

Effects of External pH or Substrate Binding and on the Inward Chloride Translocation Rate Constant of Band 3

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ABSTRACT To test the hypothesis that amino acid residues in band 3 with titratable positive charges play a role in the binding of anions to the outside-facing transport site, we measured the effects of changing external pH (pH_o) on the dissociation constant for binding of external iodide to the transport site, K_o^I . K_o^I increased with increasing pH_o , and a significant increase was seen even at pH_o values as low as 9.9. The dependence of K_o^I on pH_o can be explained by a model with one titratable site with $\text{pK } 9.5 \pm 0.2$ (probably lysine), which increases anion affinity for the external transport site when it is in the positively charged form. A more complex model, analogous to one recently proposed by Bjerrum (1992), with two titratable sites, one with $\text{pK } 9.3 \pm 0.3$ (probably lysine) and another with $\text{pK} > 11$ (probably arginine), gives a slightly better fit to the data. Thus, titratable positively charged residues seem to be functionally important for the binding of substrate anions to the outward-facing anion transport site. In addition, analysis of Dixon plot slopes for I^- inhibition of Cl^- exchange at different pH_o values, coupled with the assumption that pH_o has parallel effects on external I^- and Cl^- binding, indicates that k' , the rate-constant for inward translocation of the complex of Cl^- with the extracellular transport site, decreases with increasing pH_o . The data are compatible with a model in which titration of the $\text{pK } 9.3$ residue decreases k' to $14 \pm 10\%$ of its value at neutral pH_o . This result, however, together with Bjerrum's (1992) observation that the maximum flux (J_m) increases 1.6-fold when this residue is deprotonated, makes quantitative predictions that raise significant questions about the adequacy of the two titratable site ping-pong model or the assumptions used in analyzing the data.

INTRODUCTION

Band 3, a transmembrane protein in the human erythrocyte membrane with a molecular weight of ~ 101.7 kD (Lux et al., 1989), catalyzes one-for-one anion exchange across the membrane. The most widely accepted mechanism for anion transport by band 3 is the ping-pong model, in which band 3 can be either in a conformation (E_i) with the anion-binding site facing inward (cytoplasmic side) or a conformation (E_o) with the transport site facing outward (extracellular side) (Gunn and Fröhlich, 1979; for review see Knauf, 1989; Jennings, 1992).

Structural models deduced from the amino acid sequence of murine and human erythrocyte band 3 suggest that many positively charged residues are located near the external and internal surface of the mem-

brane (Jay and Cantley, 1986; Reithmeier et al., 1989; Lux et al., 1989; Wood, 1992). Some of these positively charged residues might form the substrate binding site or take part in the translocation of anions (Passow, 1986; Jennings, 1989a). The other possible role of these positively charged residues is to form a positive fixed-charged region which attracts anions from the medium, thereby increasing the local anion concentration around the substrate binding site (Passow, 1969; Wieth and Bjerrum, 1982).

By examining the dependence of Cl^- flux (J) on external pH (pH_o), Wieth and Bjerrum (1982) first demonstrated the existence of some functionally essential titratable residue(s) with an apparent pK value of 12 at $[\text{Cl}_o]$ higher than 0.1 M, that are most likely arginine residue(s), which we will designate Arg-a. The fact that the arginine-selective reagent phenylglyoxal (PG) inhibits anion transport also indicates that some arginine residue(s), which will be designated Arg-b, are essential for anion binding and translocation (Wieth et al., 1982; Bjerrum et al., 1983; Falke and Chan, 1986a). Wieth et

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al. (1982) have provided evidence that the Arg-b site(s) are probably identical to Arg-a. In contrast to these inhibitory effects, at $\text{pH}_o \sim 11$ the anion exchange rate is activated as compared with the flux at lower pH_o (Wieth and Bjerrum, 1982). This was explained as a result of titration of an external modifier site, suggesting that some residue(s) other than Arg-a are involved in regulating anion transport.

Dalmark (1975) and Kaufmann et al. (1986) reported that the apparent half-saturation constant of chloride with equal $[\text{Cl}^-]$ on both sides of the membrane, $K_{1/2}$, increases with increasing pH ($\text{pH}_i = \text{pH}_o$). Recently, Bjerrum (1992) and O. Fröhlich (personal communication) observed that the apparent external Cl^- half saturation constant, $K_{1/2o}$, increases with pH_o . According to the ping-pong model, the changes in $K_{1/2o}$ and $K_{1/2}$ with external pH could be due to changes in the true substrate binding affinity and/or to changes in the translocation rate (Fröhlich and Gunn, 1986; Knauf and Brahm, 1989).

