The Kdp-ATPase of *Escherichia coli* is a four-subunit P-type ATPase that accumulates K\(^+\) with high affinity and specificity. Residues clustered in four regions of the KdpA subunit of Kdp were implicated as critical for K\(^+\) binding from the analysis of mutants with reduced affinity for K\(^+\) (Buurman, E., Kim, K.-T., and Epstein, W. (1995) *J. Biol. Chem.* 270, 6678–6685). K\(^+\) binding by this pump has been analyzed in detail by site-directed mutagenesis. We have examined 83 of the 557 residues in KdpA, from 11 to 34 residues in each of four binding clusters known to affect K\(^+\) binding. Amber mutations were constructed in a plasmid carrying the *kdpFABC* structural genes. Transferring these plasmids to 12 suppressor strains, each inserting a different amino acid at amber codons, created 12 different substitutions at the mutated codons. This study delineates the four clusters and confirms that they are important for K\(^+\) affinity but have little effect on the rate of transport. At only 21 of the residues studied did at least three substitutions alter affinity for K\(^+\), an indication that a residue is in or very near a K\(^+\) binding site. At many residues lysine was the only substitution that altered its affinity. The effect of lysine is most likely a repulsive effect of this cationic residue on K\(^+\) and thus reflects the effective distance between a residue and the site of binding or passage of K\(^+\) in KdpA. Once a crystallographic structure of Kdp is available, this measure of effective distance will help identify the path of K\(^+\) as it moves through the KdpA subunit to cross the membrane.

The high specificity of transport systems is believed to be governed by the structures of the sites where the transported substrate binds, just as substrate binding sites account for much of the specificity of enzymes. The large numbers of enzymes whose high resolution crystallographic structures and hence substrate binding sites are well known contrasts dramatically with the limited structural information available for integral membrane proteins. Instead, details related to substrate-binding sites have been inferred from mutations and from covalent modifications that alter transport or from the ability of substrates to block such covalent modification. These methods allow for the identification of residues involved in substrate binding.

In this work, a genetic approach was used to analyze substrate binding by Kdp, a P-type ATPase in *Escherichia coli* and many other bacteria. Kdp consists of three large protein subunits: KdpA, 59 kDa; KdpB, 72 kDa; KdpC, 20 kDa; and the KdpF peptide, 3.2 kDa (1, 2). All but KdpF are required for transport. The KdpB subunit is the site of acylphosphorylation and is homologous to the large subunit of other P-type ATPases. KdpA and KdpC have no homologs other than Kdp genes in other bacteria. *E. coli* has four distinct K\(^+\) transport systems: three saturable systems now referred to as Kdp, Trk (formerly TrkA or TrkG/TrkH), and Kup (formerly TrkD), and a low rate nonsaturable activity referred to as TrkF\(^+\) (3). Recent work strongly suggests that TrkF activity is illicit transport of K\(^+\) through a variety of other transport systems. In this study of Kdp, we have used mutants in which the other two saturable systems, Trk and Kup, are absent so the only activity besides Kdp is the low rate TrkF system.

Residues involved in binding of K\(^+\) were previously identified from the analysis of mutants in which the *K_{mK}\) for K\(^+\) had increased from the wild type value of about 2 μM to values from 0.2 to 90 mM (4). Of the 36 mutants analyzed, 32 altered the KdpA subunit and retained high rates of transport with marked reductions in K\(^+\) affinity. The 32 mutants that altered KdpA represent 15 different mutations of 13 residues. These residues are in four clusters widely distributed in KdpA (Fig. 1). Clusters I, II, and IV were predicted by topologic analysis to face the periplasm, where they can form an initial K\(^+\)-binding site. Cluster III, predicted to begin within a membrane span and extend into a rather hydrophobic cytoplasmic region of KdpA, may be the site where cations are occluded in the process of transport. Cation occlusion has been demonstrated for several other P-type ATPases but not yet for Kdp (5–7).

Each of the four clusters was previously identified by only two to four residues, providing little information about the size of each cluster or which residues within a cluster were important for binding. Therefore, in this study, we analyzed 83 of the 557 residues in KdpA to identify those that are critical for substrate binding. To test comprehensively the importance of each residue, we examined at least 11 substitutions at each residue using the amber suppression technique (8, 9). This technique creates up to 12 amino acid substitutions at an introduced amber stop codon. The results 1) confirm that each of the four clusters is critical in determining substrate binding; 2) better define the size of each cluster; and 3) highlight the unique effect of cationic substitutions in this high affinity cation transport system.

