Lysine 539 of Human Band 3 Is Not Essential for Ion Transport or Inhibition by Stilbene Disulfonates*

Ana Maria Garcia‡ and Harvey F. Lodish‡‡

From the ‡Whitehead Institute for Biomedical Research and ‡Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142

The anion transporter from human red blood cells, band 3, has been expressed in Xenopus laevis frog oocytes microinjected with mRNA prepared from the cDNA clone. About 10% of the protein is present at the plasma membrane as determined by immunoprecipitation of covalently bound 4,4'bis(diisothiocynato)-2,2'-disulfonic acid stilbene (DIDS) with anti-DIDS antibody. The expressed band 3 transport chloride at a rate comparable to that in erythrocytes. Transport of chloride is inhibited by stilbene disulfonates, niflumic acid, and dipyrismide at concentrations similar to those that inhibit transport in red blood cells: DIDS and 4,4'-dinitro-2,2'-stilbene disulfonate inhibit chloride uptake with Ki's of 34 nM and 2.5 μM, respectively. Lysine 539 has been tentatively identified as the site of stilbene disulfonate binding. Site-directed mutagenesis of this lysine to five different amino acids has no effect on transport. Inhibition by stilbene disulfonates or their covalent binding was not affected when Lys-539 was substituted by Gin, Pro, Leu, or His. However, substitution by Ala resulted in weaker inhibition and covalent binding. These results indicate that lysine 539 is not part of the anion transport site and that it is not essential for stilbene disulfonate binding and inhibition.

Band 3 (Mr = 95,000), the predominant red blood cell membrane glycoprotein, has two domains. The cytoplasmic, amino-terminal segment (40 kDa) binds ankyrin and other cytoskeletal and cytosolic proteins. The membrane-bound domain (55 kDa) catalyzes an obligatory one-to-one exchange of anions, at a rate of 108 ions/s (for reviews, see Refs. 1–5). Anion transport can be explained by a ping-pong mechanism in which a single transport site is alternatively exposed to each side of the membrane. However, details about the structure of the anion binding and transport site are not available.

A great deal of information about band 3 structure and transport mechanism has been obtained by the use of transport inhibitors. The most widely used are a group of stilbene disulfonates which inhibit transport with high affinity. The binding of stilbene disulfonates is stabilized at the binding site by the negative charges of the sulfonates as well as by the presence of a hydrophobic moiety (6). Binding can be reversible or irreversible (covalent), although the latter does not seem to be necessary for inhibition (6, 7). Regardless of the type of binding, inhibition by stilbene disulfonates is competitive with substrate, suggesting interaction at or near the transport site. The presence of an isothiocyanate group in DIDS, H2-DIDS, and SITS, allows covalent reaction with the amino group of a lysine side chain which is accessible only from the outside of the red blood cell membrane and only when the transport site is facing outward. These two pieces of evidence, competitive inhibition and orientation of the transport site for covalent reaction with DIDS, have suggested that this lysine may be part of the anion transport site. However, modification of presumably the same lysine by reductive methylation (8), BSSS (9), or pyridoxal phosphate (10) does not affect the anion transport properties of band 3. Reductive methylation and BSSS prevent covalent but not reversible binding of stilbene disulfonates. Although it remains to be shown whether the same lysine which reacts with DIDS and other stilbene disulfonates is modified in the three cases mentioned above, these results strongly suggest that this lysine may not be directly involved in anion transport. Nevertheless, DIDS and other stilbene disulfonates are also effective inhibitors of a number of other anion transporters, including chloride channels (11). Thus, the structure of the DIDS binding site and by extension that of the transport site could be conserved among different anion transporters. For this reason, DIDS has been used in attempts to identify, purify, and clone putative anion transporters (12–14). It is thus important to identify the DIDS-binding lysine and to determine its role in anion transport.

