Characterization of the Phosphorylated Intermediate of the K⁺-translocating Kdp-ATPase from Escherichia coli

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The Kdp system is a derepressible high affinity K⁺ uptake system (Kₘ for transport = 2 μM, Ref. 1) in Escherichia coli (for reviews, see Refs. 2, 3). It is expressed as an emergency system under growth conditions when the constitutive high capacity system Trk (4) is unable to satisfy the cells’ need for K⁺. The kdpABC operon which has been cloned and sequenced (5) encodes the three cytoplasmic membrane proteins KdpA (59, 189 Da), KdpB (72, 112 Da) and KdpC (20, 267 Da). Energy to build up and maintain large K⁺ concentration gradients (up to 4 × 10⁵: 1) stems exclusively from the hydrolysis of ATP (6). As this mode of energy coupling implies, a K⁺-stimulated ATPase activity has been identified in E. coli membranes with [γ-³²P]ATP (41). These findings pointed to an acid-stable KdpB phosphoprotein that is completely discharged by ATP and ADP and partially dissipated by K⁺. Furthermore, Anes and Nikaido (13) reported on alkali-labile phosphate-containing proteins in E. coli. However, their tentative identification of a Kdp-associated phosphoprotein resided only on the fact that the appearance of the putative candidate was correalted with cell growth under conditions of K⁺ starvation.

In this paper, we propose a minimum scheme for the reaction cycle of the Kdp-ATPase comprising the participation of a phosphorylated intermediate. For these studies, the influence of effectors (ions, ATP, ADP) and inhibitors on the reaction was examined by simultaneous analysis of the level of phosphoprotein and the ATPase activity. In most of the experiments shown, purified Kdp-ATPase from a KdpA mutant with lowered affinity for K⁺ (Kₘ for ATPase activity = 6 mM, Ref. 10) was used instead of the high affinity wild-type enzyme (apparent Kₘ = 10 μM, Ref. 10). Thus, the interfering influence of micromolar K⁺ contaminations inevitably present in all assay media was excluded and, consequently, authentic K⁺-independent effects within the cycle could be detected. KdpB was identified as the phosphorylated intermediate by electrophoretic separation of both, ³²P-labeled crude cytoplasmic membranes and ³²P-labeled purified Kdp complex, in a gel system allowing salvage of alkali-labile acyl phosphates.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The homozygous diploid E. coli Kdp wild-type strain TK2240-40 carrying an F-100 episome (7), the mutant strain TK2242-42 (kdpA42 F' kdpA42, Ref. 7) as well as the deletion strain TK2281 (ΔkdpABCDE, Ref. 14) were kindly donated by Dr. W. Epstein, University of Chicago. Aside from the

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Dedicated to Professor Dr. A. Trebst on the occasion of his 60th birthday.

¹The abbreviations used are: NEM, N-ethylmaleimide; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
genetic markers stated above, all three strains have the common genotype thi rha lacZ napA trkA405 trkD1. The unc (ΔIBFHA) derivatives of TK2242-42 and TK2281 named TKR1000 and TKR2000, respectively, were constructed by P1 transduction as described previously (10). Cells were grown aerobically at 37 °C in minimal medium supplemented with thiamine (1 μg/ml) and containing 1% glucose as energy source. The media were designated according to their K⁺ content (14): K0 for nominally K⁺ free medium; K0.5, K15, and K115 for media containing 0.5, 15, and 115 mM K⁺, respectively. The Kdp system was repressed by growth in K115. Kdp- and Ktr-synthesizing strains were grown in K0.5; cultivation of Kdp-expressing wild-type cells was described previously (9). TKR2000, a strain devoid of the Kdp and Trk K⁺-uptake systems, was grown in K15 under conditions allowing only half-maximal growth rates due to K⁺ limitation.

Preparative Procedures—Bacterial cytoplasmic membranes were prepared according to Osborne and Munson (16). Purification of the Kdp-ATPase from membranes of the wild-type strain TK2240-40 and the KdpA mutant TK2242-42 was performed as described (9, 10). For the phosphorylation experiments, isolated Kdp-ATPase of highest specific activity as obtained after the two ion exchange chromatography columns was employed.

Phosphorylation and Simultaneous Determination of ATPase Activity by Charcoal Assay—The procedure employed was a modification of the method of Ray and Forte (17). The labeling reaction (9) was routinely carried out at 37 °C in a total volume of 0.2 ml. Cytoplasmic bacterial membranes (100 μg of membrane protein) or purified Kdp-ATPase (50 μg) were preincubated for 5 min in the phosphorylation buffer, consisting of 50 mM Hepes-Tris, pH 7.8 (pH adjusted to 7.8 with Tris base), 1 mM MgCl₂, and, occasionally, 10 mM KCl. The phosphorylation reaction was started by the addition of 100–200 μM [γ-³²P]ATP (about 2.7 mCi/μl 0.2 ml). The incubation was stopped after 5–15 s with 0.3 ml of 35% (v/v) ice-cold trichloroacetic acid.