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1 E. T. Buurman, D. McLaggan, J. Napretek, and W. Epstein, unpublished observations.
Cytoplasm

**FIG. 1. Schematic drawing of the topology of KdpA as proposed by Buurman et al.** (4). The boxes drawn in dashed lines identify the four regions studied in this work. Asterisks mark previously identified affinity mutants. A bracket indicates the location of membrane span 7 in the model of Durell et al. (22).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—The strain used in this work, TK2474 (F thi nagA argR lacZm5KdFAB5 trkD1 trkA405) is defective for all saturable K+ transport systems, making it possible to score for the properties of the Kdp system expressed from a plasmid by growth tests (10). The argE lac mutation was introduced to select for and maintain the presence of an amber suppressor. A streptomycin-resistant (rpsL) derivative of TK2474, strain TK2497 made by transduction, served to examine the basis of weak suppression of some amber mutations by strain TK2474.

**Strains with Amber Suppressors**—The amber suppressing strains utilized are all derivatives of TK2474 carrying either a chromosomally encoded or a plasmid-encoded suppressor tRNA. Twelve strains, each utilizing a different amber suppressor tRNA gene, were obtained as the Interchange Kit (Promega). The chromosomally encoded suppressors, supD, supE, supF, supG, and supP, were introduced into strain TK2474 by transduction, using P1 lysates made from the strains provided in the Interchange kit. Four of the suppressors also suppressed the amber lac mutation in the strain. Failure to do so by supG suggests that Lys is not an acceptable substitution at the site of the lac mutation.

The plasmid carrying the kdp genes has a colE1 replication origin and therefore is incompatible with the plasmid-borne suppressors in the interchanged kit, which have the same replication origin. We therefore transferred the suppressors to a derivative of the p15 origin plasmid, pACYC184, which is compatible with pBR322. Included in the interchanged kit was a pACYC184 derivative in which the gene encoding chloramphenicol transacylase has an amber mutation. This plasmid was digested with the EcoRI and HindIII restriction enzymes, the ends were blunt with the Klenow fragment of DNA polymerase, and then the PvuII restriction fragment of the suppressor plasmids (the fragment comprising the suppressor tRNA gene and its promoter) was inserted. The resulting plasmid carries the suppressor and lacks the tet gene, which mediates a low rate of K+ uptake that would have confounded accurate scoring (11). Only six of the seven plasmid-borne suppressors in the interchanged kit were functional. No functional Arg-inserting suppressor could be obtained because all constructs screened had multiple sequence alterations in the suppressor tRNA sequence. Instead, we used an Ala-inserting suppressor obtained from Jeffrey Miller.

**The Kdp Plasmid**—The plasmid carrying the wild type kdpFABC operon used for mutagenesis, pSD104, is a derivative of pBR322 from which most of the tet gene has been deleted, a P1 origin has been inserted, and kdp sequences beginning just upstream of the promoter of the kdpFABC operon and ending about 500 base pairs past the end of the kdpC gene are present. The kdp genes in this construct are under physiological regulation, requiring the KdpD and KdpE regulatory proteins as well as a signal to express Kdp, which is believed to be a reduction in turgor pressure (12). The kdpFAB5 deletion in host strain TK2474 ends near the border of the kdpB and kdpC genes and expresses the intact kdpD and kdpE genes (13).

**Growth, Genetic Manipulations, and Transformation**—Complex KML medium and minimal medium have been described (14). 0 mM K+ medium has no added K+ and generally contains about 20 μM K+ because of contamination of the sodium phosphates. The carbon and energy source in minimal medium was glucose, 0.2% in liquid cultures and 0.5–1% in solid medium. Ticarcillin, a stable penicillin, was used at 100 μg/ml, chloramphenicol at 15 μg/ml, and streptomycin sulfate at 200 μg/ml. Growth was at 37 °C except for transport experiments, for which cells were grown at 30 °C. Transductions with bacteriophage P1 and transformations were performed as described (14, 15).

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed by the method of Kunkel (16) on single-stranded DNA. Plasmid pSD104 was transformed into F ung dut strain CJ236, and single-stranded copies in phage coats were obtained by infecting with replica-impaired M13K07 helper phage (17). Mutant plasmids were created by using mutagenic primers of 23 bases in which the amber codon (TAG) is flanked by 10 wild type nucleotides on each side, T4 DNA polymerase and T4 ligase. The resulting plasmids were transformed into TK2474 with selection for resistance to ticarcillin.

Mutagenesis efficiency was extremely variable in this work. Between 5 and 60% of the clones obtained were unable to grow on medium containing 1 mM K+. Putative mutants were screened by transformation into the set of TK2474 derivatives each carrying a different suppressor. Amber mutations were not introduced at six residues where mutagenesis failed on three independent attempts.

Arg substitutions were made directly, starting with a plasmid already carrying an amber mutation at the residue to be mutated. The resulting constructs with the CCG codon for Arg were screened for growth properties under the expectation that they would resemble those of Lys substitutions. DNA sequence analysis showed that all 10 had the correct sequence. DNA sequencing was performed by the automated dye termination method with Applied Bioscience model 377 instruments.

