Effects of External pH on Binding of External Sulfate, 4,4'-dinitro-stilbene-2,2'-disulfonate (DNDS), and Chloride to the Band 3 Anion Exchange Protein

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ABSTRACT A model in which two positively-charged titratable sites enhance the affinity for anionic substrates can explain the increase in external iodide dissociation constant (K_i^n) with increasing pHe (Liu, S. J., F.-Y. Law, and P. A. Knauf. 1996. J. Gen. Physiol. 107:271-291). If sulfate binds to the same external site as I-, this model predicts that the SO_4^{2-} dissociation constant (K_{SO_4}^{2-}) should also increase. The data at pHe 8.5 to 10 fit this prediction, and the pK for the titration is not significantly different from that (pK_c) for the low-pK group that affects K_i^n. The dissociation constant for the apparently competitive inhibitor, DNDS (4,4'-dinitrostilbene-2,2'-disulfonate), also increases greatly as pHe increases. Particularly at high pHe, a noncompetitive inhibition by DNDS is also evident. Increasing pHe from 7.2 to 11.2 increases the competitive dissociation constant by 700-fold, but the noncompetitive is only increased ~20-fold. The pK values for these effects are similar to pK_c for K_i^n, as expected if DNDS binds near the external transport site, but it seems likely that additional titratable groups also affect DNDS binding. The apparent affinity for external Cl^- is also affected by pHe, in a manner similar to that observed for I-. Pretreatment with the amino-selective reagent, bis-sulfosuccinimidyl suberate (BSSS), decreases the apparent Cl^- affinity at pH 8.5, but two titrations are still evident, the first (lower) of which decreases the apparent Cl^- affinity, and the second of which surprisingly increases it. Thus, the BSSS-reactive amino groups (probably Lys-539 and Lys-851) do not seem to be involved in the titrations that affect Cl^- affinity. In general, the data support the concept that a positively charged amino group (or groups), together with a guanidino group, plays an important role in the binding of substrates and inhibitors at or near the external transport site.

INTRODUCTION

In the preceding paper (Liu et al., 1996), we have shown that the I^- dissociation constant (K_i^n) for the E_o form of band 3 (the conformation with the transport site facing outward) increases dramatically with increasing external pH (pHe). The data can be most simply interpreted in terms of a model in which a positively charged amino acid residue with a pK of 9.5 ± 0.2 (probably lysine) contributes to binding of external substrates (Liu et al., 1996). The data are also compatible with a model similar to that proposed by Bjerrum (1992), with two titratable sites, one with a pK value of 9.3 ± 0.5 and another with a pK > 11. The lower pK residue is probably a lysine, while the higher pK site is likely arginine. Bjerrum also proposed an alternate model in which both sites are arginines.

In addition to monovalent anions such as Cl^- and I^-, the red cell anion exchange system also transports divalent anions such as SO_4^{2-} (Schnell et al., 1977; Ku et al., 1979; Milanick and Gunn, 1982). Competition experiments demonstrate that SO_4^{2-} ions bind at or near the same external transport site as do Cl^- and I^-.

External disulfonic stilbenes such as DNDS (4,4'-dinitrostilbene-2,2'-disulfonate) and H_2DIDS (4,4'-diisothiocyanato-dihydro-stilbene-2,2'-disulfonate) appear to act primarily as competitive inhibitors of Cl^- exchange (Fröhlich, 1982; Shami et al., 1978), suggesting that they also bind to the external-facing transport site.

If titratable positive charges contribute to the free energy of binding of monovalent anions to the external transport site, then these charges should also have an effect on the binding of divalent anions or competitive inhibitors to the same site. The affinities of these latter
agents should be affected by external pH in a manner which is predictable from the pK values determined from the I\(^-\) affinity data.

In this paper, we test these predictions by examining the pH\(_o\) dependence of SO\(_4^{2-}\), Cl\(^-\), and DNDS affinity. To test the hypothesis that certain amino groups are involved in external substrate binding, we also examine the effects of an amino-selective reagent, bis-sulfosuccinimidyl suberate (BSSS), on the apparent external Cl\(^-\) affinity. The results of these tests in general support the titratable substrate binding site model (Bjerrem, 1992; Liu et al., 1996), but do not rule out the possibility that other titratable sites may play a role in the binding of at least some of these anions.

**METHODS**

**Cell Preparation and Flux Measurement**

Red blood cells were obtained from freshly drawn human blood and washed three times with 150 KH (150 mM KCl, 24 mM sucrose and 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid), pH 7.2 at 0\(^\circ\)C. The Cl\(^-\) exchange fluxes (J) were determined by measuring \(^36\)Cl efflux, as described in the accompanying paper (Liu et al., 1996).

**Sulfate and DNDS (4,4'-dinitrostibene-2,2'-disulfonate) Inhibition**

SO\(_4^{2-}\) is a substrate for band 3 but is transported at a much lower rate than Cl\(^-\) (~20,000 times slower at 8\(^\circ\)C and pH 6.3 [Ku et al., 1979]). It acts as a competitive inhibitor of Cl\(^-\) exchange (Milanick and Gunn, 1982). Therefore the apparent external SO\(_4^{2-}\) dissociation constant, \(K_{a}^{\text{E}}\), for the conformation (\(E_{o}\)) of band 3 with unloaded transport site facing outward was determined by the method of Milanick and Gunn (1982) from the x value of the intersection point of two Dixon plots for SO\(_4^{2-}\) inhibition with different [Cl\(_i\)], as described in detail for I\(^-\) in the preceding paper (Liu et al., 1996). This same method was also used to determine \(K_{a}^{\text{E}}\) for the nontransported inhibitor DNDS (ICN Pharmaceuticals, Plainview, NY).

The flux media were a combination of 0 KH (339 mM sucrose and 5 mM buffer), 165 KH (165 mM KCl, 9 mM sucrose, and 5 mM buffer), and 110 mM K\(_2\)SO\(_4\) or 0.1 mM DNDS. HEPES was used as the buffer for pH\(_i\) 7.2 to 8.5; CHES (2[N-cyclohexylamino]ethane sulfonic acid, from Research Organics, Inc., Cleveland, OH) for pH\(_i\) 9 to 10.5, and CAPS (3-[cyclohexylamino]-1-propane sulfonic acid, from Sigma Chemical Co., St. Louis, MO) for pH\(_i\) values higher than 10.5. The flux media were titrated with 1 M bicarbonate-free KOH.

**Measurement of Transport Parameters for External Chloride**

The unidirectional Cl\(^-\) efflux of control cells with ~150 mM [Cl\(_i\)] was measured in media with different [Cl\(_i\)], with sucrose replacing Cl\(^-\), as described in the preceding paper. The data were fitted to the Michaelis-Menten equation by nonlinear least squares to calculate \(J_{	ext{max}}\), the flux with maximal (saturating) [Cl\(_i\)], and \(K_{i}/2V_{o}\) the [Cl\(_i\)] that gives half-maximal flux.