For the ATPase assay an aliquot of 0.1 ml was transferred to a 1.5 ml microcentrifuge tube containing 1.0 ml of ice-cold 5% (v/v) trichloroacetic acid. The precipitated protein was removed by centrifugation in a microcentrifuge for 2 min at 15,500 × g. The supernatant was treated with charcoal for 30 min at room temperature to adsorb ATP. Then, the total suspension was filtered through a Pasteur pipette stuffed with a Millipore AP25 microfilter glass prefilter, using gentle pressure to accelerate the filtration and discarding the first and last few drops of the filtrate. The [γ-³²P]P liberated was determined by counting 0.1 ml aliquots of the supernatant before charcoal treatment and of the clear filtrate in a liquid scintillation counter. The ATPase activity was expressed in nmol P0.5/min, or milliunits of protein and minute.

The residual 0.4 ml of the denatured suspension were used for quantification of the phosphorylated compounds. The mixture was made 10 mM with respect to carrier ATP and F. Then the precipitated proteins were filtered on a microfilter glass prefilter (Millipore, type GF/F) using a suction device. Prior to use, the filter discs were incubated for at least 2 h at 60 °C in a solution of 10 mM Na₂ATP and 10 mM P0.5 in order to minimize unspecific binding of radioactivity to the filters. The trichloroacetic acid precipitates were washed three times with 2 ml of 10% (v/v) trichloroacetic acid containing 10 mM P0.5 and 2 ml carrier ATP followed by three washings with 2 ml of ice-cold ethanol to remove most of the lipids. The filters were dried, placed in vials, and after radioactivity determination, the DNA was solubilized by sonication using a sonicator. 

The dried ether-extracted membrane pellet and the trichloroacetic acid-precipitated Kdp-ATPase were solubilized and subjected to electrophoresis at pH 2.4 and 4 °C essentially as described by Lichtner and Wold (18). To render the solubilization of the trichloroacetic acid-precipitated protein more effective, the authors suggested increasing the pH of the protein solubilization solution to pH 9.3 (19). We avoided the dodecyl sulfate concentration was temporarily increased and the suspension rapidly cleared after stirring the trichloroacetic acid pellet with a glass rod. The final protein samples (including β-mercaptoethanol and sucrose) contained 1–2 μg of protein/ml for membranes and about 0.5 μg/ml for purified Kdp-ATPase. For electrophoretic separation on the lithium dodecyl sulfate-polyacrylamide gel system we used tube gels (50-mm length and 6-mm diameter) formed from 9% acrylamide and 0.18% bisacrylamide. Protein bands were visualized by staining with Coomassie Blue (19), and stained gels were scanned with a Bio-Rad model 1600 Densitometer (Bio-Rad, Munich, Federal Republic of Germany (F. R. G.).)

The distribution of radioactivity in the gels was analyzed by two different methods. First, gels were cut in 1.3-ml slices with an Nytong knife and after counting in a scintillation counter (Beckman, San Francisco). Each slice was solubilized by overnight incubation in 1 ml of 30% H₂O₂ at 60 °C before addition of 10 ml of Hydroluma and quantitation of radioactivity. Second, a flat-faced central segment was cut out of the cylindrical gels with a longitudinal gel slicer (20), dried on filter paper, and subjected to direct autoradiography.

**Results**—Protein and total phosphate concentrations were measured by the method of Hartree (21) or, if detergent was present, by that of Duliey and Grieve (22). Stock solutions of orthovanadate in water (23) and bafilomycin A₁ in methanol (24, 25) were prepared as described. The actual inhibitor concentrations were determined spectrophotometrically using the published molar extinction coefficients (23, 25).

**Materials**—Hepes, Tris, Tris-ATP, Tris-ADP (the latter two, vanadate free), hydroxylamine, NEM, and ouabain were purchased from Sigma, Deisenhofen, F. R. G. Sodium orthovanadate was from BDH, Poole, United Kingdom. Pulverized charcoal and EDTA were obtained from Merck, Darmstadt, F. R. G. Bafilomycin A₁ was a generous gift from Dr. K. N. AG, Wupper, West Germany. All chemicals used in this study were of analytical grade. [γ-³²P]P]ATP in the form of the triethylammonium salt (specific radioactivity: 3000 Ci/mmol) was supplied by Du Pont-New England Nuclear, Dreieich, F. R. G. Hydrolyma scintillation mixture was bought from Baker Chemicals, Deventer, Holland. Glass microfiltrer filters GF/F were from Whatman, Maidstone, United Kingdom and microfiber glass prefilter AP25 from Millipore, Molsheim, France.

**Relationship between the K⁺-stimulated ATPase and the Phosphorylated Intermediate**—We used a filter assay for the quantification of the phosphorylated intermediate of the Kdp-ATPase formed after incubation of the purified enzyme with [γ-³²P]P]ATP. Values for the steady-state level of phosphorylase protein ranged from 500 to 1200 nmol/g protein depending on the particular ATPase preparation used. Assuming a stoichiometry with an equal number of each subunit/complex this corresponds to phosphorylation of 10–20% of the Kdp molecules. As expected for an enzyme-catalyzed process, phosphorylation did not occur after heat- or acid-denaturation of the protein, and the incorporation of ³²P was proportional to the protein concentration in the range tested (between 5 and 100 μg of protein/0.2 ml assay volume).