**Screening Suppressed Mutants**—All colonies were tested by growth on solid medium. Master plates on which each mutant was either in the nonsuppressing parent or one of the suppressing strains were made on 115 and 1 mM K+ plates. These were replica plated, the former to 3, 5, and 10 mM K+ plates and the latter to 0, 0.1, and 0.3 mM K+ plates (scoring growth on 0 mM K+ would be unreliable using only the 115 mM K+ master plate, because there is some transfer of K+ during replica plating). Growth was scored as positive if there was visible growth after 20 h of incubation at 37 °C.

**Transport Properties of Suppressed Mutations**—Kinetics of K+ uptake were measured by the rate of net increase in cell K+ produced by osmotic upshock (18). Kdp was induced by growing cells in medium of a K+ concentration at which growth was 70% or less of the rate in high K+ medium (12). Cells were centrifuged and transferred to 70 mM NaPO4 buffer (pH 7.1) containing 0.1% (w/v) glucose at a concentration of about 1.4 × 109 cells/ml. K+ uptake was produced by diluting 1 part cell suspension with 2 parts of buffer containing 0.5 mM glucose and a mixture of K+ and NaPO4 buffers to achieve the desired final K+ concentration. Good aeration was maintained by vigorous shaking. Where growth data indicated high affinity, uptake was measured at only two concentrations, 30 μM and either 2 or 5 mM. Where affinity was not high, uptake...
Table I

Growth screening of suppressed amber substitutions

Entries indicate lowest $K^+$ concentration (mM) at which growth was observed (no entry indicates wild type phenotype, growth on 0 mM $K^+$). + indicates suppressor inserts the wild type residue.

| CLUSTER I | Inserted amino acid | Val$^{110}$ | Thr$^{111}$ | Asn$^{112}$ | Thr$^{113}$ | Asn$^{114}$ | Trp$^{115}$ | Gln$^{116}$ | Ser$^{117}$ | Tyr$^{118}$ | Ser$^{119}$ | Gly$^{120}$ |
|-----------|---------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Ala       |                     |             |             |             |             |             |             |             |             |             |             |             |
| Gly       |                     |             |             |             |             |             |             |             |             |             |             |             |
| Cys       |                     |             |             |             |             |             |             |             |             |             |             | +           |
| Ser       |                     |             |             |             |             |             |             |             |             |             |             | +           |
| Phe       |                     |             |             |             |             |             |             |             |             |             |             | +           |
| Tyr       |                     |             |             |             |             |             |             |             |             |             |             | +           |
| Leu       |                     |             |             |             |             |             |             |             |             |             |             | +           |
| Gln       |                     |             |             |             |             |             |             |             |             |             |             | +           |
| Glu       |                     |             |             |             |             |             |             |             |             |             |             | +           |
| Pro       |                     |             |             |             |             |             |             |             |             |             |             | +           |
| His       |                     |             |             |             |             |             |             |             |             |             |             | +           |
| Lys       | 0.1                 | 0.1         | 5           | 5           | 1           | 3           | 1           | 1           | 1           | 0.1         |             | 1           |
| No suppressor |             | 115$^b$    | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         |

| CLUSTER II | Inserted amino acid | Pro$^{215}$ | Met$^{216}$ | Glu$^{217}$ | Pro$^{218}$ | Val$^{219}$ | Ala$^{220}$ | Ser$^{221}$ | Glu$^{222}$ | Gln$^{223}$ | Ala$^{224}$ | Gln$^{225}$ | Lys$^{226}$ | Met$^{227}$ | Leu$^{228}$ | Gly$^{229}$ | Thr$^{230}$ | Asn$^{231}$ | Gly$^{232}$ | Gly$^{233}$ | Gly$^{234}$ | Phc$^{235}$ | Phc$^{236}$ | Asn$^{237}$ | Ala$^{238}$ | Asn$^{239}$ |
|------------|---------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Ala       |                     | 0.3         | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | 10          | +           |             | +           |             | +           | +           | +           | +           | +           | +           |
| Gly       |                     |             | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           | +           |             | +           |             | +           | +           | +           | +           | +           | +           | +           | +           |
| Cys       |                     |             |             |             |             |             |             |             |             |             |             |             |             | +           |             | 0.1         | +           |             |             |             |             |             |             |             |             |
| Ser       |                     |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
| Phe       |                     |             |             |             |             |             |             |             |             |             |             |             |             | 1           |             |             |             |             |             |             |             |             |             |             |             |
| Tyr       |                     |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
| Leu       |                     |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
| Gln       |                     |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
| Glu       |                     |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
| Pro       |                     |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
| His       |                     |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
| Lys       | 0.3                  | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         | 0.1         |
| No suppressor |             | 115$^b$    | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         | 115         |
K⁺ Binding by the Kdp ATPase