Recently, the human (15), murine (16), and chicken (17, 18) band 3 cDNAs have been cloned. The deduced amino acid sequences show a very high degree of identity in the membrane-associated domain. The homology is less in the cytoplasmic domain, but regions such as the putative ankyrin binding site are highly conserved in all three proteins (15–18). Several sites of chemical modification or proteolytic cleavage have been assigned to the primary sequence of HB3 (15). Thus, the site of stilbene disulfonate covalent binding, localized near the carboxyl terminus of the 17-kDa tryptic fragment (see reviews, Refs. 1–4), has been identified as residues 539 or 542 of human band 3. These 2 lysines are conserved in human and murine band 3, as well as in a related anion transporter cloned from kidney (19). However, only one of these, Lys-539, is present in avian band 3 (17–18), making it the most likely candidate for the DIDS-binding site.

*The abbreviations used are: DIDS, 4,4'-bis(diisothiocynato)-2,2'-stilbene disulfonate; H2-DIDS, 4,4'-bis(diisothiocynato)-1,2'-diphenylethane-2,2'-disulfonate; SITS, 4-acetamido-4'-bis(isothiocynato)-2,2'-stilbene disulfonate; DNDS, 4,4'-disnitro-2,2'-stilbene disulfonate; BSSS, bis(sulfosuccinimidyl) suberate; KLH, keyhole limpet hemocyanin; 8'-GpppG, 5,5'-diguanosine triphosphate; HB3, human band 3; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

19607
Previous reports using mouse spleen mRNA (20, 21) and our own preliminary studies with a murine band 3 clone (22) have shown that band 3 can be functionally expressed in frog oocytes. Here we show that human band 3 is expressed in oocytes injected with mRNA prepared in vitro and that the properties of the expressed protein are similar to those of native band 3 in red blood cell membranes. Furthermore, we mutated Lys-539 to five different residues and found that anion transport is not affected. The reversible and irreversible binding of DIDS was not affected when Lys-539 was substituted by Ala, Glu, Leu, or Pro, although when Lys was substituted by Ala, the inhibitory potency of stilbene disulfonates was markedly decreased. We conclude that Lys-539 is not an essential part of the anion transport site and that it is not the primary site of stilbene disulfonate binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—cDNA clones encoding the full coding sequence of human band 3 were isolated by S. Lux in our laboratory; the sequence is identical to that reported by Tanner et al. (15). The following inhibitors of band 3 transport were used: DIDS (Pierce Chemical Co.); H2-DIDS, and SITS (Molecular Probes); ND96 (Fahlz and Bauer); niflumic acid and dipyriramole (Sigma). Radiosioptopes were from Amersham Corp. All reagents were of the highest grade available.

**In Vitro Transcription**—A 3.4-kilobase band 3 clone (pHB3) comprising the full length coding sequence plus 5'- and 3'-noncoding sequences of 30 and 630 base pairs, respectively, was subcloned into the Acl and Sacl sites of Bluescribe (Strategene). It was linearized with HindIII and mRNA was prepared according to the manufacturer's instructions, using T7 RNA polymerase (Strategene) and 0.5 mM 5'-GpppG (Pharmacal, LKB Biotechnology Inc.) as the mRNA 5'-cap (23). After 2 h at 37 °C, the reaction was stopped by the addition of RNase-free Dnase (Cappel) for 10 min at 37 °C. The mRNA was purified free of unincorporated nucleotides by centrifugation through a 1.5-ml Spinco 50 column equilibrated with water. The mRNA (200 μl) was concentrated by centrifugation for 15 min in a Centricon-30 concentrator (Amicon), separated into 10-μl aliquots, and stored frozen at -70 °C.

**Site-directed Mutagenesis**—The internal 1.6-kilobase KpnI fragment of HB3 was subcloned into Bluescribe. From this, a 300-base pair XbaI fragment (residues 1561-1855) was digested and subcloned into the XbaI site of M13mp18. The following mutagenic 20-mer oligonucleotide was synthesized (Research Genetics): GATCTTGAT-CAG(T)(G,T)(A,C,T,G)(A,C,G)GGAGA. Site-directed mutagenesis of HB3 was subcloned into Bluescribe. From this, a 300-base pair fragment was inserted into the XbaI site of Bluescribe (Stratagene). The following mutagenic 20-mer oligonucleotide was synthesized (Research Genetics): GATCTTGAT-CAG(T)(G,T)(A,C,T,G)(A,C,G)GGAGA.