**BSSS (Bis-sulfosuccinimidyl suberate) Treatment**

BSSS (Pierce Chemical Co., Rockford, IL) was freshly dissolved in 150 KH at pH 7.4 at room temperature. Cells were washed once with 150 KH (pH 7.4 at room temperature), and then incubated with 2.7 mM BSSS in 150 KH at ~50% hematocrit at pH 7.25 and 37\(^\circ\)C for 1 h. The reaction was stopped by adding 40 ml of 50 mM glycine in 150 KH (pH 7.2 at 0\(^\circ\)C) to the reaction medium. Then the BSSS treated cells were washed three times with 150 KH (pH 7.2 at 0\(^\circ\)C) containing 0.2% BSA (BSA, Sigma Chemical Co.). After each wash, the resuspended cells were left at room temperature for 10 min to allow the pH\(_i\) to reach equilibrium.

**Data Analysis**

Enzfitter (Elsevier Biosoft), a nonlinear regression data analysis program for the IBM PC, was used to fit the data for Cl\(^-\) flux, J, as a function of inhibitor concentration, [Inh], to the equation for hyperbolic inhibition, \(J = J_o/(1 + [\text{Inh}]/I_{D50})\), where \(J_o\) is the uninhibited flux and \(I_{D50}\) is the [Inh] which causes 50% inhibition of the flux. Simple weighting (same error assumed for all points) was used. \(K_{a}^{\text{E}}\) was then calculated as the negative of the x value of the intersection point of Dixon plot lines (1/J vs [Inh]) with different [Cl\(_i\)], using the equations of the lines resulting from the \(I_{D50}\) and \(J_o\) values obtained by nonlinear fitting.

The dissociation constants for SO\(_4^{2-}\), DNDS, and Cl\(^-\) at various pH\(_i\) values were fitted to a model in which the affinity change is related to titration of one or two sites, as described by Eqs. 1 and 2 of the preceding paper (Liu et al., 1996). Because the variations in the dissociation constants in general increase with the size of the constants, the data were weighted according to the inverse of their values, and nonlinear least squares estimates of the parameters were obtained by using Origin (MicroCal Software, Northampton, MA).

**RESULTS AND DISCUSSION**

**Effects of pH\(_o\) on Anions Other Than Iodide**

The titratable substrate binding site (SBS) model postulates that the electrostatic field of positive charges near the external transport site contributes significantly to the binding affinity for anionic substrates, such as I\(^-\) (Liu et al., 1996). According to this model, titration of the positive charges should also affect the binding of other substrates or competitive inhibitors to the external transport site. Because of the different charges or locations of these other ions, however, the effects of pH\(_i\) may show quantitative differences, although the pK values for the titrations should not be significantly different.

The dissociation constant for binding, \(K_{a}^{\text{E}}\), is related to the free energy of binding by the equation:

\[
AG = RT\ln K_{a}^{\text{E}}.
\]  

In general, \(AG\) is a complicated function of the interactions between the ion and the binding site. In the present context, however, we are primarily concerned with the effect of addition or removal of a positive
charge near the binding site. For a single positive charge, the electrical contribution to the free energy (in kilocalories per mole) of an anion located at a distance \( r \) from the center of the titratable positive charge is equal to the electrostatic work and is given by (Gabler, 1978):

\[
\Delta G_{el} = -331.9 \, n \, \Delta r,
\]

(2)

where \( n \) is the charge of the anion (e.g., one for a univalent anion) and \( D \) is the dielectric constant. If we write Eq. 1 for the condition when the residue is protonated (subscript +) and when it is deprotonated (subscript 0), the dissociation constant for the protonated form, \( K_+ \), and for the deprotonated form, \( K_0 \), have the following relationship, provided that the entire free energy difference between the two forms is given by \( \Delta G_{el} \):

\[
\Delta G_{el} = RT \ln \left( \frac{K_+}{K_0} \right).
\]

(3)

If a different anion, designated by \(*\), is bound near the titratable charge, we can write equations for this ion similar to Eqs. 2 and 3. When these equations are solved simultaneously, we obtain:

\[
\frac{K_+}{K_0} = \left( \frac{K_{+*}}{K_{0*}} \right)^{n/n*}(r/r*). \]

(4)

That is, the ratio of dissociation constants in the two states for one anion is proportional to the same ratio for the other anion (*) raised to a power that is directly proportional to the ratio of the charges of the anions \((n/n*)\) and inversely proportional to the ratio of the distance between each anion and the positive charge \((r/r*)\).

**Effects of pH on Sulfate Affinity**

According to Eq. 4, if a divalent anion such as \( SO_4^{2-} \) is bound to the external transport site, and if the distance between the center of the \( SO_4^{2-} \) and the titratable positive charge is less than twice the distance between the charge and bound \( I^- \), the doubling of \( n \) (for \( SO_4^{2-} \)) relative to \( n^* \) (for \( I^- \)) in Eq. 4 predicts that the ratio of neutral and alkaline form dissociation constants for \( SO_4^{2-} \) should be larger than that for \( I^- \). Thus, qualitatively \( pH_0 \) would be expected to have a larger effect on the dissociation constant for \( SO_4^{2-}, K^S \), than that for \( I^-, K^I \). Because of the complex nature of the equation describing the titration (see Liu et al., 1996), however, the apparent values of \( K_0 \) and \( K^S \) at each \( pH_0 \) will not follow Eq. 4. As mentioned in the preceding paper (Liu et al., 1996), larger values of \( K_0 \) relative to \( K^I \) will cause the inflection point for the \( pH_0 \) titration to shift to higher \( pH_0 \) values. At \( pH_0 < 10.5 \), therefore, the predicted results for divalent and monovalent ions are quite similar (e.g., compare solid triangles for \( I^- \) in Fig. 3 with dashed line predicted for a divalent ion at the same position as \( I^- \)). Despite this apparent \( pK \) shift, numerical fits to the one-site or two-site SBS model should give \( pK \) values for \( SO_4^{2-} \) similar to those seen for \( I^- \).

To test the SBS model, therefore, we measured \( K^S \) at various \( pH_0 \) values. Like \( K^I \), \( K^S \) was determined from the negative of the \( x \) value of the intersection point of Dixon plots \((1/flux \ vs \ [SO_4^0])\) at different \([Cl_0]\) (Milanick and Gunn, 1986; Fröhlich and Gunn, 1986; Knauf and Brahm, 1989). Fig. 1 shows that the Dixon plots for \( SO_4^{2-} \) inhibition were nearly perfectly linear, indicating a single site of inhibition, as required for the determination of \( K^S \).

Table I shows \( K^S \) as a function of \( pH_0 \). \( K^S \) increases markedly as \( pH_0 \) is raised from 8.5 to 10, which is qualitatively consistent with the \( pH_0 \) dependence of \( K^I \). This increase in \( K^S \) is not related to the titratable site with a \( pK \) of 5.03, described by Milanick and Gunn (1982), which causes a ninefold increase in \( SO_4^{2-} \) affinity when it is protonated. Because of this site, the apparent \( K^S \) increases with an apparent \( pK \) of 5.99. By \( pH 8.5 \), however, the lowest \( pH_0 \) shown in Table I, this titration should be over 99% complete. Thus, the titratable site characterized by Milanick and Gunn (1982) has no effect on \( K^S \) in the \( pH_0 \) range examined here.