Table I—continued

Cluster III

| Inserted amino acid | Leu<sup>330</sup> | Val<sup>331</sup> | Ser<sup>332</sup> | Ser<sup>333</sup> | Leu<sup>334</sup> | Phe<sup>335</sup> | Ala<sup>336</sup> | Val<sup>337</sup> | Val<sup>338</sup> | Thr<sup>339</sup> | Thr<sup>340</sup> | Ala<sup>341</sup> | Ala<sup>342</sup> | Ser<sup>343</sup> | Cys<sup>344c</sup> | Ala<sup>346c</sup> | Le<sup>348c</sup> |
|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Ala                 |                 |                 |                 |                 |                 |                 |               |               |               |               |               |               |               |               |               |               |
| Gly                 |                 |                 |                 |                 |                 |                 |               |               |               |               |               |               |               |               |               |               |
| Cys                 | +               | +               | +               | +               |                 |                 |               |               |               |               |               |               |               |               |               |               |
| Ser                 |                 |                 |                 |                 | +               | +               |               |               |               |               |               |               |               |               |               |               |
| Phe                 |                 |                 |                 |                 |                 | +               | +             |               |               |               |               |               |               |               |               |               |
| Tyr                 |                 |                 |                 |                 |                 |                 | +             |               |               |               |               |               |               |               |               |               |
| Leu                 | +               |                 | +               | +               |                 | +               +             |               |               |               |               |               |               |               |               |               |
| Gln                 |                 |                 |                 |                 |                 |                 |               | +             |               |               |               |               |               |               |               |               |
| Glu                 |                 |                 |                 |                 |                 |                 |               |               | +             |               |               |               |               |               |               |               |
| Pro                 |                 |                 |                 |                 |                 |                 |               |               |               | +             |               |               |               |               |               |               |
| His                 |                 |                 |                 |                 |                 |                 |               |               |               |               | +             |               |               |               |               |               |
| No suppressor       | 115             | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>a</sup> | 115<sup>a</sup> | 115<sup>a</sup> | 115<sup>a</sup> | 115<sup>a</sup> | 115<sup>a</sup> | 115<sup>a</sup> | 115<sup>a</sup> | 115<sup>a</sup> | 115<sup>a</sup> | 115<sup>a</sup> | 115<sup>a</sup> |

Table I—continued

Cluster IV

| Inserted amino acid | Tyr<sup>458</sup> | Ala<sup>459</sup> | Val<sup>460</sup> | Ser<sup>461</sup> | Ser<sup>462</sup> | Ala<sup>463</sup> | Ala<sup>464</sup> | Asn<sup>465</sup> | Asn<sup>466</sup> | Asn<sup>467</sup> | Gly<sup>468</sup> | Pro<sup>469</sup> | Met<sup>470</sup> | Pro<sup>471</sup> | Leu<sup>482</sup> | Met<sup>483</sup> | Gly<sup>484</sup> | Ser<sup>485</sup> | Phe<sup>486</sup> |
|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Ala                 |                 |                 |                 |                 |                 |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| Gly                 |                 |                 |                 |                 |                 |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| Cys                 |                 |                 |                 |                 |                 |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| Ser                 |                 |                 |                 |                 |                 | +             |               |               |               |               |               |               |               |               |               |               |               |               |               |
| Phe                 |                 |                 |                 |                 |                 |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| Tyr                 |                 |                 |                 |                 |                 |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| Leu                 | 0.1             |                 |                 |                 |                 |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| Gln                 |                 |                 |                 |                 |                 |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| Glu                 |                 |                 |                 |                 |                 |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| Pro                 |                 |                 |                 |                 |                 |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| His                 |                 |                 |                 |                 |                 |               |               |               |               |               |               |               | 3             |               |               |               |               |               |               |
| Lys                 | 1               | 1               | 0.1             | 0.3             | 0.3             | 0.3           | 0.3<sup>c</sup> | 0.3           | 0.3<sup>c</sup> | 0.3           | 0.3           | 1             | 1             | 1             | 1             | 1             | 1             | 1             | 1             |
| No suppressor       | 115             | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> | 115<sup>b</sup> |

<sup>a</sup> Recreates previously isolated mutant (3).
<sup>b</sup> Growth at lower K⁺ concentration in wild type but not in rpsL strain.
<sup>c</sup> Confirmation of amber mutation by DNA sequencing.

was measured at three well spaced K⁺ concentrations in an attempt to bracket the $K_m$ value.