**Antibodies**—An antibody against the cytoplasmic, amino-terminal domain of band 3 was a generous gift of Dr. F. Low, Purdue University.

**Expression of Human Band 3**—Total membranes prepared from oocytes microinjected with water (control) or mRNA prepared in vitro from the pHB3 clone coding for human band 3 (HB3) were immunoprecipitated with an antibody against the cytoplasmic, amino-terminal domain of HB3. After autoradiography of proteins separated by gel electrophoresis, a major protein of molecular mass 90-95 kDa was observed in mRNA-injected oocytes (Fig. 1, lane 2) that was absent in control oocytes (lane 1). A large number of nonspecific polyproteins are present in both control and band 3 injected oocytes; the background varies from experiment to experi-
Transport protein in red blood cells (7), and it has recently been shown that the anion transporters (12-14) of band 3 has been used to identify the anion transporters of band 3.

In order to determine the relative amount of newly synthesized band 3 present at the plasma membrane, intact oocytes were covalently labeled with DIDS. After washing the oocytes to remove unreacted DIDS, band 3 was isolated by immunoprecipitation of oocyte membranes. This material was reimmunoprecipitated with anti-DIDS antibody (lane 3) and subsequently with anti-HB3 antibody (lane 4). Each lane corresponds to 20 microinjected oocytes, labeled with radioactive methionine for 16 h.

Properties of Human Band 3 in Frog Oocytes—In red blood cells, the physiological role of band 3 is to catalyze a one-for-one exchange of chloride for bicarbonate. However, a great deal of kinetic information has been obtained by studying chloride self-exchange (for reviews, see Refs. 1-3). In order to study anion transport properties of human band 3 expressed in frog oocytes, we measured uptake of chloride under steady state conditions, i.e. oocytes in ND-96 at room temperature (Fig. 2). Control, mock-injected oocytes show two phases of chloride accumulation: a rapid uptake during the first half-hour (our first time point), and a slower accumulation which continues for up to 8 h at a constant rate of 0.1-0.3 nmol of Cl-/oocyte/h. In contrast, the amount of chloride taken up by band 3-injected oocytes increases rapidly for up to 4 h and then continues at the same rate as control oocytes. During the initial phase of transport, the total amount of chloride taken up by mRNA-injected oocytes is about 5 times higher than the control. In most experiments, the rate of band 3-mediated chloride uptake is constant for the first 1 or 2 h. However, in some experiments (like that shown here) uptake can be linear for up to 4 h. Both the rate and total amount of chloride accumulated are variable, and this seems to be related to individual frog differences, seasonal variations, and other factors out of our control. In all subsequent experiments the rate of chloride uptake was determined within the first hour.

As indicated, the initial rate of band 3-mediated chloride accumulation varied among experiments, ranging from 0.5 to 2.5 nmol of chloride/oocyte/h and occasionally higher. However, in most experiments the rate of transport was between 1 and 1.5 nmol of Cl-/oocyte/h. For purposes of calculation, we will use 1 nmol of Cl-/oocyte/h (1.7 × 10⁻¹⁰ ions/s/oocyte) as the average rate. To compare this rate to the rate of chloride self-exchange by HB3 in erythrocyte membranes, we determined the number of surface band 3 molecules per oocyte. The amount of band 3 immunoprecipitated by the specific antibody was determined by cutting the slice of gel after autoradiography and measuring its radioactivity by scintillation counting. The specific activity of methionine was calculated from the total radioactivity of the oocyte homogenate and a methionine pool size of 30 pmol/oocyte (27). In one particular experiment, the methionine specific activity was 3.2 × 10⁸ cpm/pmol and the amount of HB3 immunoprecipitated from 10 oocytes was 300 cpm. Since each band 3 mole-