The time course of phosphorylation and ATP hydrolysis is depicted in Fig. 1. The phospho-enzyme was rapidly formed, reaching peak levels within 10 s. In the presence of K⁺, a lower phosphorylation level and, consequently, a corresponding acceleration of ATP hydrolysis were observed. However, a basal ATPase activity (approximately 15% of the K⁺-stimulated rate) was found in the absence of K⁺. This basal rate was not caused by K⁺ contaminations in the assay buffer since the KdpA mutant enzyme, isolated from strain TK2242-42, was...
used for the experiment. The simplest explanation for the correlation between the dissipation of the phosphoprotein and the course of the ATP hydrolysis is that the level of the transient intermediate drops because ATP is progressively consumed. In fact, the maximum phosphorylation level was maintained for longer time periods when labeling was performed with higher concentrations of ATP or lower amounts of protein, respectively.

**Role of Ions in the Reaction Cycle**—The steady-state level of the phosphoenzyme and the ATPase activity were measured at various concentrations of KCl and NaCl (Fig. 2). Increasing the KCl concentration up to 10 mM progressively reduced the steady-state level of phosphoprotein and simultaneously stimulated the rate of ATP hydrolysis. Half-maximal effects for both processes were observed at 2.5 mM K⁺. At higher K⁺ concentrations, however, the ATPase activity was impaired. The same reduction in ³²P incorporation was obtained irrespective of whether KCl was present during the phosphorylation or added after the enzyme phosphorylation had reached a maximum (compare Figs. 2 and 3). Consequently, the K⁺-dependent decrease in labeling represents a change in the steady-state level of the phosphoenzyme.

Na⁺ (Fig. 2) failed to stimulate ATP hydrolysis. In the presence of optimal amounts of K⁺ (16 mM) in the phosphorylation assay, Na⁺ concentrations exceeding 15 mM progressively inhibited ATPase activity. Thus, the reduction in the phosphoenzyme level produced by NaCl appears to be due to inhibition of phosphorylation rather than stimulation of the dephosphorylation.

Magnesium ions were required for the phosphorylation as revealed by titration experiments in which the concentration of free Mg²⁺ was varied by addition of EDTA to the incubation medium (data not shown). At a stoichiometric EDTA to Mg²⁺ ratio of 1:1, the formation of phosphoprotein was reduced to 5%, and at a molar 1.5:1 ratio phosphorylation was completely prevented (see the respective value indicated by × in Fig. 3). In order to determine whether the dephosphorylation step also requires Mg²⁺, we first labeled the enzyme in the presence of Mg²⁺, then chelated all Mg²⁺ by adding excess of EDTA, and followed the fate of the intermediate. Under steady-state conditions the level of phosphoenzyme did not fall when EDTA was added either alone or together with K⁺ (Fig. 3). By contrast, addition of K⁺ alone led to rapid decay of the intermediate; a new low level of phosphorylation was achieved

**Fig. 1.** Time course of phosphorylation and ATP hydrolysis. Purified Kdp-ATPase from strain TK2242-42 (50 µg) was labeled with 20 µM [γ-³²P]ATP (2.7 µCi/0.2 ml) at 37 °C. The phosphorylation was carried out for the indicated time periods in 50 mM Hepes-Tris, pH 7.8, and 1 mM MgCl₂ either in the absence (open symbols) or presence (closed symbols) of 10 mM KCl. The formation of phosphoprotein (O—O, without K⁺; —•—•, with K⁺) and the ATPase activity (Δ—Δ, without K⁺; △—△, with K⁺) were determined by the filter assay and by the charcoal assay, respectively, starting from identical reaction tubes.

**Fig. 2.** Effects of K⁺ and Na⁺ on the level of phosphoprotein and the ATPase activity. Experimental conditions were as described in the legend to Fig. 1 except that labeling was carried out for 5 s in standard buffer (see "Experimental Procedures") containing the indicated concentrations of KCl (O—O), of NaCl (Δ—Δ), or the indicated concentration of NaCl in the presence of 10 mM KCl (■—■). The dotted lines represent control levels found without addition of K⁺ or Na⁺ (maximal phosphorylation, basal ATPase activity), and the dashed lines indicate levels observed in the presence of the optimal K⁺ concentration of 10 mM (low phosphorylation, maximal ATPase activity).

