with HindIII, ligated, and after gel purification subjected to a second run. The mutant PCR product was digested with EcoRI/StyI, and the resulting fragment was cloned into vector pTM001.

Plasmid pTM201 was generated from the predecessor pTM101, in which a double point mutation was introduced at position 24/25 to create a *SalI* restriction site in front of the *gtg* start codon. Following digestion with *SalI* and treatment with Klenow DNA polymerase and blunt end ligation yielded a 72-bp deletion encompassing the start codon. These manipulations leave 21 bp upstream of the *kdpA* gene intact including the putative ribosome binding site of *kdpA*.

Synthesis and Detection of Plasmid-encoded Proteins in Minicells—For the preparation of minicells according to the method of Reeve (31) strain DK6 was used. Cells were grown and harvested as described (23). To perform the labeling reactions 0.1 ml aliquots of minicells were diluted in 1 ml of ice-cold K115 salt solution, pelleted by centrifugation for 3 min at 10,000 × g (4 °C) and resuspended in 100 μl of K115 medium containing 0.4% glucose and 20 μg/ml n-cyloscrine. The cells were preincubated for 30 min at 30 °C. After addition of 5 μl of DIFCO methionine assay mix, the probes were shifted to 37 or 42 °C (DK6/pTM1). After 2 min of equilibration, 10 μCi of [35S]methionine were added, and the incubation was continued for 10 min. The reaction was stopped by addition of 10 μl of unlabeled methionine (0.1 μl) and centrifugation for 2 min at 8,000 × g. Pellets were resuspended in electrophoresis sample buffer (32), heated for 5 min at 100 °C, treated for 2 min in a sonifier bath, and subjected to SDS-PAGE (32). For autoradiography, the gels were pre Autoradiography, the gels were pre-treated with 5% methanol, 2% glycerol to prevent cracking, dried, and exposed for 3 days to an x-ray film (Kodak, X-OMAT AR, BIOMAX MR).

Preparations—The Kdp complex was prepared from *E. coli* strain TKW3205 harboring one of the different plasmids using the two column purification protocol described previously (9). For purification of the KdpF peptide, the protocol for the isolation of subunit c of the ATP synthase using C/M extraction, and diethyl ether precipitations were applied (33–35). Starting with 10 g of cells of strains TKW228505 or TK228505/pSR4 or TK228505/pTM001 resuspended in 12 ml of H2O. The sample, obtained by C/M extraction and two successive diethyl ether precipitations is called “KdpF extract” throughout the manuscript. In parallel, the enriched KdpF fraction (without diethyl ether precipitations) was applied (25–27) starting with 10 ml of cells of strains TKW3205/pSR4 or TK228505/pTM001. The enriched KdpF fraction (without diethyl ether precipitations) was applied (33–35) starting with 10 ml of cells of strains TKW3205/pSR4 or TK228505/pTM001. The enriched KdpF fraction (without diethyl ether precipitations) was applied (33–35) starting with 10 ml of cells of strains TKW3205/pSR4 or TK228505/pTM001.
The transfer of KdpF from organic solvents to detergent solutions was performed in the following way. A KdpF-containing sample (100 μl) was mixed end-over-end with 45 μl of chloroform and 25 μl of H2O (10 times), and after phase separation the upper methanol/H2O phase was sucked off. After doubling the volume of the samples with chloroform and treated with an ion exchange matrix (AG 50W-X8, Bio-Rad). The matrix-assisted laser desorption/ionization mass spectrometry was performed with a VISION 2000 (Finnigan, Bremen, Germany) endowed with a 2,5-dihydroxy benzoic acid matrix. The total impact energy was 20 keV, and the laser was calibrated at 337 nm. The matrix-positive ion and the visualization was performed according to Blake et al. (39). For quantification the blot was dried, and bands were cut out and directly counted in a γ-counter. For calibration a preparation of homogeneous Kdp complex was used.