![Fig. 1. Immunoprecipitation of oocyte membranes prepared from human band 3-injected oocytes and identification of surface molecules. Lane 1, control, mock-injected oocytes. Lanes 2-4, oocytes injected with 14 ng of HB3 mRNA/oocyte. Lanes 1 and 2, oocyte membranes immunoprecipitated with anti-HB3 antibody. Lanes 3 and 4, after radioactive labeling, the oocytes were reacted with 1 μM DIDS for 1 h. Band 3 immunoprecipitated from these oocyte membranes was re-immunoprecipitated with anti-DIDS antibody (lane 3) and subsequently with anti-HB3 antibody (lane 4). Each lane corresponds to 20 microinjected oocytes, labeled with radioactive methionine for 16 h.](image1)

![Fig. 2. Time course of chloride uptake. Oocytes were injected with HB3 mRNA (closed circles) or mock-injected with water (open circles). Chloride uptake was measured 2 days after microinjection as indicated under “Experimental Procedures.” Each time point is the average of 10 oocytes ± S.E.](image2)
cule has 24 methionines, we calculated that $2.3 \times 10^6$ molecules of band 3 were synthesized per oocyte. Assuming that 10% is at the plasma membrane (Fig. 1, lanes 3 and 4), we estimated a turnover number of $7.4 \times 10^4$ Cl$^{-}$/s for band 3-mediated anion transport in frog oocytes. From Brahms (30), the rate of chloride transport for HB3 in red blood cells in 150 mM KCl and 20 °C is $7.7 \times 10^4$ ions/s and at 38 °C is $4.8 \times 10^4$ ions/s. Thus, although our calculation is approximate, we conclude that HB3 in the plasma membrane of an oocyte transport chloride at a rate similar to that in red blood cell membranes.

Effect of Inhibitors of Transport—To analyze in more detail the properties of HB3 expressed in frog oocytes, different inhibitors of chloride transport were studied and their effects compared to those on HB3 in erythrocytes. The inhibitors used can be divided in two groups: competitive and noncompetitive. Among the competitive inhibitors, DIDS (7), H$_2$DIDS (31), and SITS (6) are stilbene disulfonates which covalently react with band 3, although covalent reaction is not necessary for inhibition. Another stilbene disulfonate, DNDS (32), does not react covalently. Two other inhibitors, dipyriramole (33) and niflumic acid (34), are reversible inhibitors whose effect does not depend on chloride concentration. Thus, these probably do not bind to the transport site and, therefore, have a different mechanism of inhibition from that of stilbene disulfonates.

Results of two experiments are summarized in Table I. The concentration of each inhibitor used corresponds to $K_{pp}$ values determined for erythrocytes (1). At the concentrations used, none of the inhibitors had a significant effect on the basal anion transport by the oocytes, indicating that the endogenous frog oocyte anion transport systems have properties different from those of band 3. In contrast, all six drugs inhibited band 3-mediated chloride transport. At the concentrations tested, DIDS, H$_2$DIDS, DNDS, and dipyriramole reduced the initial rate of chloride uptake by approximately 50%. SITS inhibits oocyte-expressed HB3 more than expected from the $K_{pp}$ determined in red blood cells. However, available data for SITS inhibition refer exclusively to inhibition of transport by covalently bound SITS (6). The covalent reaction with SITS is slower and less efficient than that of DIDS (6). Clearly, inhibition of transport occurs at a concentration lower than that needed for covalent reaction. Our results show that SITS inhibits chloride accumulation by HB3 in frog oocytes with an estimated $K_{pp}$ of 100 nM (data not shown). Niflumic acid inhibition is less than expected from the $K_{pp}$ measured on red blood cells (34). However, the reported $K_{pp}$ was determined for oxalate self-exchange (34). Oxalate seems to be transported by band 3, but it is not clear whether the mechanism of oxalate transport is comparable to that of chloride. Nevertheless, our data indicate that niflumic acid inhibits band 3-mediated chloride transport, although with an estimated $K_{pp}$ of 4 μM at 20 °C.