As shown in Table I, when \( K^I \) and \( K^S \) at various \( pH_0 \) are divided by their values at \( pH_0 8.5 \), the ratio for \( SO_4^{2-} \) shows a dependence on \( pH_0 \) that is similar to but somewhat stronger than that seen for \( I^- \). This is fairly consistent with the SBS model, suggesting that the reduction

**FIGURE 1.** Dixon plot of inhibition of \( Cl^- \) exchange by external \( SO_4^{2-} \) at \( pH 8.5, 0^\circ C \). Cells were loaded with 36Cl and efflux was measured into media with various \( SO_4^{2-} \) concentrations, as described in Methods. Circles represent data with \([Cl_0]= 0.55 \, mM\) and triangles data with \([Cl_0]= 148.5 \, mM\). Open symbols and solid lines are for one experiment; closed symbols and dashed lines for a different experiment. Lines were drawn using the parameters for single-site hyperbolic inhibition obtained from nonlinear least-squares fits (using Enzfitter) to the original flux vs \( SO_4^{2-} \) data. The dissociation constant for external \( SO_4^{2-} \) binding to the external-facing transport site, \( K^S \), is given by the negative of the \( x \) value of the intersection point for the lines with low and high \([Cl_0]\), and is nearly the same for the two experiments.
of local positive charge by titration may explain the decrease in both SO₄²⁻ and I⁻ binding affinity with pH₀. The effect of pH₀ on Kₛ, however, is somewhat larger than predicted for a divalent ion at the same location as I⁻ (in which case the ratios for SO₄²⁻ and I⁻ should be nearly identical at pH₀ ≈ 10), suggesting that SO₄²⁻ may interact with an additional titratable charge. The idea that SO₄²⁻ and I⁻ are not located in precisely the same place when bound to the external transport site is supported by evidence that SO₄²⁻ and Cl⁻ interact differently with an inhibitor, NAP-taurine (N-[4-azido-2-nitrophenyl]-2-aminoethylsulfonate) (Fröhlich and Gunn, 1987; Knauf et al., 1978).

If the favorable electrostatic free energy resulting from interaction of this positive charge with anions were the dominant factor in determining the total binding free energy, and if I⁻ and SO₄²⁻ were bound at exactly the same position, one would expect divalent anions such as SO₄²⁻ to have a higher apparent affinity for the external transport site than do monovalent anions such as I⁻ (Eqs. 1 and 2). The fact that the dissociation constants are higher for SO₄²⁻ than for I⁻ (Table I), indicating a lower affinity for the doubly charged ion, demonstrates that factors other than the electrostatic interaction make important contributions to the binding free energy.

pK for Dependence of Kₛ on pH₀
If the seven determinations of Kₛ between pH₀ 8.5 and 10 (Table I) are fitted to a single-site titration curve, the pK value is 8.7 (± 1.0). A fit to a two-site model, with the pK for the higher pK residue (D) set to 11.35 (Bjerrum, 1992), also gives a pK for the lower pK (C) residue of 8.7 ± 1.0. Because of the limited data range, these pK's are not very accurately determined, but they are not far from the pK values (9.5 ± 0.2 or 9.3 ± 0.3) determined by fitting similar models to the data for the pH₀ dependence of Kᵢ (Liu et al., 1996). The pK value is very close to that (8.7) determined by Bjerrum (1992) from measurements of Cl⁻ fluxes under conditions of low ionic strength. The simplest interpretation of the similar pK values would be that SO₄²⁻, I⁻, and Cl⁻ interact with the same titratable positive charge.

Although the data fit well with the SBS model, this is not the only model which could explain the increase of SO₄²⁻ dissociation constants with pH₀. A model in which positive fixed charges act to increase the local concentration of anions near the transport site, without themselves participating in substrate binding, predicts qualitatively similar results. In this case, the ratio of local anion concentration to that in the bulk medium is governed by the electrical potential between the local region and the bulk medium. The equilibrium distribution ratio of divalent anions (SO₄²⁻) between the local and bulk regions, given by the Nernst equation, will thus be the square of the corresponding distribution for monovalent anions (I⁻) at each value of pH₀. Table I shows that this relationship does not hold for pH₀ 10 as compared to pH₀ 8.5, providing evidence against this simple version of the fixed charge model. As discussed

### TABLE I

Comparison of pH₀ Dependence of the Extracellular Sulfate Dissociation Constant, Kₛ, with that of the Extracellular Iodide Dissociation Constant, Kᵢ,

<table>
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<th>pH₀</th>
<th>Kᵢ</th>
<th>Kᵢ/Kᵢ₈.5</th>
<th>(Kᵢ/Kᵢ₈.5)²</th>
<th>Kₛ</th>
<th>Kₛ/Kₛ₈.5</th>
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<td>2.89</td>
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<td>8.11</td>
<td>65.7</td>
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*The average value of the parameters in the column at each pH₀.

†Significantly different from (Kᵢ/Kᵢ₈.5)², determined by an unpaired t test (P < 0.05), but not significantly different from Kᵢ/Kᵢ₈.5 (P = 0.11).
in the previous paper, however, precise calculations of the expected results for variations of such fixed charge models are impossible at present, and the model in general involves complex interactions of Cl− and SO₄²⁻ ions in the local region which would probably lead to nonlinearities in the Dixon plots. Because this model is more complex and less predictive than the SBS model, and because it does not seem to fit the data as well, it seems that the alternative SBS model provides a better explanation of the pHo effects.

**pH Dependence of DNDS Inhibition**

DNDS, a stilbene derivative with two negative charges, inhibits anion transport by reversibly binding to band 3. At neutral pHo DNDS seems to act primarily as a competitive (transport site) inhibitor (Fröhlich, 1982). If DNDS binds at or near the substrate binding site, one would expect that the titration of charged residues in or around the substrate binding site would have effects on the dissociation constant for DNDS binding to the E₀ conformation, K_CDNDSD. Therefore, we tested the effects of external pH on K_CDNDSD with constant 150 mM [Cl⁻] and pH₀ 7.2.

To determine ID₀ (the concentration which half-inhibits Cl⁻ exchange) and K_CDNDSD, ⁸⁶Cl effluxes were measured in media of different [Clo] with sucrose replacing Cl⁻ with various [DNDS]s. The results were plotted on Dixon plots (1/flux vs [DNDS]) such as Fig. 2, which shows data for pH₀ 10.9. The ID₀ is equal to the negative of the x intercept of the Dixon plot. K_CDNDSD is given by the negative of the x coordinate of the intersection point of the Dixon plot lines with different [Cl₀].

The values of ID₀ and K_CDNDSD at various pHo are shown in Table II. At pH₀ 7.2 to 8.5 the K_CDNDSD values range from 0.038 to 0.097 μM, very similar to the value of 0.084 ± 0.004 μM reported by Fröhlich (1982), using an optical absorbance technique to measure DNDS binding. Because Fröhlich’s data were obtained by using citrate to replace Cl⁻ and to maintain constant ionic strength, whereas the experiments in Table II had varying ionic strength and no citrate, it seems likely, as discussed in the preceding paper (Liu et al., 1996) in connection with K₀, that the effects of ionic strength on K_CDNDSD are small, or else that effects of ionic strength on the dissociation constant and on the pH values for the positive sites tend to cancel out in this pHo range. K_CDNDSD increases with pH₀, indicating that the inhibitory potency decreases at higher pH₀. This is consistent with the dramatic reduction in the inhibition of Cl⁻ exchange by DNDS at pH₀ values > 10 observed by Wieth and Bjerrum (1983).