**Assays**—Protein was determined by the method of Hartree (40). High resolution SDS-PAGE was performed according to Schagger and von Jagow (32) using 16.5% T and 3% C. To obtain strongly focussed bands, urea was replaced by glycerol (10%). Proteins were visualized by silver staining (41). The phospholipid content in samples obtained from C/M extraction or chromatography on CM 23-cellulose or from E. coli lipid solutions (purchased from Avanti) was determined according to Ames (42). The ATPase activity of the Kdp complex was determined as described by Altendorf et al. (11).

**RESULTS**

The kdpABC operon contains an unusually large stretch of 116 bp between the kdp regulatory sites and the start codon of the kdpA gene. Analysis of this sequence revealed that an additional open reading frame, kdpF, exists starting at position 29 with a GTG and ending with a stop codon, which overlaps by one nucleotide with the start codon of the kdpA gene. The protein sequence deduced from the DNA sequence calls for a small putative protein consisting of 29 amino acids with a calculated molecular mass of 3072 Da (Fig. 2). A hydrophobicity plot according to the algorithm of Kyte and Doolittle (44) predicts that this protein is very hydrophobic in nature and thus very likely to be membrane-embedded (data not shown).

**Detection of the kdpF Gene Product and Verification of the Start Codon of kdpF by Site-directed Mutagenesis**—The expres-
sion of the kdpF gene by plasmid pTM1 was investigated using \(^{35}\text{S}\)methionine-labeled minicells derived from strain DK6 (Fig. 3). In a second approach the presence of the KdpF peptide could be verified in the purified Kdp complex by SDS-PAGE visualizing the proteins by silver staining (Fig. 4). As can be taken from SDS-PAGE (Figs. 3 and 4), KdpF exhibits a molecular mass of 4000–6000 Da, considerably higher than expected from the amino acid composition. Such a behavior has also been observed for other proteins of this class. This difference seems to be dependent on the protein itself and/or on the lipid content of the sample (compare Figs. 4 and 9).

To substantiate further that the GTG at position 29 represents the start codon for kdpF, the plasmid series pTMX02 was used, in which the genes of the kdpFABC operon were expressed constitutively from a promoter belonging to the regulatory sites of the tetracycline resistance gene (Fig. 1). Replacing the GTG by a CTG codon (pTM302), gene expression in minicells revealed that kdpF was not detectable any more (Fig. 3). This was also the case for pTM202, which carries a deletion of kdpF encompassing the GTG start codon, leaving the putative ribosomal binding site for kdpA intact. In contrast, changing the GTG to an ATG (pTM402), the expression of kdpF was enhanced as expected. Taken together, these data provide strong evidence that the GTG codon at position 29 represents the translational start site for kdpF.

**Effect of Mutations within kdpF on Cell Growth under K\(^{+}\) Limiting Conditions**—To address the question whether mutations in kdpF give rise to a detectable phenotype, strain TK22285 (ΔkdpFABC5) was transformed with plasmids of the series pTMX01. In this case the expression of kdpFABC genes was under control of the chromosomally encoded regulatory proteins KdpD and KdpE. When cells of TK22285/pTMX01 (start codon: CTG) or TK22285/pTM401 (start codon: ATG) were grown in K0.1 minimal medium, where the limiting K\(^{+}\) concentrations served as the environmental stimulus for the expression of the plasmid-encoded kdpF genes, no significant change in growth could be observed compared with TK22285/pTM001 (wild type start codon: GTG) (data not shown). To avoid overproduction from the altered kdpF gene, the mutations were transferred to the chromosome using strain TK22285/pTM001 (ΔkdpFACS5 polA). In comparison with the control (TK22285–001, rate of growth (μ) = 0.60·h\(^{-1}\)), the growth of strains TK22285–301 (μ = 0.62·h\(^{-1}\)) and TK22285–401 (μ = 0.62·h\(^{-1}\)) was hardly affected, whereas the growth rate of TK22285–201 (μ = 0.56·h\(^{-1}\)) was significantly impaired (Fig. 5). Because in the case of strain DK6/pTM201 the expression of the kdpABC genes was also affected by the deletion of kdpF (Fig. 3), probably produced by polarity effects, a smaller kdpF deletion of only 24 bp was generated (pTM501), leaving the GTG start codon and most of the kdpF gene intact. A transfer of this smaller deletion to the chromosome revealed that the growth rate of TK22285–501 (μ = 0.60·h\(^{-1}\)) was identical to that of the control (Fig. 5). These data clearly demonstrate that the inactivation of kdpF (pTM501), which does not affect the expression of the kdpABC operon, still leads to a functional KdpABC complex in vivo.