We studied in detail the inhibition of chloride transport by two stilbene disulfonates, DIDS, which reacts covalently with band 3 in red blood cells (7), and DNDS, a reversible inhibitor (32). Fig. 3 shows that in agreement with previous data (20), uptake of chloride by control oocytes is not affected by concentrations of DIDS up to 1 μM (Fig. 3, open circles). In contrast, increasing concentrations of DIDS decreases the initial rate of chloride uptake by mRNA-injected oocytes to the same value as control oocytes: 1 μM DIDS inhibits 99% of the band 3-mediated chloride transport. A plot of the reciprocal of the inhibitor concentration versus the reciprocal of the fractional inhibition generates a straight line (inset, Fig. 3), indicating that under the conditions of our experiments DIDS acts at a single site (1, 31). The x-intercept corresponds to the reciprocal of the $K_{pp}$ for DIDS: 34 nM (see also Fig. 6A, which gives a $K_{pp}$ of 18 nm). These values are in very good agreement with the value of 40 nM determined in erythrocytes (1). DNDS also inhibited HB3 expressed in oocytes with a $K_{pp}$ of 2.5 μM (see Fig. 6B), in very good agreement with published data (32).

Expression and Properties of Lys-539 Mutants—Fig. 4 shows that oocytes injected with mRNA coding for HB3 with five different substitutions of Lys-539 (K/A, K/H, K/L, K/P, and K/Q lanes), make comparable amounts of a polypeptide that is indistinguishable from wild type HB3 (HB3 lane), both in terms of its mobility as well as its reactivity toward the anti-band 3 antibody. These mutants are all capable of transporting chloride at a rate similar to wild type band 3, i.e. 2.8-3.8 nmol of Cl$^{-}$/oocyte/h (Fig. 5, -DIDS). In a different experiment, the rate of transport was normalized for the amount of protein made: all the mutants show the same rate of chloride accumulation as wild type (data not shown).

In order to characterize these mutants further, the effect of inhibitors of transport was examined. Fig. 5 (+DIDS), shows the effect of 100 nM DIDS on the initial rate of chloride uptake. As determined from the $K_{pp}$ this concentration of DIDS was expected to inhibit the rate of transport about 70%.

| Table I | Effect of inhibitors of chloride uptake: control vs. HB3 mRNA-injected frog oocytes |
|---------|----------------------------------|
| Expt.   | Inhibitor | Chloride uptake rate | Specific uptake | Inhibition |
|         | μM | Control* | HB3* |          |          |
| 1.      |     |          |      |          |          |
| None    | 0.03 | 0.64 ± 0.06 | 1.90 ± 0.18 | 1.26 | 57.8 |
| DIDS    | 0.03 | 0.60 ± 0.04 | 1.14 ± 0.08 | 0.54 | 58.7 |
| H$_2$DIDS | 0.34  | 0.70 ± 0.05 | 1.23 ± 0.11 | 0.53 | 88.8 |
| SITS    | 10$^a$ | 0.69 ± 0.05 | 0.82 ± 0.07 | 0.12 | 75.0 |
| DNDS    | 2$^a$ | 0.58 ± 0.03 | 1.24 ± 0.06 | 0.06 | 48.4 |
| Dipyriramole | 5$^a$ | 0.61 ± 0.03 | 0.93 ± 0.09 | 0.32 | 75.0 |
| 2.      |     |          |      |          |          |
| None    | 2.5$^a$ | 0.64 ± 0.02 | 5.18 ± 0.86 | 4.54 | 10.1 |
| Niflumic acid | 25 | 0.62 ± 0.03 | 4.75 ± 0.86 | 4.13 | 61.7 |

* Average of 10 oocytes, expressed as nanomoles of Cl$^{-}$/oocyte/h ± S.E.
1 Nanomoles of Cl$^{-}$/oocyte/h.
$^a$ Concentration of inhibitor corresponding to the $K_{pp}$ in erythrocytes (1).
$^b$ $K_{pp}$ for inhibition by covalently bound SITS (6).
$^c$ $K_{pp}$ for oxalate self-exchange at 22 °C (34).
FIG. 3. DIDS inhibition of band 3-mediated chloride uptake. Summary of three experiments measuring chloride uptake by frog oocytes in the presence of the indicated concentrations of DIDS (see "Experimental Procedures"). The data are expressed as percent inhibition of the initial rate of band 3-mediated chloride uptake (closed circles) or of the rate of chloride uptake by control oocytes (open circles). Inset, the data have been plotted as fractional inhibition versus the reciprocal of the inhibitor concentration. The extrapolated x value corresponds to the \( K^{\text{app}} \) for DIDS in 96 mM NaCl.