**Fig. 3.** Effects of K⁺, EDTA, ATP, and ADP on the steady-state level of the phosphorylated intermediate. In deviation to the standard protocol, the phosphorylation of the purified Kdp-ATPase from strain TK2242-42 was carried out with 25 µg of protein and 100 µM [γ-³²P]ATP (2.7 µCi/0.2 ml) so that the maximal phosphorylation level was maintained for longer time periods. At 15 s, when the steady-state concentration of the ³²P intermediate had been reached, different substances were added. The mixtures were incubated for further 15 and 30 s and, then, subjected to the filter assay. Additions at the time point indicated by arrow were: H₂O (control, ○—○); 10 mM EDTA (O—O); 10 mM EDTA and 10 mM KCl (■—■); 10 mM KCl (■—■); 5 mM ATP (△—△); 5 mM ADP (△—△). When 1.5 mM EDTA was already present prior to the addition of [γ-³²P]ATP, the formation of the phosphoprotein was suppressed as shown by the respective control value (×) at 10 s.
within 15 s. From the stability of the phosphoprotein in the presence of K⁺ and excess EDTA, it can be concluded that Mg²⁺, besides its importance for phosphorylation, is also necessary for a subsequent step in the reaction cycle.

**Role of ATP and ADP in the Reaction Cycle**—We tested the relationship between the phosphorylated intermediate and the ATP hydrolysis rate as a function of various ATP concentrations (Fig. 4). Since the high affinity wild-type enzyme was used for the experiment, ATPase activity in the absence of added K⁺ was artifically high (45% of the K⁺-stimulated rate) and the K⁺-dependent reduction in the phosphorylintermediate level correspondingly low (15% K⁺-stimulated dephosphorylation), presumably due to contaminating K⁺ in the assay buffer. These results underline the necessity of using the KdpA mutant enzyme from strain TK2242-42 for determination of reliable K⁺ effects. There existed a clear difference in the ATP saturation of both simultaneously examined processes. In the presence of optimal K⁺ concentrations half-maximal steady-state levels of phosphoprotein were reached at 2.5 μM whereas the Km value for the ATPase reaction was determined as 25 μM.

In a pulse-chase experiment, purified Kdp-ATPase was labeled to a constant level in the absence of KCl and then a 50-fold excess of unlabeled ATP was added to dilute the specific activity of the [γ-32P]ATP already present (Fig. 3). This addition resulted in the rapid loss of 32P from the enzyme leaving a residual 32P-protein fraction of only 3%. Studies on the kinetics of the chase (data not shown) revealed that the dephosphorylation rate is beyond the time resolution of the manual detection technique applied. Based on a rough estimate, half-maximal dissipation of the phosphoprotein in the absence of K⁺ occurred within 1 s. Therefore, differences in the turnover rate of 32P-enzyme in the presence and absence of K⁺ could not be resolved. The results obtained after isotopic dilution of [γ-32P]ATP confirm that the Pi on the protein is continuously turning over.

The breakdown of the 32P-protein could also be accomplished by addition of a 50-fold excess of ADP over ATP (Fig. 3). This finding provided evidence that the reverse reaction of the ATP-dependent phosphorylation step occurred and, hence, an ADP-sensitive form of phosphoenzyme existed. ADP was acting as a product inhibitor of the overall ATPase reaction. The enzymatic activity of the Kdp-ATPase was inhibited by approximately 50% when ADP was added to the assay medium in a 2-fold molar excess over ATP (data not shown).

**Test of Inhibitors**—In order to obtain information on their site of action in the ATPase reaction sequence, several inhibitors were tested for their effect on the level of phosphorylated intermediate and on the hydrolysis of ATP in the presence of K⁺ (Fig. 5). Orthovanadate showed half-maximal inhibition of ATPase activity at 30 μM (Fig. 5A). This confirms our former results that the KdpA mutant enzyme exhibited a reduced sensitivity to vanadate as compared with the wild-type ATPase (10). Progressive inhibition of the ATPase activity is accompanied by a correlated elevation in the phosphoprotein. Inhibition by the SH-group-specific reagent NEM (Kᵣ = 0.25 mM) was likewise accompanied by a concomitant increase in the level of phosphointermediate (Fig. 5B). The macrocyclic antibiotic basiflucin A (24) also impaired ATP hydrolytic activity (Kᵣ = 2 μM). The transient decrease in 32P incorporation was small (15% decomposition) but reproducible; the minimum was reached at half-inhibitory concentrations of the agent (Fig. 5C). Ouabain, the classical inhibitor of the Na⁺,K⁺-ATPase exerted no significant effect in the concentration range up to 1 mM (Fig. 5D).

**Chemical Nature of the Phosphorylated Intermediate**—Preliminary evidence on the chemical nature of an enzyme-phosphate linkage can be obtained by investigation of its pH dependence (26). The pH stability profile of the Kdp phosphoprotein bond (Fig. 6) reflected progressive hydrolysis of the 32P intermediate at alkaline pH; a marked drop in stability occurred above pH 9. The acid-denatured, filter-bound phosphointermediate was treated with hydroxylamine, a reagent known to cleave acylphosphate linkages (27). After incubation with a 28% hydroxylamine solution, pH 6.5, for 30 min at 0 °C, more than 80% of the protein-bound phosphate was liberated in form of 32P; and appeared in the supernatant. These chemical properties strongly suggest that the phosphoryl group of the phosphoenzyme is linked covalently to a carboxyl group of the enzyme.