If DNDS binds to the transport site, the increase in K_CDNDSD with increasing pH₀ could be partially or completely the result of the same titration which causes the increase in K₀. Fig. 3 shows a comparison of the values of K_CDNDSD and K₀, divided by their respective values at pH 7.2. From Fig. 3 A, it is clear that increasing pH₀ from 8 to 10.5 has a much larger effect on K_CDNDSD (open triangles) than on K₀ (solid triangles). Bjerrum (1992) has also reported unpublished data showing that DNDS affinity is affected at lower pH₀ values than Cl⁻ affinity. At higher pH₀ values, K_CDNDSD increases to such an extent that it is difficult to present on a linear scale, and is better seen on a logarithmic plot such as Fig. 3 B. By pH₀ 11.2, there is a ~700-fold increase in K_CDNDSD compared to only a ~40-fold increase in K₀.

**Competitive and Noncompetitive Effects of DNDS**

According to the ping-pong mechanism, the distribution of band 3 among its various forms is a function of [Cl] and [Clo]. In general, the reciprocal of the ID₀ for an inhibitor such as DNDS is the average of the reciprocals of the dissociation constants of DNDS for each form of band 3 (Fig. 4 A), weighted according to the fraction of band 3 in each form in the absence of inhibitor (Knauf et al., 1992):

\[
\frac{1}{ID₀} = \left( \frac{E₀}{K₀} + \frac{E_i}{K_i} + \frac{EC_i}{K_{EC_i}} \right) \left( \frac{H_i}{K_h} \right) \right) / E_i,
\]

(5)

where Eᵢ is the total concentration of band 3 and Kᵢ, K₀, and K_ECᵢ are dissociation constants of inhibitor for the ECᵢ, Eᵢ, and EClᵢ conformations respectively (Fig. 4 A). Thus, the ID₀ will vary with [Cl₀], as the distribution of band 3 among the various conformations changes. If DNDS is an external competitive inhibitor, it can bind only to the E₀ conformation of band 3. That is, Kₐ, Kᵢ,
Table II

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<th>pH&lt;sub&gt;o&lt;/sub&gt;</th>
<th>K&lt;sub&gt;1/2o&lt;/sub&gt; (SE)</th>
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<td>161.7</td>
<td>14.2 (2.2)</td>
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<tr>
<td>10.6</td>
<td>25.1 (2.5)</td>
<td>16.5</td>
<td>8.39 (1.51)</td>
<td>5.38&lt;sup&gt;1&lt;/sup&gt;</td>
<td>JE1</td>
</tr>
<tr>
<td></td>
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<td>160.1</td>
<td>23.4 (3.5)</td>
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<td></td>
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<td>160.1</td>
<td>25.0 (5.0)</td>
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<td></td>
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<td>11.3 (1.4)</td>
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<td>48.2 (7.9)</td>
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<td>35.0 (2.7)</td>
<td>32.4&lt;sup&gt;1&lt;/sup&gt;</td>
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<td></td>
<td>160.1</td>
<td>52.1 (1.6)</td>
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<td></td>
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</table>

* At pH<sub>o</sub> 10.9, the ID<sub>50</sub> values for low [Cl<sub>o</sub>] in experiment 66 and 66a are not given because the highest DNDS concentration (0.3 μM) did not significantly inhibit anion transport.

<sup>1</sup> Significantly different (<i>P</i> < 0.005) from the grouped data at pH<sub>o</sub> 7.2 and 8.5 according to an unpaired <i>t</i> test.

The results in Table II show strong effects of [Cl<sub>o</sub>] on the DNDS ID<sub>50</sub> at pH<sub>o</sub> 7.2, as expected. In the low Cl<sup>-</sup> (0.5–1 mM) media, the ID<sub>50</sub> for DNDS is ~25 times lower with low [Cl<sub>o</sub>] than with high [Cl<sub>o</sub>], due to the increase in <i>E</i><sub>o</sub> under these conditions (Fröhlich et al., 1983; Fröhlich and Gunn, 1986). For example, when [Cl<sub>o</sub>] = K<sub>1/2o</sub> (the concentration of external Cl<sup>-</sup> that gives half-maximal flux), <i>E</i><sub>o</sub> = <i>E</i><sub>i</sub>/2.

and K<sub>o</sub> are infinite or very large, so Eq. 5 reduces to ID<sub>50</sub> = K<sub>e</sub>(<i>E</i><sub>e</sub>/<i>E</i><sub>o</sub>). Thus, the ID<sub>50</sub> will be much lower with low [Cl<sub>o</sub>] than with high [Cl<sub>o</sub>], due to the increase in <i>E</i><sub>e</sub> under these conditions (Fröhlich et al., 1983; Fröhlich and Gunn, 1986). For example, when [Cl<sub>o</sub>] = K<sub>1/2o</sub> (the concentration of external Cl<sup>-</sup> that gives half-maximal flux), <i>E</i><sub>e</sub> = <i>E</i><sub>i</sub>/2.

Published February 1, 1996
lower than the ID$_{50}$ in 149 mM Cl$^-$ medium, suggesting that DNDS binds preferentially to the $E_0$ conformation, which is consistent with the idea that DNDS is a competitive inhibitor (Fröhlich, 1982). At higher pH$_o$, the ID$_{50}$ for DNDS does not increase as much with increasing [Cl$^-$]. In part this is due to the increase in $K_{1/2o}$, which makes Cl$^-$ a less effective competitor, but it also suggests that DNDS can bind to other conformations of band 3 as well as to $E_0$.

The possibility that DNDS displays some noncompetitive inhibition at high pH$_o$ (and even at lower pH$_o$ values) was tested as follows: if one assumes (Fig. 4 B) that DNDS, besides binding to $E_0$ as a competitive inhibitor with dissociation constant $K_c^c$, can also bind noncompetitively to all forms of band 3 with the same dissociation constant $K_c^n$, and that the competitive and noncompetitive binding of DNDS to $E_0$ are mutually exclusive, a linear Dixon plot will be obtained as in Fig. 2 and $1/ID_{50}$ will be given by (see Appendix):

$$1/ID_{50} = 1/K_c^n + 1/(K_c^c [1 + [Cl^-]/K_{1/2o}]),$$

where $K_{1/2o}$ is the external half saturation concentration for Cl$^-$ with [Cl$^-$] kept constant at each pH$_o$, determined by fitting the Michaelis-Menten equation to the data for flux as a function of [Cl$^-$]. The values of $K_c^c$ and $K_c^n$, determined by nonlinear fitting of ID$_{50}$ vs [Cl$^-$], as well as the experimentally measured $K_c^{DNDS}$, are presented in Table III. $K_c^c$ is approximately equal to $K_c^{DNDS}$, but is much lower than $K_c^n$ (~120 times lower at pH$_o$ 7.2 and 16 times lower at pH$_o$ 10.4), as expected if the competitive component dominates the overall inhibition.