![Graph showing DIDS inhibition of chloride uptake.](image)

FIG. 4. Immunoprecipitation of membranes from oocytes injected with mRNA coding for wild type band 3 (HB3) or the mutants K539A (K/A), K539H (K/H), K539Q (K/Q), K539L (K/L), and K539P (K/P). C corresponds to control, mock-injected oocytes and MW to molecular weight markers. Each lane corresponds to membranes from 15 oocytes.

The accumulation of chloride by wild type HB3 as well as by the K539Q, K539L and K539P mutants was inhibited 90%, higher than expected but within experimental variation. In contrast, chloride transport by the K539A mutant was inhibited only 30%. Table II shows a summary of two experiments (different from the one shown in Fig. 5) in which several inhibitors were tested. Despite some variability in the absolute extent of inhibition of chloride transport, K539A mutant consistently shows less inhibition of chloride uptake by all these stilbene disulfonates, while the K539Q mutant is inhibited to the same extent as is the wild type. However, the noncompetitive inhibitor dipyridamole inhibits anion transport by all the mutants to the same extent as wild type band 3 (Table II).

Further characterization of ion transport by K539A generates a \( K^{\text{pp}} \) of 42.5 nM for DIDS inhibition and 6.1 \( \mu \)M for DNDS (Fig. 6, A and B). In contrast, in this particular experiment wild type HB3 is inhibited with a \( K^{\text{pp}} \) of 18.4 nM for DIDS and 2.5 \( \mu \)M for DNDS (Fig. 6, A and B). Thus, the K539A mutant is inhibited by these stilbene disulfonates 2-fold less than the wild type, regardless of whether the inhibitor binds reversibly or irreversibly.

Since only reversible binding of stilbene disulfonates is necessary for inhibition of anion transport, we needed to...
determine whether the mutants are capable of binding DIDS covalently. To this end, 2 days after injection the oocytes were treated with 10 or 100 μM DIDS for 1 h at 20 °C, conditions that irreversibly inhibit anion exchange by erythrocytes. After thoroughly washing to remove unreacted DIDS, the oocytes were assayed for their ability to accumulate 36Cl⁻ (Table III). Uptake of chloride was almost completely inhibited in oocytes injected with wild type HB3 or the K539Q mutant, indicating that even after washing the oocytes three times with a solution containing 0.5% bovine serum albumin, DIDS remains bound, presumably covalently. In contrast, K539A is inhibited much less, probably reflecting a lower binding affinity for DIDS. It remains to be seen whether longer incubations or higher DIDS concentration would promote complete inhibition by DIDS.

**Figure 6.** Effect of inhibitors of transport on chloride uptake by wild type HB3 or mutant K539A. Chloride uptake was measured in the presence of the indicated concentrations of DIDS (A) or DNDS (B), for 1 h, for either wild type HB3 (HB3, closed circles) or the mutant K539A (K/A, open triangles). The data are expressed as the percent inhibition of the specific chloride transport for groups of 10 oocytes.

**Table III**

Chloride uptake by wild type HB3 and Lys-539 mutants after covalent binding of DIDS

| Labeling conditions | Inhibition |
|---------------------|------------|
|                     | HB3        | K/A* | K/Q* |
| 10 μM DIDS          | 84.6%      | 0%   | 73.5% |
| 100 μM DIDS (Expt. 1)| 100%       | ND*  | 80.0% |
| 100 μM DIDS (Expt. 2)| 89.4%      | 56.8%| 78.2% |

* Mutant K539A.
* Mutant K539Q.
* Not determined.

**Discussion**

The work presented here clearly shows that frog oocytes injected with in vitro prepared human band 3 mRNA synthesize a functional protein which is recognized by anti-band 3 antibodies. All properties of the protein we tested, its rate of chloride transport as well as the effect of several transport inhibitors, are similar to those of band 3 in erythrocyte membranes. In particular, the ability of oocyte-synthetized band 3 to react covalently with DIDS and most likely with other stilbene disulfonates indicates that it is in a native conformation. Thus, within its limits and despite its variability, frog oocytes seem to be an appropriate system in which to test the effect of mutations on the transport properties of band 3.