**Identification of the Phosphorylated Kdp Subunit**—For the identification of the alkaline- and temperature-labile phosphointermediate, we used the lithium dodecyl sulfate-polyacrylamide gel system of Lichtner and Wolf (18) that resolves proteins according to their molecular weights (see inset of Fig. 3). This finding provided evidence that the reverse reaction of the ATP-dependent phosphorylation step occurred and, hence, an ADP-sensitive form of phosphoenzyme existed. ADP was acting as a product inhibitor of the overall ATPase reaction. The enzymatic activity of the Kdp-ATPase was inhibited by approximately 50% when ADP was added to the assay medium in a 2-fold molar excess over ATP (data not shown).

![Figure 4](image-url)  
**Figure 4.** Effect of ATP concentration on the level of phosphoprotein and the hydrolysis rate of ATP. 50 μg of purified wild-type Kdp-ATPase from strain TK2240-40 were phosphorylated for 10 s under standard conditions using [γ-32P]ATP of constant specific radioactivity (0.7 μCi/nmol). For explanation of the symbols, see legend to Fig. 1.
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**FIG. 5.** Effects of inhibitors on the level of phosphoprotein and the ATPase activity. 50 µg of purified Kdp-ATPase from strain TK2242-42 were preincubated at 37°C in 50 mM Hepes-Tris, pH 7.8, 1 mM MgCl₂, 10 mM KCl with A, vanadate for 10 min; B, NEM, 30 min; C, bafilomycin A₁, 1 min; D, ouabain, 10 min. The phosphorylation reaction was started by addition of 100 µM [γ-³²P]ATP (2.7 µCi/0.2 ml) and stopped after 10 s. 100% values of ATPase activity corresponded to hydrolysis rates of approximately 300 nmol × g⁻¹ × min⁻¹.

**FIG. 6.** Effect of pH on the stability of the phosphoprotein. Purified Kdp-ATPase from strain TK2240-40 (50 µg) was phosphorylated with 20 µM [γ-³²P]ATP (2.7 µCi/0.2 ml) for 10 s in the absence of KCl. The trichloroacetic acid-precipitated protein was collected and washed on glass microfiber filters (see "Experimental Procedures"). The dried filter discs containing the ³²P intermediate were transferred to 4.0-ml buffers of different pH values and incubated for 60 min at room temperature. The following 0.05 M buffer systems were used: pH 1-2, HCl/KCl; pH 3, glycine/HCl; pH 4-5, acetate buffer; pH 6-8, phosphate buffer; pH 9-10.5, glycine/NaOH. After incubation, the filters and aliquots of the supernatants were counted. The 100% value of total ³²P incorporation corresponded to 745 nmol × g⁻¹.

with the values found for purified Kdp-ATPase. Densitometric gel scans of cytoplasmic membranes allowed us to estimate that KdpB constitutes approximately 5% of the total membrane protein. Based on this assumption theoretical labeling values of about 500 nmol × g⁻¹ must be postulated for the purified enzyme, what is in good agreement with the actually found ³²P incorporation (Figs. 1-5). The Kdp-independent background phosphorylation was dependent on active enzymes or native conformation since it did not occur in trichloroacetic acid-denatured membranes. The phosphate groups did not turn over rapidly since the ³²P incorporated was not released by a 1-min chase with unlabeled ATP. These observations point to an enzyme-catalyzed process of low turnover-number or to saturation of ATP-binding sites with high binding constants. The background phosphorylation might be attributed to the weakly labeled, Kdp-independent 10-kDa protein visible after electrophoretic separation of phosphorylated membranes (see Fig. 7, lanes e–g). In the filter assay the bulk of phospholipids was removed by extensive washing of filter-bound precipitated membranes with ethanol (see "Experimental Procedures").

After separation of the purified Kdp-ATPase on the acidic gel system (see Fig. 8a and the densitometric scan), the three subunits could be unequivocally identified. In contrast to common alkaline gel systems (10), KdpA is well stained and KdpC is only weakly stained by Coomassie Blue after separation under acidic conditions. Calibration of the gel with proteins of known molecular weights yielded molecular weights for KdpB and KdpC that were in accordance with the values derived from the DNA sequence. KdpA, as with common alkaline gel systems, showed the aberrant high mobility of hydrophobic proteins (10). Superimposition of the densitometric scan and the radioactivity profile of the sliced gel revealed that the radioactive peak was associated with the KdpB subunit. Neither by liquid scintillation counting of gel slices nor by autoradiography (Fig. 8b) was any other labeled compound detected. From these results it can be deduced that KdpB is the catalytic subunit of the Kdp complex which is continuously phosphorylated and dephosphorylated during the ATPase reaction cycle.