To avoid this ambiguity, we examined the pH_o dependence of substrate binding affinity and translocation rate separately. Unfortunately, we cannot determine the intrinsic Cl^- dissociation constant independent of the translocation rate by means of kinetic techniques presently available. Instead, by using iodide, a band 3 substrate that is transported at a much slower rate than Cl^- (Dalmark and Wieth, 1972), we measured the dissociation constant for I^- binding to the outward-facing transport site, K_o^1 , at various pH_o values (Milanick and Gunn, 1986; Knauf and Brahm, 1989). In addition, to see the effects of the external pH change on the inward translocation rate constant for Cl^- , k' , we used an indirect method to estimate the ratio of k' at various pH_o relative to the k' value at a certain reference pH_o (since a method for direct measurement of k' is not available at present). A preliminary report of parts of this work has been presented in abstract form (Liu and Knauf, 1990).

METHODS

Cell Preparation

Freshly drawn human blood with heparin as anticoagulant was washed three times with 150 KH (150 mM KCl, 24 mM sucrose and 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.2 at 0°C), and the white cells were removed by aspiration.

Nystatin treatment. To measure K_o^1 at $\text{pH}_o > 11$, the cells were first loaded with 300 mM Cl^- by nystatin treatment, as previously described (Knauf and Brahm, 1989). Cells were washed once with 300 KH (300 mM KCl, 20 mM Hepes, 24 mM sucrose, pH 6.9 at room temperature). The cells were resuspended in ice-cold 300 KH at 10% hematocrit with 30 $\mu\text{g}/\text{ml}$ nystatin (Sigma Chemical Company, St. Louis, MO) for 30 min and then centrifuged. The same nystatin treatment procedure was repeated twice more.

Then the cells were washed five times with 300 KH at room temperature and each time after resuspension the cells were left at room temperature for 3 min.

Measurement of Chloride Flux and Cell Chloride

The cells were loaded with ^{36}Cl (ICN Radiochemicals, Irvine, CA) (2.3 $\mu\text{Ci}/\text{ml}$) in 150 KH (or 300 KH for nystatin treated cells) and the ^{36}Cl exchange rate was determined at 0°C by using the rapid filtration technique of Dalmark and Wieth (1972), as described previously (Knauf and Mann, 1984; Knauf et al., 1989).

The ^{36}Cl exchange rate constant was calculated from the slope of the linear regression of $\ln\{1 - (\text{cpm}[t]/\text{cpm}[\text{inf}])\}$ vs time (t), where $\text{cpm}(t)$ is the radioactivity in the medium at time t and $\text{cpm}(\text{inf})$ is the radioactivity of an equal volume of cell suspension (cells and medium). When the flux was measured under conditions where $[\text{Cl}_o] \ll [\text{Cl}_i]$, the internal and external compartment sizes were determined and used to calculate a corrected value for $\text{cpm}(\text{inf})$ and for the rate constant as described previously (Knauf et al., 1989; Knauf and Brahm, 1989). Solutions were titrated with HCO_3^- -free KOH and were kept tightly stoppered; no corrections were made for the traces of HCO_3^- that were probably present. The unidirectional efflux of Cl^- was calculated by multiplying the rate constant by the amount of cell chloride per kilogram dry weight, which was determined as described by Knauf and Mann (1986).

Measurement of K_c , the Dissociation Constant of an Inhibitor for the Conformation (E_o) of Band 3 with the Unloaded Transport Site Facing Outward

To determine K_c for an inhibitor, the Cl^- flux is measured in media with two or more different $[\text{Cl}_o]$ containing various inhibitor concentrations, with constant $[\text{Cl}_i]$ (~ 150 mM or 300 mM). The results are plotted on a Dixon plot ($1/\text{flux}$ vs inhibitor concentration) for each $[\text{Cl}_o]$. K_c is given by the negative of the x coordinate of the intersection point of Dixon plot lines with different $[\text{Cl}_o]$ (Fröhlich and Gunn, 1986; Knauf et al., 1987).

K_c for citrate. The citrate K_c was measured at pH_o 6.8 and 8.0 for intact red blood cells with 150 mM Cl_i^- and pH_i 7.2 at 0°C. Flux measurements were carried out in media with 1 mM or 10 mM Cl^- and various citrate concentrations, with citrate replacing sucrose.