Collection and analysis of cell samples for measurement of K⁺ uptake and expression of the values per unit dry weight based on turbidity measurements have been described (3). Initial rates of uptake were based on a visual best fit to the early part of the uptake curves, which were linear for at least 3 min. Kinetics of K⁺ uptake for the two strains tested that grew in 5 mM K⁺ medium in the absence of a suppressor and the Lys replacement at residue 110 were performed after K⁺ depletion to increase accuracy in measuring the low rate of uptake in these strains. Kinetics in all cases are based on transport data after correction for the contribution of TrkF activity (3).

Protein Alignment and Analysis—Nucleotide sequences were downloaded from GenBank<sup>TM</sup>, translated into protein sequences, and aligned using the Clustal method (DNASTAR software package). Accession numbers are K02670 (E. coli), AL123456 (Mycobacterium tuberculosis), AL078610 (Streptomyces coelicolor), U44892 (Clostridium acetobutylicum), AJ243194 (Alicyclobacillus acidocaldarius), AB001339 (Synechocystis PCC6803), and AP213466 (unidentified Anabaena species).

RESULTS

The Miller-Abelson Interchange System—This system allows large scale and rapid phenotypic screening of a large number of changes at each amino acid residue. Identification of amber mutations can be done without DNA sequence analysis by simple growth tests in suppressing or nonsuppressing strains. For this study, we made amber mutations in 83 of the 557 amino acid residues of KdpA: 11 residues in cluster I, 25 in cluster II, 34 in cluster III, and 13 in cluster IV. Although suppression confirms the presence of these amber mutations, adjacent residues might also have been altered. To determine the frequency of such an event, the 21 amber mutations
marked by footnote c in Table I were subjected to DNA sequencing of ~400 base pairs spanning the site of the mutation. All sequenced constructs had the expected amber mutation and no other change.

**Screening by Growth**—Each combination of mutation and suppressor was tested for growth on solid medium containing a range of K\(^+\) concentrations. Rapid growth of these strains in medium with K\(^+\) below about 20 mM depends on Kdp activity. Kdp activity is essential for visible growth below 12–15 mM K\(^+\).

A summary of the results of screening substitutions by growth is presented in Fig. 2. The height of each column indicates the number of substitutions affecting K\(^+\) binding. Effects of Lys substitutions on binding, a unique result of this study, are indicated by shaded boxes. The specific results summarized in Fig. 2 are based on detailed data shown in the Table I. Numeric entries in the Table I indicate the lowest K\(^+\) concentration on which the strain could grow; no entry in the table means that substitution restored the wild type phenotype (i.e. growth on 0 mM K\(^+\) medium).

The criterion we have chosen to implicate residues in a binding site is a change in affinity by three or more different amino acid substitutions. Even subtle changes in a binding site should have significant effects on binding. By this criterion 21 of the residues studied are in binding sites: 113, 114, and 116 in cluster I, 220, 224, 231, 232, 233 in cluster II, 339, 340, 343, 348, 352, 354, and 355 in cluster III, and 461, 464, 469, and 471 in cluster IV. Six of these residues, 114, 231, 232, 352, 461, and 469, had been previously identified as sites of affinity mutations (4). Only one of the residues tested was fastidious in its requirements; at residue 232 only Phe in addition to the resto-
ration of the normal Gly by one of the suppressors resulted in wild type growth.

The Lys substitution altered affinity at over 70% of the sites tested. At many sites, it was the only residue that did so. This effect appears to be due to its positive charge, because anionic, large neutral polar, and large hydrophobic residues were generally well tolerated at these residues. To confirm the effects of introducing a positive charge, we constructed Arg substitutions at 10 residues in clusters I and II (Table II). The results showed that Arg can be as disruptive as Lys. However, the effect of these two substitutions were somewhat different in that at some sites only one or the other abolished wild type growth. His tended to be disruptive at sites where Lys was disruptive (Table I).

Positive and Negative Controls—At 53 of the 83 sites, one of the suppressors inserted the wild type residue. This resulted in wild type growth in all cases, shown by a + in Table I, providing a positive control. In seven cases, suppression created a mutation studied earlier (4), and all of these substitutions had reduced affinity. In several cases the quantitative K

| Inserted amino acid | Val
| Thr
| Thr
| Trp
| Gln
| Ser
| Ser
| Met
| Leu
| Gly
| Km

rate (shown by curve B in Fig. 3) is sufficient to allow growth at all K

1

concentrations and hence to score as wild type. Thus, a reduction in rate of up to 97% with retention of normal affinity will not appear mutant by the growth tests. However, a reduction in affinity without drastic change in rate (shown by curves C and D in Fig. 3) will abolish growth at the lowest K

1

concentrations. A slight bit of leakiness, in which the affinity is not greatly altered, will also shift the growth curve (as illustrated in curve E in Fig. 3). Thus, the growth data cannot distinguish between effects of a large change in affinity from a dramatic reduction in the rate to less than 3% of the wild type rate.