**Effect of the Mutations within kdpF on the Kdp Complex in Vitro**—To study the influence of the mutations in kdpF at the level of the Kdp complex, the enzyme complex was purified, subjected to SDS-PAGE, and probed with polyclonal antibodies raised against the Kdp complex. The yield of Kdp complexes...
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Table II

ATPase activity of the Kdp complex at different stages of purification in the presence or absence of KdpF

The specific ATPase activity is based on the amount of KdpABC determined by [125I]protein A labeling taking into account the variations in the amount of enzyme complex derived from strain TKW3205/pSR4 and TKW3205/pTM501. The KdpF extract used was obtained by C/M extraction and two subsequent diethyl ether precipitations as described under "Experimental Procedures."

| Preparation step                      | TKW3205/pSR4 | TKW3205/pTM501 | TKW3205/pTM501 + KdpF extract |
|---------------------------------------|--------------|----------------|-------------------------------|
| Everted vesicles                      | 3.4          | 2.2            | 3.9                           |
| EDTA-washed vesicles                  | 4.4          | 5.3            | 7.3                           |
| Aminoxide solubilization membrane extract | 8.4        | 2.0            | 6.0                           |
| Fractogel TSK AF-Red Kdp fraction pool | 11.5        | 0.7            | 10.9                          |

FIG. 6. Extraction of KdpF with C/M followed by two successive diethyl ether precipitations. Samples of the different steps of the extraction procedure as described under "Experimental Procedures" were separated by SDS-PAGE and visualized by silver staining. Lane 1, whole cells; lane 2, filter backlog; lane 3, water/methanol phase; lane 4, chloroform phase; lane 5, concentrated chloroform phase; lane 6, precipitate after the first diethyl ether precipitation; lane 6b, precipitate after the second diethyl ether precipitation. The organic solvent of the samples was removed as described under "Experimental Procedures," and the dried samples were dissolved in electrophoresis sample buffer. Myoglobin fragments (st1; Merck) and Mark12 (st2; Novex) were used as standards. The KdpF peptide and the molecular masses of the marker proteins are indicated.

FIG. 7. Recovery of the ATPase activity of the KdpABC complex in the presence of KdpF or E. coli lipids. The KdpABC complex was prepared from strain TKW3205/pTM501 and mixed with KdpF extract or E. coli lipids as described under "Experimental Procedures." In both KdpF extract and the lipids, the amount added was standardized on the concentration of phospholipids present in the sample. The K+ -stimulated ATPase activity was determined in the presence of 1 mM KCl. □, KdpABC complex mixed with KdpF extract; ○, KdpABC complex mixed with E. coli lipids.

intact KdpFABC complex after the column steps, probably because of the loss of the natural lipid environment. Addition of KdpF extract brings this residual activity back to the level of the intact KdpFABC complex (Table II). Because the ATPase activity is stimulated by K+ (Fig. 8A) and completely inhibited by ortho-vanadate (Fig. 8B), the native conformation of the Kdp
complex is only maintained in the presence of the four subunits KdpA, KdpB, KdpC, and KdpF.

Purification of C/M-extracted KdpF by Ion Exchange Chromatography—To differentiate between the effect caused by KdpF itself on the one hand and by the lipids on the other in more detail, KdpF obtained by C/M extraction was further purified by chromatography on CM 23-cellulose. In accord with the deduced amino acid composition of the KdpF peptide, no absorption could be detected at 280 nm (Fig. 9, A and C). Therefore, each fraction containing proteins of the appropriate size (Fig. 9, B and D) was tested for the ability to recover the K⁺-stimulated ATPase activity of the purified KdpABC complex. Fig. 9 clearly demonstrates that only fractions 18–28 (with the maximum at fraction 25) derived from strain TKW3205/pSR4 (kdpFABC), in contrast to that of strain TKW3205/pTM501 (kdpABC), were capable to recover the K⁺-stimulated ATPase activity. A determination of the phospholipid content within those fractions revealed that it was below the detection limit. These results provide evidence that the recovery of the ATPase activity is due to the addition of the KdpF peptide.