Our aim was to identify unequivocally the putative DIDS-binding site and to determine its role in chloride transport. Chemical modification data together with knowledge of the primary sequence have localized this site to lysines 539 or 542 of the human band 3 sequence. However, since only Lys-539 is found in all three band 3 proteins cloned so far, it was considered the most likely candidate for being the DIDS-binding site. Our site-directed mutagenesis strategy provided several mutants from which five were randomly chosen and tested. The rate of chloride accumulation was not affected, regardless of whether Lys-539 was substituted for a smaller, uncharged residue (Ala or Leu) or a bulkier one (His, Pro, Glu) (Fig. 5). This indicates clearly that Lys-539 is not part of the anion transport site or at least that it does not participate in a limiting step in the transport of anions, corroborating previous information (8–10).

The results of inhibition by stilbene disulfonates were more surprising. For the K539Q, K539L, and K539P mutants, inhibition of chloride transport by the reversible inhibitor DNDS or by the irreversible inhibitors DIDS and SITS was not affected (Fig. 5 and Table II). Furthermore, inhibition by DIDS was accompanied by irreversible binding (Table III), suggesting that Lys-539 is not part of the DIDS-binding site. Our site-directed mutagenesis strategy provided several mutants from which five were randomly chosen and tested. The rate of chloride accumulation was not affected, regardless of whether Lys-539 was substituted for a smaller, uncharged residue (Ala or Leu) or a bulkier one (His, Pro, Glu) (Fig. 5). This indicates clearly that Lys-539 is not part of the anion transport site or at least that it does not participate in a limiting step in the transport of anions, corroborating previous information (8–10).

The above results can be interpreted in either of two ways: (a) Lys-539 is not the DIDS-binding lysine, in which case Lys-542 is the most likely candidate for it; or (b) DIDS can bind to either Lys-539 or Lys-542. The lack of effect on stilbene disulfonate inhibition observed with mutants K539Q, K539L, and K539P favors the first possibility, although if both lysines are equally reactive, DIDS could randomly bind to either Lys-539 or Lys-542. The fact that the K539A mutant shows an effect on stilbene disulfonate inhibition indicates that Lys-539 is near the DIDS-binding site. It is possible that the presence of Ala instead of Lys induces a conformational change which renders the DIDS-binding lysine less accessible. In fact, according to the Chou-Fasman prediction program...
(35), the stretch of 10 amino acids around Lys-539 in HB3 as well as in four of the five mutants has a higher probability of being in a β-sheet conformation. The only exception is the K539A substitution, for which the probability of the same stretch of folding in an α-helix configuration is higher. It is possible that in an α-helix conformation the binding of DIDS to a lysine residue such as Lys-542 is constrained. However, since there is very little information about the secondary and tertiary structure of band 3, such prediction values are speculative.

In summary, our results indicate that Lys-539 has no specific role in anion transport. According to secondary structure prediction models, Lys-539 is in an external loop between transmembrane helices 5 and 6. There is a cluster of about 20 positive charges lining the mouth of the transport pathway on both sides of the membrane. These charges are part of the loops between transmembrane segments and their role seems to be to attract anions to the transport site. Thus, the role of Lys-539 in transport could be mainly to contribute a positive charge. Also, Lys-539 does not seem to be the primary DIDS-binding residue. However, it must be kept in mind that the DIDS-binding site is localized in an area of the protein which, after inward translocation of an anion, becomes inaccessible to external DIDS. Lys-539 may be located in a flexible structure, possibly β-sheet, which can accommodate a small conformational change, either a rotation or a packing of the peptide chain. This conformational change could result in the accessibility of a lysine side chain for binding to stilbene disulfonates. Thus, it will be important to mutate Lys-542 alone and in combination with Lys-539 and study the resultant transport and inhibition properties.

Acknowledgments—We thank Drs. Seth Alper, Frank Brosius, Kevin Davies, Phil Knauf, and Sam Lux for helpful advice and gifts of materials.