To confirm the reliability of the growth data in reflecting primarily changes in affinity, we determined the transport kinetics of 29 substitutions in cluster I (Table III). All but one of the substitutions made by the amber suppression method have a rate that is at least 25% of the control. These rates are about what is expected for Kdp, given that the efficiency of suppression ranges from about 10 to 70% (20). The relatively low rates of the Lys substitutions are consistent with the relatively low efficiency of this suppressor. The three Arg substitutions for which transport was analyzed, made directly and not dependent on the efficiency of suppression, have rates in good agreement with the control. Thus, dependence of growth on K

1

concentration reflects changes in affinity. Substitution of Lys at residue 110 is the one exception. Here the
**TABLE III**

| Substitutions | Wild type residue | Replaced by | $K_a$ | $K_m$ | $V_{max}$ |
|---------------|-------------------|-------------|-------|-------|----------|
| Control       |                   | +           | <0.01 | 73    | 73       |
| Val$^{110}$   | Arg +             | <0.01       | 98    |       |          |
|               | Gly +             | <0.01       | 50    |       |          |
|               | Ser +             | 0.03        | 52    |       |          |
|               | Tyr +             | <0.01       | 36    |       |          |
| Lys           | 0.1               | ~5'         | <2'   |       |          |
| Asn$^{112}$   | Cys +             | <0.01       | 40    |       |          |
|               | Glu +             | <0.01       | 62    |       |          |
|               | Glu +             | <0.01       | 51    |       |          |
|               | Gly +             | <0.01       | 65    |       |          |
|               | His +             | 0.04        | 17    |       |          |
|               | Phe +             | 0.04        | 54    |       |          |
|               | Pro +             | 0.1         | 33    |       |          |
|               | Ser +             | 0.015       | 50    |       |          |
|               | Tyr +             | 0.015       | 49    |       |          |
| Thr$^{113}$   | Arg 10            | ~100$^d$    | ~80$^d$ |       |          |
|               | Glu +             | 0.045       | 35    |       |          |
|               | Lys 5             | 30          | 28    |       |          |
|               | Phe 0.1           | 4           | 50    |       |          |
|               | Ser +             | 0.04        | 45    |       |          |
| Asn$^{114}$   | Gly +             | 0.08        | 25    |       |          |
| Gln$^{116}$   | Arg 0.1           | 1           | 70    |       |          |
|               | Gln +             | <0.01       | 37    |       |          |
|               | Lys 1             | 18          | 22    |       |          |

$^a$ Lowest $K^+$ concentration where growth was observed. + indicates wild type growth (growth on 0 mM K$^+$ plates).

$^d$ Lower limit determinable by transport measurements with nonradioactive K$^+$ is 0.01 mM.

Rate too low to obtain more exact values.

$^d$ Kinetic data with greater errors because extrapolated from rates measured up to a K$^+$ concentration of 75 mM.

The effect is a drastic reduction in rate. The low rate of transport is so close to that of the TrkF system alone that a reliable estimate of the affinity could not be determined.

The measurements of apparent affinity, the $K_m$ in Table III, show that growth data are a conservative indicator of changes from wild type affinity. Of the 15 displaying wild type growth, only seven had a wild type affinity; the other eight showed $K_m$ values ranging from 15 to 80 $\mu$M. Thus, the growth data are generally reliable in that reductions in affinity of more than 40-fold always appear mutant, whereas smaller reductions in affinity may not be detected by growth tests. The limited power of growth tests is expected, because the lowest $K^+$ concentration easily tested by growth is 20 $\mu$M because of contamination by K$^+$ of the medium without added K$^+$.

**DISCUSSION**

The analysis of the effects of at least 11 substitutions at 83 residues in KdpA has resulted in the delineation of regions critical for K$^+$ binding. The high affinity of Kdp enabled us to analyze substitutions that remain fully functional for transport but drastically reduce affinity, this parameter changing by more than a factor of 10$^4$ for some substitutions. Furthermore, the wide range of amino acid substitutions ensured a comprehensive analysis of each of the residues examined in this study. The functional information provided by genetic manipulation of transport proteins can also be used to infer structural features of integral membrane proteins in transport systems, a class of proteins for which crystallographic structures are generally lacking. The identification of residues in K$^+$-binding sites in conjunction with the effects of cationic substitutions further supports the role of KdpA in binding K$^+$ and mediating its transmembrane movement.