K_o^1 for iodide. Since I^- is a substrate for band 3 but is transported at a much lower rate than Cl^- (260 times slower at 0°C and pH 7.4, Dalmark and Wieth, 1972), I^- acts as a nontransported competitive inhibitor of Cl^- exchange (Dalmark, 1976; Milanick and Gunn, 1986; Knauf et al., 1986). Therefore, the same method used to measure K_c for citrate was applied to determine K_c for iodide, designated K_o^1 .

The flux media for 150 mM $[\text{Cl}_i]$ cells were a combination of 0KH (339 mM sucrose and 5 mM buffer), 165 KH (165 mM KCl, 9 mM sucrose and 5 mM buffer), and 165 mM potassium iodide solutions. At $\text{pH}_o > 11$, the flux medium was composed of 0 KH-300 (639 mM sucrose and 5 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid, Sigma Chemical Co.), 300 KH-CAPS (300 mM KCl, 20 mM CAPS and 24 mM sucrose), and 325 mM KI. Hepes was used as buffer for the solutions between pH_o 7.2 and 8.5, CHES (2-[*N*-cyclohexylamino]ethane sulfonic acid, from Research Organics, Inc., Cleveland, OH) for pH 9 to 10.5 and CAPS for pH values > 10.5 . The solutions were titrated with 1 M bicar-

TABLE I
Effects of pH_o on External Iodide Dissociation Constant, K_oⁱ

Experiment	pH _o	K _o ⁱ	Buffer	Cl _i	Cl _o		ID ₅₀ [*]	Average K _o ⁱ (SE)
					mM			
No.	°C	mM		mM	high	low	mM	mM
60	7.2	0.70	Hepes	129	150	2.0	2.77	0.83 (0.11)
60a	7.2	0.65	Hepes	149	150	1.0	2.44	
70	7.2	0.66	Hepes	147	110	0.6	1.47	0.45 (0.002) [†]
85	7.2	1.24	Hepes	133	5.5	0.3	1.80	
85	7.2	0.89	Hepes	133	5.5	0.6	0.89	1.01 (0.11)
72	8.0	0.45	Hepes	129	154	0.9	1.82	
84	8.0	0.45	Hepes	130	154	0.9	2.05	1.26 (0.47)
67	8.5	1.24	CHES	144	154	0.6	2.18	
70	8.5	0.98	CHES	147	110	0.6	1.82	2.60 (0.30)
74	8.5	0.71	CHES	156	121	1.1	2.10	
81	8.5	1.08	Hepes	142	149	0.6	2.31	5.63 (0.02) [§]
64	8.66	1.73	CHES	138	154	0.9	3.06	
64a	8.66	0.79	CHES	137	154	0.9	2.24	6.48 (0.75) [§]
72	9.2	2.90	CHES	129	154	0.9	4.19	
80	9.2	2.30	CHES	142	110	0.9	3.88	18.4
64	9.9	5.61	CHES	138	154	2.2	7.48	
64a	9.9	5.66	CHES	137	154	2.2	7.57	16.4 (4.9)
67	10	8.13	CHES	137	154	2.2	7.57	
81	10	5.66	CHES	142	116	1.7	7.81	35.8 (4.8) [‡]
82	10	7.31	CHES	138	116	1.7	9.85	
83	10	4.82	CHES	129	116	1.7	6.58	75.6 (9.3)
82	10.6	18.4	CHES	138	116	1.7	19.8	
60	10.7	11.5	CHES	129	150	2.0	12.9	73.4 (0.8) [§]
60a	10.7	21.3	CHES	149	150	2.0	22.6	
61	11.6	28.6	CAPS	131	150	50	34.9	110.5
61a	11.6	34.0	CAPS	144	150	28	46.6	
86	11.6	44.8	CAPS	295	200	30	63.3	110.5
87	11.9	66.3	CAPS	294	200	30	83.8	
89	11.9	84.8	CAPS	304	200	30	91.8	110.5
86	12.4	72.9	CAPS	295	200	30	88.5	
87	12.4	74.0	CAPS	294	200	30	92.5	110.5
89	12	110.5	No buffer	304	200	30	128.5	

*ID₅₀ for iodide in the medium with low Cl⁻ concentration.

[†] t test results show the K_oⁱ is significantly different from the K_oⁱ at pH_o 7.2. (P < 0.5).