The competitive component has a different pH dependence from the noncompetitive one, as shown in Table III and in Fig. 3, where $K_c^c$ and $K_c^n$, divided by their values at pH 7.2, are plotted for comparison with similarly normalized values of $K_c^{DNDS}$ and $K_c^l$. Like $K_c^{DNDS}$, $K_c^c$ begins to increase between pH$_o$ 8 and 10 (Fig. 3 A), and by pH$_o$ 11.2 reaches a value ~1,000 times larger than its value at pH$_o$ 7.2 (Fig. 3 B). In contrast, $K_c^n$ does not increase until pH$_o$ is raised to 9.9, as

---

**Figure 3.** Effect of pH$_o$ on $K_c^{DNDS}$, $K_c^c$, $K_c^n$, and $K_c^l$. (A) Linear ordinate; (B) logarithmic ordinate. The various constants are expressed as ratios relative to their values at pH$_o$ 7.2. Data for $K_c^{DNDS}$ are from Table II, for $K_c^c$ and $K_c^n$ are from Table III, and for $K_c^l$ are from Liu et al. (1996). The dashed line represents predictions based on a model in which a doubly charged anion is located at the same distance from the titratable sites as I$^-$, calculated using the two titratable site model in which the completely deprotonated form has negligible affinity for anions, with the pK values given in the preceding paper (Liu et al., 1996).

**Figure 4.** (A) Model for inhibition of Cl$^-$ exchange by an inhibitor (D), which can bind to all of the various forms of band 3. (B) Inhibition of Cl$^-$ exchange, assuming that DNDS (D) binds to the transport site with dissociation constant $K_c^c$, and to a noncompetitive site with dissociation constant $K_c^n$, which is the same regardless of the conformation of band 3.
shown in Fig. 3 B, and the maximum change by pHo of 11.2 is only ~20-fold (Fig. 3 A). The different titration profiles for Ke and Kn fit well with the concept that DNDS binds at different sites in the band 3 molecule for competitive and noncompetitive inhibition.

Because Kn (~5 μM at pHo 7.2) is much higher than Ke (Table III), at low concentrations DNDS acts primarily as a competitive inhibitor and the noncompetitive component does not play an important role. However, when DNDS is used at relatively high concentrations, for example, to protect the substrate binding site from chemical modification, the experiments must be interpreted cautiously, because under these conditions DNDS binds to at least one other site (the noncompetitive binding site) besides the transport site. Two recent brief reports provide further evidence that there is a noncompetitive component of DNDS inhibition (Knauf et al., 1993; Aranibar et al., 1994).

**Table III**

<table>
<thead>
<tr>
<th>pHo</th>
<th>KeDNDS</th>
<th>Ke</th>
<th>Kn</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2</td>
<td>0.0485</td>
<td>0.0453</td>
<td>5.44</td>
</tr>
<tr>
<td>8.5</td>
<td>0.0705</td>
<td>0.129</td>
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<td>9.2</td>
<td>0.305</td>
<td>0.389</td>
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<td>9.9</td>
<td>1.20</td>
<td>1.60</td>
<td>11.7</td>
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<tr>
<td>10.4</td>
<td>1.69</td>
<td>1.83</td>
<td>30.3</td>
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<td>10.6</td>
<td>4.74</td>
<td>4.99</td>
<td>90.9</td>
</tr>
<tr>
<td>10.9</td>
<td>13.6</td>
<td>10.1</td>
<td>103.</td>
</tr>
<tr>
<td>11.2</td>
<td>33.8</td>
<td>45.1</td>
<td>110</td>
</tr>
</tbody>
</table>

Standard errors for Ke and Kn were < 30% of the mean, except for one Ke value at pH 10.9. Average KeDNDS values for each pHo were calculated from Table II; standard errors for pHo > 9 were < 38% of the mean.

As another test of the hypothesis that DNDS and I- are affected by the same titratable sites, we calculated the expected pHo dependence of the dissociation constant for a doubly charged anion that is bound at precisely the same location as I-. This was done by taking the best-fit parameters for the pHo dependence of I- binding, based on a model in which titration of the high-pK site (D) abolishes anion binding affinity (Bjerrum, 1992; Liu et al., 1996), and squaring the value of the dissociation constant with the C residue deprotonated (corresponding to Ke), as predicted by Eq. 4. The prediction, shown by the dashed lines in Fig. 3, a and b, falls well below the observed KeDNDS and Ke values over the entire pHo range, suggesting that additional titratable positive charges besides those that affect I- binding influence the competitive binding of DNDS. Evidence that DNDS binds in a region with several positive charges (Passow, 1986) makes this possibility more likely. Moreover, site-directed mutations of Lys-558 and Lys 869 of mouse band 3 (corresponding to Lys-539 and Lys-851 of human band 3) have much larger effects on DNDS affinity than on Cl- affinity (Wood et al., 1992), consistent with the idea that additional charge interactions are involved in DNDS binding.

The lower-affinity noncompetitive component of DNDS inhibition, Kn, fits better to a one or two-titratable site model, with pK's of 9.4 ± 0.5 in either case, although the affinities for the protonated and deprotonated forms are very poorly defined. This is not significantly different from the pK value obtained from the Kn data (Liu et al., 1996) or from Bjerrum’s model, so the data do not disprove the hypothesis that the same (probably Lys) site may affect both binding of transported substrates and the noncompetitive binding of DNDS. This might seem an unlikely situation, but it is easier to envision if the amino group is actually located several Å from the transport site (see below). On the other hand, a separate group with a similar pK may be responsible for the pH dependence of Kn.

**pHo Dependence of External Cl- Half Saturation K1/2o and Maximum Flux Jmo**

The data in Table II for the effect of pHo on K1/2o are plotted in Fig. 5 (solid circles). It is apparent that K1/2o increases dramatically at pH values > 9.1 According to the ping-pong model for anion exchange (see Appendix and Fröhlich and Gunn, 1986), K1/2o is given by:

\[ K_{1/2o} = \frac{1}{K_{eDNDS} + K_{c}} \]

The values of K1/2o at pH 7–8.5 are somewhat lower than those previously reported by Gunn and Fröhlich (1979) at pH 7.8. This may be due to the fact that citrate was not used in these experiments to maintain ionic strength. Thus, as discussed in the preceding paper (Liu et al., 1996), competitive effects of citrate were avoided, but the constants are “apparent” in the sense that they apply strictly only to conditions where the ionic strength is allowed to vary with [Cl-].
Published February 1, 1996

\[ K_{1/2o} = K_o (k/k') / \left\{ 1 + (K'_i/[Cl_i]) + (k/k') \right\} , \]  

where \( K_o \) and \( K_i \) are the dissociation constants for binding of Cl\(^-\) to the outward-facing or inward-facing transport sites, respectively, and where \( k \) is the rate constant for conversion from the inward-facing ECl\(_i\) form to the outward-facing ECl\(_o\) form, while \( k' \) is the rate constant for the reverse conformational change (see Fig. 4). Because [Cl\(_i\)] is high and constant, the term \( K'_i/[Cl_i] \) is unlikely to strongly influence \( K_{1/2o} \). An increase in \( K_{1/2o} \) could, however, reflect an increase in \( K_o \) with \( \text{pH}_{o} \), or could be related to either an increase in \( k \) or a decrease in \( k' \), any one of which would increase the numerator in Eq. 7.