**DISCUSSION**

In this study we extended the characterization of the Kdp-ATPase from *E. coli* by demonstrating a phosphorylated intermediate of this enzyme. Therefore we confirmed implications drawn from homology data (5, 12) and from the sensitivity of this K⁺-ATPase to vanadate (10, 28), the characteristic inhibitor of P-type ATPases. Moreover, we confirmed preliminary experimental findings concerning this subject.² The kinetic properties of the phosphoprotein were analyzed by a manual detection technique ("filter assay") and supported the notion of a transient catalytic intermediate. Within the limitations given by the restricted time resolution of this method, we found a rapid formation of phosphoenzyme (Fig.
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FIG. 7. Analysis of phosphorylated bacterial membranes by gel electrophoresis at pH 2.4. Cytoplasmic membranes (2.5 mg) of strains TKR1000 and TKR2000 were labeled with 250 μCi [γ-32P]ATP (50 μCi/ml) for 15 s, resolved on acidic polyacrylamide gels (18), and the labeled proteins were visualized by autoradiography. Details of the labeling, of the washing steps, including the ether extraction of the membranes, and of the electrophoresis are given under “Experimental Procedures.” The left-hand part of the figure (lanes a-d) shows the protein pattern after staining with Coomassie Blue, the right-hand part (lanes e-g) shows the autoradiographic image. a, standard proteins, numbers give the corresponding molecular masses in kDa; b and e, membranes of TKR1000, Kdp repressed, 150 μg; c and f membranes of TKR1000, Kdp derepressed, 150 μg; d and g, membranes of TKR2000, Kdp deleted, 150 μg. The position of the single Kdp subunits and of the dye front is marked.

TABLE I
Phosphorylation of cytoplasmic membranes from different E. coli strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Status of Kdp system (growth media)</th>
<th>32P incorporation nmoles (x g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TKR1000 Repressed (K115)</td>
<td></td>
<td>6.5</td>
</tr>
<tr>
<td>TKR1000 Derepressed (K0.5)</td>
<td></td>
<td>32.4</td>
</tr>
<tr>
<td>TKR2000 Deleted (K15)</td>
<td></td>
<td>20.3</td>
</tr>
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1) and a fast turnover induced by K⁺ (Fig. 3) and by ATP after isotopic dilution of [γ-32P]ATP (Fig. 3).

For most of the experiments described, the purified KdpA mutant ATPase from strain TK2242-42 was used instead of the wild-type enzyme. Comparative control experiments ensured that, except for the known differences in K⁺ affinity and vanadate sensitivity (7, 10), the basic characteristics and kinetic behavior of the wild-type and mutant phosphointermediates were identical. Therefore, the possibility that results and deduced conclusions on the principles of the reaction cycle might be attributed to artifacts resulting from the mutation was excluded. Aside from the strain-specific differences, preparation-dependent fluctuations were observed. First, the values for the steady-state levels of phosphoprotein ranged from 500 to 1200 nmoles x g⁻¹ indicating variations in the fraction of phosphorylatable enzyme molecules from 10 to 20%. Since this low phosphorylation was also observed for membrane-bound enzyme (see Table I and derived calculations in the text), it is unlikely to be due to damage of the Kdp-ATPase by the solubilization or purification procedure. Secondly, the percentage of K⁺-dependent dephosphorylation varied widely from 30 to 70%, supporting the idea of variations in the tightness of coupling between the processes of K⁺ binding and ATP hydrolysis.

Our studies prompt us to propose the following minimum reaction scheme:

\[ \begin{array}{c}
\text{K⁺ stimulated the rate of dephosphorylation, reducing the} \\
\text{steady-state level of the intermediate (Figs. 1–3). Experiments} \\
\text{with the chelating agent EDTA added either in the} \\
\text{phosphorylation assay or under steady-state conditions (Fig. 3)} \\
\text{revealed that Mg²⁺ is necessary for the phosphorylation as well} \\
\text{as for the dephosphorylation step. A basal, solely Mg²⁺-} \\
\text{dependent, ATPase activity was observed in the absence of K⁺} \\
\text{(Figs. 1 and 2; see the dashed line in the scheme). From} \\
\text{the fact that in pulse-chase experiments addition of excess unlabeled} \\
\text{ATP evoked the dissipation of the 32P intermediate level in} \\
\text{the presence (data not shown) as well as in the absence of} \\
\text{K⁺ (Fig. 3), we concluded that phosphointermediates are} \\
\text{associated with both the basal and the K⁺-stimulated ATPase.} \\
\text{In our tentative model which also takes into account analogous} \\
\text{interrelations in the mechanistically very similar gastric} \\
\text{H⁺,K⁺-ATPase (17, 29), the basal ATPase activity is ex-}
\end{array} \]