[§] K_oⁱ is significantly different from that at pH_o 7.2 with P < 0.01.

bonate-free KOH (J. T. Baker, Phillipsburg, NJ). At pH_o 8.5, CHES buffered solution gives nearly the same K_oⁱ as does Hepes buffered medium. CAPS does not show any inhibitory effects on anion transport or on K_oⁱ at pH_o ~12, as compared with data obtained without buffer (Table I). Therefore, under the experimental conditions these buffers do not seem to have significant effects on the results.

Measurement of iodide influx. Red blood cells were obtained, washed, and made up to 50% hematocrit in 150 KH as described above. To a 0.6 ml portion of the 50% hematocrit cells, 9 μl of 2 mM DIDS (4,4'-diisothiocyano-stilbene-2,2'-disulfonate) was added, and the reaction with DIDS was allowed to proceed for 1 h at room temperature. A 200 μl portion of either control or DIDS-treated cells (at 50% hematocrit) was centrifuged, the supernatant was removed, and the cells were resuspended at 0°C in 10 ml of medium prepared by combining 0 KH, 165 KH, and 165 mM

KI as described above. 30 s after addition of cells, ~2-3 μCi of ¹²⁵I (NEZ033A, New England Nuclear, Boston, MA) was added. Beginning 30 s after addition of ¹²⁵I and at 1-min intervals thereafter, 1-ml samples of the suspension were added to 2 ml of ice-cold stop solution (110 mM K₂SO₄, 0.5 mM Na metabisulfite, 0.2 mM DNDS (4,4'-dinitrostilbene-2,2'-disulfonate), and 10 mM Hepes, pH 7.4 at room temperature) and centrifuged in a microcentrifuge (Eppendorf). The time of the sample was taken as the time of addition of cell suspension to the stop solution. The cell pellets were then washed 4 times, each time with 2 ml of ice-cold stop solution, and then were counted for radioactivity. Duplicate 0.5 ml samples of the cell suspension were added to 2 ml of Drabkin's solution containing 15 μl of 10 mg/ml digitonin (dissolved in DMSO), and the hemoglobin content of the suspension was determined from the optical density at 540 nm, based on the value of 1.465 g hemoglobin per liter per absorbance unit (Gunn

and Fröhlich, 1989). The radioactivity of duplicate 0.1-ml aliquots of the suspension was measured for determination of the specific activity of I^- in the flux medium.

The slope of a plot of ^{125}I counts per minute vs time was determined by least squares fitting using the program Enzfitter (Elsevier Biosoft), and the I^- flux was then calculated in millimoles per kilogram of hemoglobin per minute from the hemoglobin and specific activity data. Since ~92% of the dry weight of red blood cells is hemoglobin (Gunn and Fröhlich, 1989), fluxes expressed in these units are ~8.7% higher than fluxes expressed in terms of kilograms of dry weight (as are the Cl^- fluxes reported here).

Data Analysis

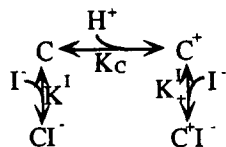
The confidence intervals for K_o^I , given by the negative of the x value of the intersection point of Dixon plot lines for different $[Cl_o]$, were determined by the method of covariance (Walker and Lev, 1953), using straight line fits to the $1/\text{flux}$ vs inhibitor plots. K_o^I values in the tables were determined by using Enzfitter, a nonlinear regression data analysis program for the IBM PC, to fit the data for Cl^- flux, J , as a function of $[I_o]$, to the equation for hyperbolic inhibition, $J = J_u / (1 + [I_o]/ID_{50})$, where J_u is the uninhibited flux and ID_{50} is the $[I_o]$ which causes 50% inhibition of the flux. K_o^I was then calculated as the negative of the x value of the intersection point of Dixon plot lines ($1/J$ vs $[I_o]$) with different $[Cl_o]$, calculated from the ID_{50} and J_u values obtained by nonlinear fitting. Calculations of Dixon slope were also made with Enzfitter. Simple weighting (same error assumed for all points) was used, except where otherwise indicated (see footnote 1). Similar methods were used to determine K_c for citrate.

THEORY

Substrate Binding Site Model

To model the behavior of the substrate binding site model, in which the titratable groups interact directly with substrate anions, we assume simply that the local concentration of anions and cations near the external transport site is equal to their concentration in the bulk solution. Titration (deprotonation) of one or more residues in or very near the transport site causes a decrease in the binding affinity of the transport site for substrate anions.