The maximum Cl\(^-\) exchange flux, with constant [Cl\(_i\)] and with saturating [Cl\(_o\)], is given by:

\[ J^{mo} = kE_i / \left\{ 1 + (K'_i/[Cl_i]) + (k/k') \right\} . \]  

\( J^{mo} \) is plotted as a function of \( \text{pH}_{o} \) in Fig. 5 (open circles). Like \( K_{1/2o} \) \( J^{mo} \) also increases with increasing \( \text{pH}_{o} \), but in this case the increase is smaller and occurs only at \( \text{pH}_{o} \) around 11. If Eq. 7 is divided by Eq. 8, one is left with:

\[ K_{1/2o}/J^{mo} = K_o/(k'_{E_i}) , \]  

because the identical denominators of Eq. 7 and 8 cancel out. (This is the reason why, in a ping-pong model [Gunn and Fröhlich, 1979], the ratio \( K_{1/2o}/J^{mo} \) is independent of [Cl\(_i\)].) When the ratio \( K_{1/2o}/J^{mo} \) is plotted against \( \text{pH}_{o} \) (Fig. 6, solid circles), there is a large increase with increasing \( \text{pH}_{o} \). This increase must be related either to an increase in \( K_o \) or to a decrease in \( k' \) or both, because \( E_i \), the total amount of band 3, remains constant.

The solid line through the open circles is a least-squares best-fit (using Origin) to a two titratable site model, with each point weighted as its inverse, including both our data and Bjerrum’s (1992) data, as shown in Fig. 7.

In the preceding paper (Liu et al., 1996), we calculated the \( \text{pH}_{o} \) dependence of \( k' \), relative to its value at \( \text{pH} \) 8.5, \( k'_{o} \), based on the assumption that external pH has the same effects on Cl\(^-\) binding as on I\(^-\) binding. If we multiply equation 6 by \( k'/k'_{o} \), we obtain:

\[ (K_{1/2o}/J^{mo}) (k'/k'_{o}) = K_o/(k'_{E_i}) \]  

Because all of the terms on the right-hand side are constants except \( K_o \), a plot of this function (Fig. 6, open circles) should indicate the effects of \( \text{pH}_{o} \) on \( K_o \).

These observations complement and are consistent with Bjerrum’s (1992) report that \( J^{mo} \) increases slightly as \( \text{pH}_{o} \) is raised \( >1 \), that \( K_{1/2o} \) increases dramatically with \( \text{pH}_{o} \), and that \( K_{1/2o}/J^{mo} \) tends to increase 10-fold for a 1-U change in \( \text{pH} \) at high \( \text{pH}_{o} \). In fact, when Bjerrum’s data for \( K_{1/2o}/J^{mo} \) are multiplied by the \( k'/k'_{o} \) data from the preceding paper (Liu et al., 1996), the resulting \( K_o/(k'_{E_i}) \) values (Fig. 7, solid circles) fit well with those in Fig. 6 (open circles in Fig. 7).

When all of the \( K_o/(k'_{E_i}) \) data are fitted to a two titratable site model (assuming no Cl\(^-\) binding to the completely deprotonated form), keeping the dissociation constant for the neutral form at the measured value of 0.02, we obtain \( pK_c = 8.8 \pm 0.6 \) and \( pK_o = 12.1 \pm 0.5 \), in reasonable agreement with the \( pK \) values (9.3 \pm 0.3 and 12.7 \pm 0.3) determined by fitting the \( K_o/(k'_{E_i}) \) data to the same model (Liu et al., 1996). The \( K_o/(k'_{E_i}) \) value for the alkaline form, with one residue deprotonated, is very poorly determined but is \( \sim 1.8 \pm 1.3 \). The mean value

\[ 301 \quad \text{LIU ET AL.} \]
Published February 1, 1996

justed for the different magnitude of each parameter at
with the C residue deprotonated is 1.8 ± 1.3 mM s kg mmo1-1, the
factor 0.38 pmol/cm -2 s -1 = 1 mmol kg -1 min -l (Gunn and
from Fig. 13 of Bjerrum (1992)
The left axis shows
shows values of
Table I of the preceding paper (Liu et al., 1996). The right axis
is fixed at 0.02 mM s kg mmo1-1, the value for the alkaline form
the pKc value is 8.8 ± 0.6, and the pIgv value is 12.1 ± 0.5. The axes for
Ko/(k'+Et), the value for the protonated form is fixed at 0.02 mM s kg mmo1-1, the value for the alkaline form
with the C residue deprotonated is 1.8 ± 1.3 mM s kg mmo1-1, the
pKc value is 8.8 ± 0.6, and the pKo value is 12.1 ± 0.5. The axes for
Ko/(k'+Et) and K\text{O} are scaled so that the values match at pHo 7.2.
corresponds to a 90-fold increase in Ko, not significantly different from the 70-fold increase seen for Ko\text{I}.

Fig. 7 also shows a direct comparison of the Ko/(k'+Et) data with the Ko\text{I} data. When the scales are adjusted for the different magnitude of each parameter at pHo 7.2, it appears that the major difference is that some of the Ko\text{I} values, particularly at pHo 11.6 and 12.4, fall somewhat below the Ko/(k'+Et) values. Because Ko should be the only pH-dependent variable in Ko/(k'+Et), this seems to violate the assumption that pHo has parallel effects on Cl- and I- dissociation constants. Most of the apparent discrepancy, however, seems to be related to scatter in the Ko\text{I} values; for example, the fit of the Ko\text{I} data to the Cl- data is much better around pHo 12 than at pHo 12.4. Also, as mentioned above, fits to the data indicate no significant difference in the effect of deprotonation of the low-pK C residue on Ko/(k'+Et) as compared to Ko\text{I}, so the assumption that I- and Cl- affinities are affected similarly by pHo is at least approximately valid.

Even if the data in Fig. 7 are taken at face value, the additional increase in Ko/(k'+Et) relative to that for Ko\text{I} would be only about twofold. If we make the corresponding assumption that the alkaline form of band 3 (with the C group deprotonated) exhibits twice as large an increase in Ko (for Cl-) as in Ko\text{I}, Eq. T2.6 in the preceding paper (Liu et al., 1996) must be replaced by:

\[ T_b = \frac{K_o}{K_i} = 2T = 2\frac{K_o}{K_i} \] (11)

where T\text{b} represents the ratio of Cl- to I- affinities for the alkaline state and the other terms have the meanings defined in the preceding paper. Insertion of this equation yields the final result that the term \((k'+/k'+_+)\) in Eq. 4 of the preceding paper is replaced by \((k'+/k'+_+)/2\). Thus, the fit to the Dixon plot slope data gives half of \((k'+/k'+_+)\), rather than \((k'+/k'+_+)\). In general, the value obtained from the fit is equal to \(T/T_b\) \((k'+/k'+_+)\). Thus, the true value of \((k'+/k'+_+)\) would be 0.28 instead of 0.14. Such a change would not substantially affect the conclusions drawn in the preceding paper.