K⁺ stimulated the rate of dephosphorylation, reducing the steady-state level of the intermediate (Figs. 1–3). Experiments with the chelating agent EDTA added either in the phosphorylation assay or under steady-state conditions (Fig. 3) revealed that Mg²⁺ is necessary for the phosphorylation as well as for the dephosphorylation step. A basal, solely Mg²⁺-dependent, ATPase activity was observed in the absence of K⁺ (Figs. 1 and 2; see the dashed line in the scheme). From the fact that in pulse-chase experiments addition of excess unlabeled ATP evoked the dissipation of the ³²P intermediate level in the presence (data not shown) as well as in the absence of K⁺ (Fig. 3), we concluded that phosphointermediates are associated with both the basal and the K⁺-stimulated ATPase. In our tentative model which also takes into account analogous interrelations in the mechanistically very similar gastric H⁺,K⁺-ATPase (17, 29), the basal ATPase activity is ex-
FIG. 8. Analysis of phosphorylated purified Kdp-ATPase by gel electrophoresis at pH 2.4. Purified Kdp-ATPase (1 mg) from strain TK2242-42 was labeled with 400 μM [γ-32P]ATP (90 μCi/ml) for 15 s and processed as described under “Experimental Procedures.” A photo of a Coomassie Blue-stained acidic polyacrylamide gel after resolution of approximately 50 μg of protein is shown in part a, and the corresponding densitometric scan is also depicted. Distribution of radioactivity was analyzed by liquid scintillation counting of solubilized gel slices (see the 32P profile) or by autoradiography of a longitudinal flat-faced central gel segment (part b). The inset depicts the calibration curve for the mobility of standard proteins. The position of the Kdp subunits is identified by arrows and the derived molecular weights are indicated in brackets.

Compared through the turnover of $E_1$-P and the K$^+$-stimulated activity is expressed through the turnover of $E_2$-P. Complete decomposition of the 32P intermediates was also achieved by addition of high ADP concentrations (Fig. 3), indicating that, with an excess of product, the ATP-specific phosphorylation reaction could be driven backward. Summing up, the total sequence can be subdivided into the following partial reactions: (i) a Mg$^{2+}$-dependent protein kinase leading to the formation of the phosphorylated intermediate, (ii) a Mg$^{2+}$-dependent and K$^+$-stimulated phosphoprotein phosphatase promoting the breakdown of the phosphointermediate, and (iii) a K$^+$-uncoupled basal phosphoprotein phosphatase.

As indicated by the question marks in the reaction scheme, concrete experimental evidence for $E_1$-$E_2$ conformational transitions in the Kdp-ATPase is lacking so far. A suggestive finding for such an interconversion is the occurrence of a K$^+$-insensitive fraction of the Kdp phosphoenzyme. In addition, many mechanistic analogies to the eukaryotic K$^+$-translocating $E_1E_2$-ATPases support this view (for a detailed discussion, see below). For these better-characterized animal ATPases, the existence of two principal conformational states has been verified by fluorescence measurements and proteolytic studies (11, 30).

Several theoretical possibilities exist for the action of inhibitors within the cycle. (i) When the phosphorylation reaction (the binding of ATP or the catalytic cleavage of ATP) is affected, a parallel inhibition of the ATPase activity and the phosphointermediate formation is expected. (ii) When the dephosphorylation reaction is impaired and the rate of dephosphorylation is diminished, an accumulation of the phosphoenzyme will be the consequence. The latter phenomenon was observed after treatment of the enzyme with vanadate (Fig. 5A) or NEM (Fig. 5B). The reduction of the K$^+$-stimulated ATPase activity was correlated with a simultaneous elevation of the phosphointermediate level in the presence of K$^+$, suggesting that the inhibitor blocks occurred in the K$^+$-stimulated dephosphorylation step. Bafilomycin A$_1$, a novel class-specific ATPase inhibitor (24), seemed to interfere with another step of the cycle since, in contrast to vanadate and NEM, its effect was characterized by a small transient decrease in the phosphoprotein level (Fig. 5C). As its site of action obviously lay subsequent to the initial phosphorylation step and was probably different from the dephosphorylation step, this substance might act on the interconversion between $E_1$-P and $E_2$-P.

Comparisons with published inhibitor data for other P-type ATPases revealed some variations in the inhibitory mechanism of vanadate and NEM. A common characteristic is the preferential binding of vanadate to the $E_2$ conformation whereas fundamental differences reside in the question whether vanadate displaces 32P at the catalytic phosphorylation site or reacts with an additional low affinity ATP-binding site. In the literature both phenomena are reported: vanadate-induced breakdown of the 32P intermediate level (for example for the Na$^+$,K$^+$-ATPase, Ref. 31; for the H$^+$,K$^+$-ATPase, Ref. 32; or for the K$^+$-ATPase from brush-border, Ref. 33) as well as vanadate-insensitive maintenance of the 32P-enzyme level (for example for the Ca$^{2+}$-ATPase from sarcoplasmic reticulum, Ref. 34, or the H$^+$-ATPase from Saccharomyces cerevisiae, Ref. 35). The Kdp-ATPase obviously belongs to the latter group, since displacement of 32P from the phosphoprotein by vanadate was not observed. For NEM different sites of action within the fundamental reaction pathway of P-type ATPases have been proposed. Whereas in the Na$^+$,K$^+$-ATPase (36) and the Ca$^{2+}$-ATPase from sarcoplasmic reticulum (37) NEM was reported to interfere with the conversion of $E_1$-P to $E_2$-P, the sulfhydryl reagent seems to act on the K$^+$-dependent dephosphorylation step in the gastric H$^+$,K$^+$-ATPase (17) and presumably the bacterial Kdp-ATPase (this work).