REFERENCES
1. Knauf, P. A. (1979) Curr. Top. Membr. Transp. 12, 249–339
2. Passow, H. (1986) Rev. Physiol. Biochem. Pharmacol. 103, 61–223
3. Knauf, P. A. (1986) Membrane Transport Disorders (Andreoli, T., Hoffman, J. F., Schultz, S. G., and Faneustil, D. D., eds) pp. 191–220, Plenum Publishing Corp., New York
4. Jay, D., and Cantley, L. (1986) Annu. Rev. Biochem. 55, 511–538
5. Low, P. S. (1986) Biochim. Biophys. Acta 864, 145–167
6. Cabantchik, Z. I., and Rothstein, A. (1972) J. Membr. Biol. 10, 311–330
7. Cabantchik, Z. I., and Rothstein, A. (1974) J. Membr. Biol. 15, 207–226
8. Jennings, M. L. (1982) J. Biol. Chem. 257, 7554–7559
9. Jennings, M. L., Monaghan, R., Douglas, S. M., and Nicknich, J. S. (1985) J. Gen. Physiol. 86, 653–669
10. Nanri, H., Hamasaki, N., and Minakami, S. (1983) J. Biol. Chem. 258, 5985–5999
11. Bretag, A. H. (1987) Physiol. Rev. 67, 618–724
12. Jentsch, T., Garcia, A. M., and Lodish, H. F. (1989) Biochem. J. 261, 155–166
13. Pimplikar, S. W., and Reithmeier, R. A. F. (1988) J. Biol. Chem. 263, 4485–4493
14. Karniski, L. P., and Jennings, M. L. (1989) J. Biol. Chem. 264, 4564–4570
15. Tanner, M. J. A., Martin, P. G., and High, S. (1988) Biochem. J. 265, 703–712
16. Kopito, R. R., and Lodish, H. F. (1985) Nature 316, 234–238
17. Kim, H. C., Yew, N. S., Ansorge, W., Voss, H., Schwager, C., Vennstrom, B., Zsok, M., and Engel, J. D. (1988) Mol. Cell. Biol. 8, 4416–4424
18. Cox, J. V., and Lazarides, E. (1988) Mol. Cell. Biol. 8, 1327–1335
19. Alper, S. L., Kopito, R. R., Libresco, S. M., and Lodish, H. F. (1988) J. Biol. Chem. 263, 17092–17099
20. Morgan, M., Hanke, P., Gregorczyk, R., Tintichai, A., Fasold, H., and Passow, H. (1985) EMBO J. 4, 1927–1931
21. Hanke-Baier, P., Raiba, M., and Passow, H. (1988) Biochim. Biophys. Acta 940, 136–140
22. Garcia, A. M., Kopito, R. R., and Lodish, H. F. (1987) Biophys. J. 51, 567 (abstr.)
23. Contreras, R., Cherouette, H., Degrange, W., and Fiers, W. (1982) Nucleic Acids Res. 10, 6353–6362
24. Kunkel, T. A., Roberts, J. D., and Zabour, R. A. (1987) Methods Enzymol. 154, 367–382
25. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
26. Biggin, M. D., Gibson, T. J., and Hong, G. F. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3953–3956
27. Colman, A. (1984) in Transcription and Translation: A Practical Approach (Hames, B. D., and Higgins, S. J., eds) pp. 271–302, IRL Press, Oxford
28. Laemmli, U. K. (1970) Nature 227, 680–685
29. Schneider, C., Newman, R. A., Sutherland, D. R., Asser, U., and Sreaves, M. F. (1982) J. Biol. Chem. 257, 10766–10769
30. Braham, J. (1977) J. Gen. Physiol. 79, 293–305
31. Shami, Y., Rothstein, A., and Knauf, P. (1978) Biochim. Biophys. Acta 508, 357–363
32. Fröhlich, O. (1982) J. Membr. Biol. 65, 111–123
33. Schnell, F. (1972) Biochim. Biophys. Acta 282, 265–276
34. Cousin, J. L., and Motais, R. (1975) J. Membr. Biol. 46, 125–153
35. Chou, P. Y., and Fasman, G. D. (1978) Adv. Enzymol. 47, 45–148