Effects of BSSS on \(K_{1/20}\) and \(J^{100}\)

To see whether or not particular externally-accessible lysines are involved in external anion binding, we used BSSS, a nonpenetrating cross linking reagent (Staros and Kakkad, 1983) which probably reacts with the same Lys residues with which 
DIDS reacts (Jennings et al., 1985; Okubo et al., 1994). BSSS has the advantage that it does not introduce new charged groups into band 3, as do the disulfonic stilbenes, and that upon reaction with an amino group, the pK is changed so that the positive charge is absent at neutral pH. Also, since BSSS does not completely inhibit anion exchange, the modified transport system may be studied. In fact, although BSSS inhibits Cl-/Br exchange by at least 90% at pHo > 8, Cl-/Br exchange of BSSS treated cells could be activated by lowering external pH (Jennings et al., 1985). In BSSS treated cells the half-saturation constant for extracellular Br- was higher than the corresponding Ko/2 for control cells, indicating that reaction with BSSS decreases substrate affinity (Jennings et al., 1985).

Cl- exchange was inhibited by ~90% at pHo 7.2 in BSSS treated cells (Knauf and Spinelli, 1995), but the remaining flux could still be measured easily. Therefore, we examined how BSSS treatment affects the pH dependence of substrate binding to band 3. Because Cl-/I- exchange is stimulated by BSSS (Jennings et al., 1985), we were unable to measure Ko\text{I} in BSSS treated cells, since the Dixon plot intersection technique requires that Cl-/Cl exchange be much faster than Cl-/I exchange (Knauf and Brahm, 1989; Liu et al., 1996). We chose instead to measure the effects of pHo on the apparent half-saturation constant for external Cl- , \(K_{1/20}\), and on the apparent maximum flux, \(J^{100}\), in BSSS-treated cells with a constant pHo (7.2) and constant [Cl\text{I}] (~150 mM), as described above for control cells.

\(J^{100}\) (solid circles in Fig. 8) is much lower in BSSS-treated cells than in control cells (compare solid circles in Fig. 8 with open circles in Fig. 5; note difference in scale), and remains fairly constant above pHo 8.7, decreasing slightly above pH 11. This suggests (Eq. 8) that k is greatly reduced in BSSS-treated cells. The increase in \(J^{100}\) at low pHo (6.4) is similar to the behavior that is seen for Cl-/Br exchange, where more acidic pHo acti-
vates transport with a pK of ~7 (Jennings et al., 1985). Fig. 8 also shows data for $K_{1/2o}$ and $K_{1/2o}/J^{\infty}$ for BSSS-treated cells as a function of pH. Even at the lowest pH values shown, $K_{1/2o}$ (open squares) is much higher than in control cells (Fig. 5). This probably reflects an increase in $K_o$ (Eq. 7), which could be explained either if neutralization of an amino group reduces the favorable electrostatic free energy for Cl$^-$ binding or if BSSS sterically or allosterically hinders Cl$^-$ binding. If $k/k'$ is assumed to remain constant, the increase in $K_{1/2o}$ would correspond to a 32-fold increase in $K_o$ relative to untreated cells around pH 8.5–8.7.

When $K_{1/2o}/J^{\infty}$ is plotted (open circles in Fig. 8), it is clear that the value is much greater than for the control (solid circles in Fig. 6; note difference in scale) at all pH values. At pH 8.7, for example, with BSSS $K_{1/2o}/J^{\infty}$ is ~2,400 times larger than for the control. This could be explained by the combined effects of a 32-fold increase in $K_o$, as discussed above, and a 75-fold decrease in $k'$ (Eq. 9).

The increase in both $K_{1/2o}$ and $K_{1/2o}/J^{\infty}$ as pH$_o$ is increased from 8.7 to 10 could be due to either a further increase in $K_o$ or a decrease in $k'$ (Eq. 7 and 9). In either case, the data indicate that a low-pK titration is preserved in BSSS-treated cells, suggesting that the C residue does not react with BSSS. If we fit the $K_{1/2o}/J^{\infty}$ data to a two titratable site model, with pK$_C$ fixed at 8.8 and pK$_D$ at 11.35 (Bjerrum’s (1992) parameters for relatively low ionic strength media), the fit is not too good for the left limb of the bell-shaped curve (dashed line in Fig. 9). An even poorer fit (not shown) is obtained if the value of 9.3 from the preceding paper (Liu et al., 1996) is used for pK$_C$. If pK$_C$ is treated as a variable, the best-fit value is 7.5 ± 0.2, and a rather good fit is obtained (solid line in Fig. 9). The different value for pK$_C$ does not rule out the possibility that the C residue remains titratable after BSSS treatment, because BSSS might alter the environment of the residue and hence might lower its apparent pK.

Surprisingly, both $K_{1/2o}$ and $K_{1/2o}/J^{\infty}$ in BSSS-treated cells decrease markedly at pH$_o$ > 10 (Fig. 8). The simplest explanation for this (Eqs. 7 and 9) is that $K_o$ decreases, but an increase in $k'$ cannot be ruled out. At pH$_o$ > 11, $K_{1/2o}$ for BSSS-treated cells is actually lower than for control cells (compare open squares in Fig. 8 with solid circles in Fig. 5), implying that under these conditions the BSSS-modified band 3 has a higher apparent affinity for Cl$^-$ than does unmodified band 3. As shown in Fig. 9, this behavior is compatible with a two-site model in which the second titration lowers the apparent dissociation constant for Cl$^-$, rather than raising it as in control cells.

The ability of the cross-linker BSSS to prevent the decrease in apparent Cl$^-$ affinity that normally occurs at high pH might indicate that the increasing negative charge on band 3 causes electrostatic repulsion that "opens up" the band 3 structure and perturbs the external anion binding site. The BSSS intramolecular cross-link would act to stabilize the normal structure and prevent this effect. As the effects of pH$_o$ are completely reversible (Wieth and Bjerrum, 1982), any changes in structure which take place must also be reversible. On the other hand, it is possible that the decrease in $K_{1/2o}$ and in $K_{1/2o}/J^{\infty}$ is caused in whole or in part by an increase in $k'$, that would decrease the number of outward-facing sites and thereby decrease the apparent $K_{1/2o}$ (which is equivalent to the [Cl$_o$] at which half of the band 3 molecules are in...

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**Figure 8.** Effects of pH$_o$ on $K_{1/2o}$ (open squares), $J^{\infty}$ (solid circles), and $K_{1/2o}/J^{\infty}$ (open circles) in BSSS-treated cells. Fluxes were calculated from Cl$^-$ exchange rate-constants, using a value of 181.5 mmol Cl$^-$/kg dry weight. Bars indicate range of two experiments; other points are single experiments. (Error bars for $K_{1/2o}/J^{\infty}$ are shown in Fig. 9.) Flux vs [Cl$_o$] data were used to determine $K_{1/2o}$ and $J^{\infty}$ from nonlinear fits to the Michaelis-Menten equation as described in Fig. 5; [Cl$_o$] was ~150 mM.