Single titratable group. In this model, the substrate binding site has a single titratable group, C, with a proton dissociation constant, K_c . Deprotonation of C increases K_o^I from K_+^I to K^I ,



$$\text{where } K_+^I = [I^-] \cdot [C^+] / [C^+ I^-], \quad (1)$$

$$K^I = [I^-] \cdot [C] / [C I^-] \quad (2)$$

$$\text{and } K_c = [C] \cdot [H^+] / [C^+]. \quad (3)$$

For simplicity, only the titration of the forms without I^- bound is shown above, although the I^- -loaded forms can also be titrated. The apparent I^- dissociation constant for E_o is:

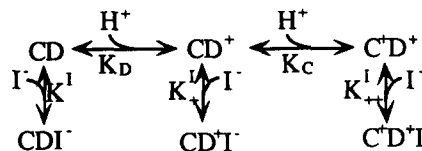
$$K_o^I = [I^-] \cdot ([C] + [C^+]) / ([C I^-] + [C^+ I^-]) \quad (4)$$

Substituting Eqs. 1–3 into the reciprocal of Eq. 4:

$$\begin{aligned} 1/K_o^I &= (1/K^I) \cdot \{1 / (1 + [H^+] / K_c)\} + \\ &(1/K_+^I) \cdot \{1 / (1 + K_c / [H^+])\} \end{aligned} \quad (5)$$

Thus, $1/K_o^I$ is average of the affinities (reciprocals of the dissociation constants) for both forms, weighted by their prevalence.

Two titratable groups. The substrate binding site is assumed to have two titratable groups, C and D, with $pK_c < pK_D$,



where

$$K_c = [C] \cdot [H^+] / [C^+] \text{ and } K_D = [D] \cdot [H^+] / [D^+]. \quad (6)$$

K_{++}^I is the K_o^I at pH_o values where all the C and D residues are in their protonated forms. K_+^I is the I^- dissociation constant for an intermediate state when C is deprotonated and D is protonated and K^I is the I^- dissociation constant when C and D are both deprotonated (uncharged). Titration of the I^- -loaded forms is not shown, although such titration can probably occur (see Fig. 6). Omission of these steps does not affect the model, so long as all steps are reversible. Also, the rare state with the C residue protonated and the D residue deprotonated is not included in the calculation. Thus,

$$\begin{aligned} K_{++}^I &= [C^+ D^+] \cdot [I^-] / [C^+ D^+ I^-], \\ K_+^I &= [CD^+] \cdot [I^-] / [CD^+ I^-] \text{ and} \\ K^I &= [CD] \cdot [I^-] / [CD I^-]. \end{aligned} \quad (7)$$

$$\begin{aligned} K_o^I &= [I^-] \cdot ([CD^+] + [C^+ D^+] + [CD]) / \\ &([CD^+ I^-] + [C^+ D^+ I^-] + [CD I^-]) \end{aligned} \quad (8)$$

Substituting Eqs. 6 and 7 into 8 and solving for the reciprocal of K_o^I , we obtain:

$$\begin{aligned} 1/K_o^I &= ([H^+]^2 / (K_c \cdot K_D \cdot K_{++}^I) + [H^+] / (K_+^I \cdot K_D) \\ &+ 1/K^I) / (1 + [H^+] / K_D + [H^+]^2 / (K_c \cdot K_D)). \end{aligned} \quad (9)$$

Ratio of Inward Translocation Rates

Bjerrum (1992) has presented a similar model with two titratable sites. Deprotonation of the first site, analogous to the "C" site, changes the transport system from the normal state to an alkaline state, with different affinities for external substrates and different rate constants for the transporting conformational change (Fig. 6). In the following, the constants that characterize the normal (neutral) state will be designated with a subscript ++ or superscript H and those that describe the alkaline state by a subscript + or no superscript or subscript. By analogy with the terms used for I^- , $K_{o,++}$ is the dissociation constant for the binding of Cl^- to the outward-facing transport site in the neutral state, while $K_{o,+}$ is the corresponding dissociation constant for the alkaline form.

In terms of such a model, it is possible to obtain information about the pH dependence of k' , the rate-constant for transloca-

tion of Cl⁻ inward, from the slope of Dixon plots such as those shown in Fig. 2. The following treatment makes the assumption that the same titration (of the C residue) that affects K_o¹ also changes k'. Further, it is assumed that deprotonating the C residue has very similar effects on Cl⁻ and I⁻ affinities for the external transport site. Calculations based on other assumptions are discussed in the text and in Liu (1990).