**Criteria for Residues in Binding Sites**—Mutations that alter the apparent affinity for a transported substrate, the $K_m$ for transport used in Table III, are considered to identify residues in a substrate-binding site. This inference is justified if the rate constants subsequent to binding are not greatly altered. In the mutants studied here the overall rate of transport is usually not significantly reduced, implying no large change in the rate constants of transport. Therefore, we conclude that large changes in $K_m$ represent predominantly changes in affinity. Our identification of the residues involved in binding is conservative because changes that reduce $K_m$ by less than ~40-fold may not be scored because of the limitations in the sensitivity of growth tests used. Of course, residues may have been misidentified as altering affinity if they dramatically altered the rate of transport (decreasing it to <3% of the wild type level), as seen for Lys$^{110}$. Based on the data of Table III and the transport data on mutations analyzed earlier (4), this is unlikely to have occurred at more than a few residues. Mutations outside a binding site can alter the site by creating a conformational change that is propagated to the site. Such an effect is very likely to reduce the rate of transport as well and was inferred to explain the three mutants altering the KdpB and KdpC subunits, which had low transport rates (4). This is concordant with the observed kinetics of transport for Lys$^{110}$ as well.

Our studies of the substitutions tested only affinity for K$^+$, but many of these mutations probably reduce the specificity of transport as well. Several of the residues studied earlier had reduced discrimination against Rb$^+$ and Cs$^+$ (4). The substitution of Gly$^{232}$ by Asp relaxes specificity so dramatically that Rb$^+$ is transported with an affinity equal to that for K$^+$, and even Na$^+$ and H$^+$ are transported (21).

**Conservation of K$^+$-binding Residues**—There is considerable divergence between the 17 KdpA protein sequences currently available but much greater conservation within the four clusters delineated in this study. Absolute residue conservation across seven widely divergent bacterial species in the four clusters is 33% (29 of 89 residues), whereas it is only 16% (73 of 468 residues) in the remainder of the protein (comparison conducted between E. coli, M. tuberculosis, S. coelicolor, A. acetobutylicum, A. acidocaldarius, Synechocystis PCC6803, and an unidentified Anabaena species). This is statistically significant utilizing a Fisher's Exact Test (two-tailed, $p = 0.0045$). The ability to bind K$^+$ with high affinity and specificity is therefore a functionally constrained property of Kdp that has been conserved over relatively large evolutionary distances (22).

**The Effect of Cationic Substitutions**—A striking finding was the large number of residues where Lys substitution reduced affinity. Neither size nor polarity alone can account for this effect, because large nonpolar, polar neutral or anionic residues only infrequently reduced affinity at sites not identified as binding sites. These effects are primarily attributable to the cationic charge of Lys, which tends to repel the cationic substrate of Kdp. Arg substitution had qualitatively similar effects to those sites where this change was made (Table II). There are differences between the effects of Arg and Lys substitutions, probably accounted for by the rather different structure and chemical properties of these two residues and the fact that the Arg substitutions, made directly and not by suppression, were expressed at a much higher rate than the Lys substitutions. Consistent with the unique effect of cationic residues is the fact that His is disruptive at a subset of sites affected by Lys. His
residues will be positively charged when the local environment is such that the alkaline pK of the His side chain is above 7.5. Local environmental effects are unlikely to shift the higher pK of the δ-amino group of Lys to such an extent that it would not be positively charged.

To what extent can the effects of Lys be attributed to conformational effects of this large and strongly charged residue? The other residue which is especially likely to alter conformation is Pro. At 62 residues in KdpA, Lys substitution alters affinity, whereas at only 19 does Pro have this effect. Furthermore, the magnitude of the effects of Lys substitution is much greater than that of Pro except at two residues. A guide to interpreting our data is provided by studies of two soluble proteins by the amber-suppression method. In the 132 residues of the Lac repressor tested, Lys altered function at 75 residues and abolished activity at 42 versus Pro, which altered function at 91 residues and abolished activity at 55 (8). In the 136 residues of T4 lysozyme, Lys altered function at 101 sites and abolished activity at 11 versus Pro, which altered function at 88 residues and abolished activity at 21 (23). These soluble proteins are an appropriate comparison, because almost all of the clusters studied here are extramembraneous and hence exposed to solvent. At a number of residues Lys effects are probably due to conformational effects, such as at residue 110 (Table III), but the marked difference between effects of Lys and Pro provide support for the importance of charge in most of these results.

Cationic charges will repel the cationic substrate of Kdp, K^+. The strength of that repulsion will be determined by the effective distance, the combined effect of distance, and the intervening dielectric constant from a residue to the site where K^+ is present. Thus, the Lys substitutions provide a rough indication of how far a residue is from a site where K^+ is bound or past which K^+ moves. The repulsive effect will be sensitive to the orientation of the Lys side chain within the structure of the protein. The many sites where Lys is disruptive confirm the importance of the KdpA subunit, and in particular the four clusters studied here, in binding.