**Figure 9.** Fits of $K_{1/2o}/J^{\infty}$ in BSSS-treated cells to a two titratable site model. Dashed line represents best fit to a model with pK$_C$ fixed at 8.8 and pK$_D$ fixed at 11.35 (Bjerrum, 1992), with $K_{1/2o}/J^{\infty}$ of 30 ± 2, 980 ± 230, and 44 ± 3 mM kg$^{-1}$ mmol$^{-1}$ for the doubly protonated, singly protonated, and unprotonated forms. Solid line depicts best fit with pK$_C$ fixed at 11.35 (Bjerrum, 1992) and with pK$_C$ = 7.45 ± 0.2, and $K_{1/2o}/J^{\infty}$ values of 6 ± 3, 370 ± 30, and 54 ± 4 mM kg$^{-1}$ mmol$^{-1}$ for the different titration states.
the $E_o$ form; this needs to be lower to increase $E_o$ sufficiently if there is less $E_o$ at high $[Cl_o]$. Identity of the Charged Residues

Structural models of band 3 (Tanner et al., 1988; Lux et al., 1989; Wood, 1992) indicate that there are many Lys and Arg residues in the transmembrane segments or in the loops adjacent to them which could function as the charges that bind external anions. At least one of these, Lys-430, seems unlikely because it is the site of reaction of eosin-5-maleimide, which is a noncompetitive inhibitor and which therefore must not bind to the external transport site (Knauf et al., 1993; Liu and Knauf, 1993; Liu et al., 1995). However, as discussed in the preceding paper (Liu et al., 1996) the amino group that increases substrate affinity could be $>4.4 \AA$ from the anion binding site (assuming a dielectric constant of 30 in the region near the transport site), so it might be possible for an inhibitor to react with the amino group and still not block the transport site. Lys-851, which reacts with a transported substrate, pyridoxal phosphate (Kawano et al., 1988) and with a competitive inhibitor, H$_2$DIDS (Okubo et al., 1994), would seem to be a good candidate. The fact that transport can proceed even if the corresponding Lys in the mouse band 3 is altered by site-directed mutation (Wood et al., 1992) fits with the idea that this charge increases anion affinity, but is not essential for transport. Jennings et al. (1985), however, have interpreted the effects of BSSS on reductive methylation of this Lys as indicating that BSSS reacts with “Lys-b,” now known to be Lys-851 (Okubo et al., 1994). The observation of a low-pK titration in BSSS-treated cells would, therefore, suggest that Lys-851 is not the titratable Lys. Passow (1986) has also argued that the H$_2$DIDS-reactive lysines are not directly involved in substrate binding. There are, however, several other candidate lysines in band 3, as well as several arginines that might be the high-pK residue. Much further work with chemical probes and site-directed mutagenesis will be necessary to identify the functionally-important residues and to determine their precise role in transport.

APPENDIX

Determination of $K_{o \times}$ and $K_{c \times}$ for DNDS from $ID_{50}$ and $[Cl_o]$ The assumption here is that DNDS inhibits anion transport by binding competitively to the $E_o$ form of band 3 with a dissociation constant of $K_{c \times}$ and noncompetitively to $E_i$, $E_c$, $ECl_{o \times}$ and $ECl$, with the same dissociation constant, $K_{o \times}$ (Fig. 4 B), where $K_{c \times} = [E_o] \cdot ([D_o] \cdot [ECl_oD])$ and

$$K_{o \times} = [E_o] \cdot [D_o]/[E_oD] = [ECl_o] \cdot [D_o]/[ECl_oD] = [ECl] \cdot [D_o]/[EClD] = [E_o] \cdot [D_o]/[E_oD],$$

(A1.1)

Subscripts $i$ and $o$ represent the band 3 conformations with the transport site facing the cytoplasmic or extracellular side of the membrane, respectively. DNDS binds to $E_o$ to form $ED_o$ (in competition with substrates) or to form $E_oD$ as a noncompetitive inhibitor (Fig. 4 B). The $Cl^-$ dissociation constants for the $E_o$ form, $K_o$, and for the $E_i$ form, $K_i$, are:

$$K_o = [E_o] \cdot [Cl_o]/[ECl_o],$$

and

$$K_i = [E_i] \cdot [Cl_i]/[ECl_i].$$

(A1.2)

The $Cl^-$ efflux and influx are:

$$J_o = k \cdot [ECl_o], J_i = k' \cdot [ECl_i],$$

and $J_o = J_i$.

(A1.3)

The maximum flux, $J_m$, is the flux when all of the band 3 molecules are either in the $ECl_o$ or $ECl_i$ form, that is, $E_i = ECl_o + ECl_i$, so:

$$J_m = k \cdot E_i/(1 + k/k').$$

(A1.4)

The total amount of band 3 molecules, $E_i$, is:

$$[E_i] = [E_o] + [ECl_o] + [ECl_i] + [EClD] + [E_oD] + [EClD] + [ECl_iD]$$

(A1.5)

Substituting Eqs. A1.1–A1.3 into A1.5:

$$E_i = [ECl_i] \cdot F,$$

where

$$F = ((1 + K_o/[Cl_o]) \cdot k/k' + 1 + K_i/[Cl_i])/$$

$$(1 + [D_o]/K_{o \times}) + k \cdot [D_o] \cdot K_o/(k' \cdot K_{c \times}[Cl_o])$$

(A1.6)

Therefore, the flux $f = k \cdot [ECl_i] = k \cdot E_i/F = J_m \cdot (1 + k/k')/F$

and

$$1/F = f/(J_m \cdot (1 + k/k')).$$  

(A1.7)

From Eq. A1.7, the ID$_{50}$ for DNDS is:

$$ID_{50} = (1 + K_o/[Cl_o]) \cdot k/k' + K_i/[Cl_i] + 1)

(1 + k/K_o/[Cl_o]) \cdot k/k' \cdot K_{i \times}$$

$$+ (1 + K_o/[Cl_o]) \cdot K_o/k' \cdot K_{o \times}.$$  

(A1.8)

Since the external $Cl^-$ half saturation constant, $K_{1/2o}$, is equal to $(K_o \cdot k/k')/(1 + k/k' + K_i/[Cl_i])$, the reciprocal of Eq. A1.8 becomes:

$$1/ID_{50} = 1/K_{o \times} + 1/[K_o]$$

(A1.9)

Factors Which Influence $K_{1/2o}$ and $J^{mo}$

For a ping-pong model of $Cl^-$ exchange such as that depicted in Fig. 4, with no inhibitors present, if the ion
binding and dissociation steps are rapid compared to the ion translocation steps, the unidirectional efflux is given by (see Fröhlich and Gunn, 1986; Knauf et al., 1989):

\[
J = \frac{kE}{\left(1 + K/[Cl_o] + k/k' \right) + \left( K_o/[Cl_o] \right) \left( k/k' \right)}
\]

where \(E\) is the total amount of band 3 and the other parameters are as defined in Fig. 4. If \([Cl_o]\) is maximal, the rightmost term in (A2.1) vanishes. Thus, \(J^{\text{max}}\), the flux with maximal \([Cl_o]\) and constant \([Cl]\) becomes:

\[
J^{\text{max}} = \frac{kE}{\left(1 + K/[Cl_i] + k/k' \right)}
\]

This is text Eq. 8.

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