In the following section, basic features of the Kdp-ATPase reaction sequence will be compared with functional properties exhibited by other K$^+$-translocating P-type ATPases, namely the Na$^+$,K$^+$-ATPase (36, 38, 39) and the gastric H$^+$,K$^+$-ATPase (17, 29, 40). All these K$^+$-ATPases have in common that Mg$^{2+}$ is required for the formation of the phosphointermediate and that K$^+$ activates the dephosphorylation step. The existence of a K$^+$-uncoupled, Mg$^{2+}$-dependent ATPase activity (cf. Figs. 1 and 2) has also been described for the H$^+$,K$^+$-ATPase (17, 29) and the Na$^+$,K$^+$-ATPase (38). This basal activity seems to reflect the spontaneous decay of the enzyme-phosphate complex in the absence of activating cation. Studies on the Kdp-ATPase revealed a dual (stimulatory and inhibitory) effect of potassium: K$^+$-concentrations up to 15 mM activated the enzyme, higher concentrations progressively reduced the extent of stimulation (Fig. 2). This phenomenon was likewise reported for the H$^+$,K$^+$- and the Na$^+$,K$^+$-ATPase (29, 40). Kinetic analyses with these eukaryotic enzymes provided strong evidence that K$^+$, in addition to its stimulatory role in the dephosphorylation step, also stabilizes a dephospho form of the enzyme, thereby decreasing the rate of phosphorylation. Binding of ATP is necessary for the release of K$^+$ and restart of the cycle. At high K$^+$/ATP ratios the displacement of K$^+$ from the enzyme is the rate-limiting step of the overall reac-
Phosphorylated Intermediate of the Kdp-ATPase

With the Kdp-ATPase we also observed that the inhibitory effect of K⁺ was reduced by elevation of the ATP concentration in the test (data not shown). Thus, the contradiction that the enzyme was already inhibited at K⁺ concentrations far below the intracellular values is explained by the unphysiologically low ATP concentrations (mostly 20 μM) in the assay designed for phosphorylation studies. A further characteristic of the Kdp reaction pathway was that for half-maximal hydrolysis rates an approximately 10-fold higher ATP concentration was needed than for half-maximal intermediate levels (Fig. 4). Since the kinetic parameters of the overall reaction are determined by the rate-limiting step, this finding implies (i) that the phosphorylation is not the restricting step of the total sequence and (ii) that ATP, at higher concentrations, exerts a positive regulatory effect aside from its role as the phosphate donor. Even higher differences in the ATP dependence of phosphorylation and of the overall ATPase reaction have been described for the Na⁺,K⁺- and the H⁺,K⁺-ATPase (17). For these enzymes, the coexistence of high affinity and low affinity catalytic and/or regulatory ATP-binding sites has been experimentally verified (29, 31). From the consensus reaction sequence of E₁E₂-ATPases, it can be deduced that two forms of the phosphorylated intermediate exist, an ADP-sensitive, K⁺-insensitive one (E₁-P) and an ADP-insensitive, K⁺-sensitive one (E₂-P). The phosphoenzyme of the native Na⁺,K⁺-ATPase is readily discharged by K⁺ and, under steady-state conditions, only a small part of the intermediate reacts with ADP in the backward reaction (36). As the rate constant for the conversion of E₂-P to E₁-P is small (38), only a small part of the phosphointermediate exists in the E₁-P form; the equilibrium is shifted towards E₁-P. In contrast, the H⁺,K⁺-ATPase is partially dephosphorylated by K⁺ (17, 29) and ADP sensitivity can only be detected under special experimental conditions (39). Based on kinetic analyses values of 22% for E₁-P and 78% for E₂-P have been calculated (39). A third situation was manifested with the Kdp-ATPase. This enzyme was partially dephosphorylated by K⁺ (Figs. 1–3) and completely dissipated by excess ADP (Fig. 3). We suppose that the interconversion of the E-P forms might be the rate-limiting step in the overall reaction and for this reason the E₁-P conformation might be strongly favored. This would explain why the equilibrium could only be forced in direction of E₂-P in the presence of K⁺ and why a rapid shift-back occurred after addition of excess ADP.

Kdp shares common functional properties with eukaryotic E₁E₂-ATPases, especially with those also mediating K⁺ transport. However, some differences exist in subunits of the catalytic process. KdpB, the subunit homologous to other representatives of this class of ATPases (5, 12) exhibited in addition structural similarities. In this study, the KdpB polypeptide was identified as the catalytic subunit forming the phosphorylated intermediate. Two chemical criteria pointed to an aclyphosphate, the alkali-lability of the phosphate bond and the sensitivity to hydroxylamine. The confirmation that we are dealing with an aspartyl phosphate located at the position predicted from the homology data (Asp 307) is still lacking, but the matter is presently under investigation.

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