Comparisons with Other P-type ATPases—Cation-binding sites in KdpA differ markedly in location and function from those in other P-type ATPases. Such sites in the Ca^{2+} and Na^{+},K^{+}-ATPases are formed by several membrane spans and are located in the membrane space. This location, inferred from mutational studies (24, 25) has now been confirmed by crystallographic analysis in the case of the Ca^{2+}-ATPase (26). The mutational studies showed that the binding sites have a dual role because most substitution in key residues abolish transport although some reduce but do not abolish binding of Ca^{2+} (27, 28). Therefore, these residues must also play a key role in one or more of the steps of the transport cycle in addition to binding Ca^{2+}. By contrast, cation-binding sites in KdpA are largely extramembranous, and mutational alteration of key binding residues generally alters only binding and not transport rates. However, to the extent that KdpA participates in major conformational changes during transport, some binding residues may be important in determining the rate of transport. The difference of Kdp from other similar transport ATPases is not unexpected because the latter have no equivalent of KdpA. In Kdp, substrate binding and presumable movement is relegated to the KdpA subunit whereas energy coupling and other related functions must be performed by the separate KdpB subunit. These two functions are performed in other ATPases by a single large subunit.

There is some similarity of KdpA to the sequence of some plant pyrophosphatases that couple H^+ transport to synthesis or hydrolysis of pyrophosphate (29). However, most of the regions of similarity are outside the clusters, and only a few residues in clusters are included. Although these pyrophosphatases can transport K^+ (30), the similarity seen suggests that it is not substrate specificity that has been conserved. Rather, KdpA might participate in the hydrolysis of the phosphorylated intermediate of the system, which is formed on the KdpB subunit.

Resemblance to K^+ Channels—A structure of Kdp modeled on that of K^+ channels has been proposed by Durell et al. (22). That model differs from ours in two important ways: 1) a topological difference is in the placement of membrane span seven, which Durell et al. move some 26–52 residues distally (Fig. 1) from the location based on topologic analysis (4), and 2) the model postulates a single K^+ “recognition” site analogous to the narrow part of a K^+ channel, whereas we postulate one binding site in the periplasm and a second largely cytoplasmic binding site that represents the site where K^+ would be occluded. The model of Durell et al. is critically dependent on a different location of membrane span 7 than that shown in Fig. 1 but accepts all other aspects of this previous topological analysis. Their shift in the location of membrane span seven is “…really only a hypothesis based on sequence analysis and limited, indirect experimental data” (22). In fact, these authors presented no experimental data about Kdp. The position of membrane span 7 in our model is well supported by data for seven fusion proteins: one in the periplasmic loop before span 7, one in the middle of span 7, two just distal to span 7, and three more distal in the loop between spans 7 and 8. Potential ambiguity in topology of the fusions because of the presence of Glu^{370} was excluded by showing that its replacement by Ala resulted in the same result for fusions at residues 375 and 378 (4). Given the absence of new experimental data and the extensive data presented previously, changing the location of membrane span 7 is unsupported. However, even if the topology were such that cluster III is in the periplasm, the mutational evidence presented here shows that the specific model of a K^+-channel like structure is not supported. The Durell model arranges the four binding clusters to form four similar P-loops symmetrically distributed around a central K^+-accepting pore.
This model implies that alterations of corresponding residues in each P-loop would have similar functional effects, whereas our data suggest otherwise. In Fig. 4, residues in the four proposed P-loops are aligned with the effects of mutations as illustrated in Fig. 2. Marked differences in sensitivity to substitutions are observed. A Gly residue, proposed to be analogous to one of the residues in the conserved Gly-Tyr-Gly sequence of K⁺ channels, is not present in cluster I and is sensitive to mutation in cluster II but indifferent in cluster IV. The distribution of key residues outside these P2 regions is also different, with cluster III showing a very large number of distant residues that are important in binding of K⁺, a distribution clearly different from that of the other clusters.

Utility of Amber Suppression Screening.—The amber substitution method allows for comprehensive screening of residues with much less effort than making each substitution individually. Originally developed when mutagenic procedures were cumbersome (9), it remains useful for genes that can be expressed in *E. coli* and poses some advantages over other screening methodologies. For example, alanine screening mutagenesis would not have identified all binding residues because in all but four cases this substitution was wild type by growth tests. One limitation of the method is the variable degree of suppression, although this is largely compensated for by the increased gene dosage of using plasmid-encoded genes. Misscoring can also occur at a low frequency but will rarely be problematic because a miscoded product will never comprise more than a small fraction of the total product.

In the absence of a detailed structure of Kdp, we must make inferences about the sites where a substrate binds based on growth and transport kinetic data. The criteria used here, that three or more substitutions alter apparent affinity, is reasonable but could be misleading in the case of residues forming a key conformation that is disrupted by most substitutions. The effects of Lys substitutions are unusual and potentially useful in giving information on the distance between a residue and sites where K⁺ binds or passes. Cationic or anionic residue substitutions may be useful as an approximate indicator of distance to binding sites or transport paths in other systems for which cations or anions, respectively, are a substrate.

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