To verify that the purified peptide is indeed KdpF, a matrix-assisted laser desorption/ionization mass spectrometry analysis was carried out of fraction 25 derived from strain TKW3205/pSR4 (Fig. 9B). With this approach we were able to detect a mass at 3100.7 Da, which corresponds well with the molecular mass of the protonated formyl-KdpF.

DISCUSSION

A number of membrane-bound complexes that mediate transport of cations across biological membranes in different organisms and tissues have been shown to contain small hydrophobic subunits, like the γ subunit of the Na⁺,K⁺-ATPase (46), sarcolipin (47, 48), plasma membrane proteolipids 1 and 2 (49), and phospholamban (50). None of these polypeptides, however, is essential for the transport function of the enzyme complexes in vivo. In this study, we report on the smallest protein found to date, KdpF from E. coli, which also belongs to this diverse class of polypeptides. Comparable putative peptides can be found in kdp sequences of other organisms, like Clostridium acetobutylicum (51), Synechocystis sp. PCC6803 (genome project), and Mycobacterium tuberculosis (genome project).

Deletion of the kdpF gene did not give rise to a phenotype. However, a purified KdpABC complex (lacking KdpF) did not exhibit ATPase activity any more. Addition of purified KdpF to the KdpABC complex restored that activity back to wild type level. These results point to a stabilizing function of KdpF in vitro, which also could be mimicked by high concentrations of lipids. In summary, KdpF seems to function as a specific, stabilizing lipid-like peptide in the Kdp complex.
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in or altered during electron or proton transfer (57), and it has been proposed that subunit IV is an evolutionary remnant (56). Another group of peptides that influence the activity/affinity of their corresponding eukaryotic enzyme complexes share with KdpF one membrane-spanning domain followed or flanked by hydrophilic regions. However, these similarities are confined to the structural level and could not be extended to the level of the amino acid sequence. The \( \gamma \) subunit (58–61 amino acids) of the Na\(^+\)/K\(^-\)-ATPase was ultimately proven to be part of the complex by Mercer et al. (58). After dissociation of the \( \gamma \) subunit the purified complex is still active, and maximal turnover of the Na\(^+\)/K\(^-\)-ATPase is not affected (59). This subunit, which is tissue specifically expressed, seems to affect the external cation-binding site of the transport complex (46). A more drastic effect could be observed for the plasma membrane H\(^+\)-ATPase of Saccharomyces cerevisiae, where two highly homologous proteolipids (plasma membrane proteolipids 1 and 2; 43 amino acids) modulate the ATPase activity. Deletion of both genes, coding for the two peptides, results in a membrane-bound enzyme complex exhibiting only 50% of the normal ATPase activity (49). Sarcolipin (31 amino acids) (47) and phospholamban (52 amino acids) (60) seem to be regulatory components of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) (48). In both cases the synthesis of these peptides is tissue-specific, and both peptides influence the affinity of the corresponding Ca\(^{2+}\)-ATPase (sarcolipin/SERCA class 1 and phospholamban/SERCA class 2a). Furthermore, phospholamban can be phosphorylated at three different sites and forms pentamers after phosphorylation (for review see Ref. 61). Although sarcolipin and KdpF are similar in size and embedded in the membrane by one membrane-spanning domain, sarcolipin is involved in regulatory control of the SERCA class 1, whereas no regulatory effects have been observed so far for KdpF. In summary, the peptides associated with eukaryotic transporters appear to function as activity/affinity-modulating subunits, whereas the peptides from the prokaryotic transporters, like KdpF, act as stabilizing elements.

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