Taking as a starting point Bjerrum's (1992) equation T14, which describes the Cl⁻ flux in the presence of I_o⁻, and ignoring effects of I⁻ binding to the extracellular noncompetitive inhibitory (modifier) site, the slope of the Dixon plot (DS) is obtained by inverting Eq. T14 of Bjerrum and taking only those terms that are factors of [I_o]:

$$DS = (1/(K_{++}^1 \cdot [Cl_0]) + K_C/([H^+] \cdot K_+^1 \cdot [Cl_0])) / (J_{m,++} \cdot (1 + k'_{++}/k_{++})/K_{o,++} + J_{m,+} (K_C/[H^+]) \cdot (1 + k'_+/k_+)/K_{o,+}) \quad (10)$$

where $K_C = [E_0] \cdot [H^+] / [E_0^H]$, $K_{o,++} = [Cl_0] \cdot [E_0^H] / [ECI_0^H]$, and $K_{o,+} = [Cl_0] \cdot [E_0] / [ECI_0]$. The maximum flux for the neutral form, $J_{m,++}$, is

$$J_{m,++} = E_t \cdot (k'_{++} \cdot k_{++} / [k'_{++} + k_{++}]) \quad (11)$$

where k_{++} and k'_{++} are the outward and inward translocation rate constants and E_t is the total amount of band 3. The maximum flux for the alkaline form, $J_{m,+}$, is

$$J_{m,+} = E_t \cdot (k'_+ \cdot k_+ / [k'_+ + k_+]) \quad (12)$$

Substituting Eqs. 11 and 12 into 10, we obtain the Dixon slope in terms of the rate constants:

$$DS = \{1/(K_{++}^1 \cdot [Cl_0]) + K_C/([H^+] \cdot K_+^1 [Cl_0])\} / \{E_t \cdot (k'_{++}/K_{o,++} + (K_C/[H^+]) \cdot k'_+/K_{o,+})\} \quad (13)$$

Thus,

$$DS \cdot Cl_0 \cdot E_t = (1/K_{++}^1 + K_C/([H^+] \cdot K_+^1)) / (k'_{++}/K_{o,++} + (K_C/[H^+]) \cdot k'_+/K_{o,+}) \quad (14)$$

Next, we use the assumption that pH_o has exactly the same effects on the external Cl⁻ dissociation constant as it does on K_o¹. That is, the ratio of the Cl⁻ to the I⁻ dissociation constants is a constant, regardless of whether the system is in the neutral or alkaline state. Thus,

$$K_{o,++}/K_{++}^1 = K_{o,+}/K_+^1 = T. \quad (15)$$

If we now multiply the numerator and denominator of Eq. 14 by K₊₊¹ and substitute T from Eq. 15, after rearranging so that T is on the left-hand side, we obtain:

$$DS \cdot Cl_0 \cdot E_t/T = \{1 + K_C \cdot K_{++}^1 / ([H^+] \cdot K_+^1)\} / \{k'_{++} + k'_+ \cdot (K_{++}^1/K_+^1) \cdot (K_C/[H^+])\}. \quad (16)$$

If we define R as K₊₊¹/K₊¹, Eq. 2.7 can be written as:

$$DS \cdot Cl_0 \cdot E_t/T = \{1 + K_C \cdot R / ([H^+])\} / \{k'_{++} + k'_+ \cdot R \cdot (K_C/[H^+])\}. \quad (17)$$

Note that R and K_c always appear as a product, so the same fit to

the data can be obtained for different values of K_c and R, so long as their product is constant.

Some more insight into the meaning of Eq. 16 can be obtained if we define the average k' value, weighted according to the prevalence of the acid and alkaline forms, as:

$$Av(k') = (k'_+ \cdot ([ECI_0]) + k'_{++} \cdot ([ECI_0^H])) / ([ECI_0] + [ECI_0^H]). \quad (18)$$

If we use Eq. 15 to eliminate the terms in K₊₊¹ and K₊¹, and then substitute the expressions for K_{o,++}, K_{o,+}, and K_c as defined above under Eq. 10, Eq. 16 can be expressed in terms of Eq. 18 as:

$$DS \cdot Cl_0 \cdot E_t/T = 1/Av(k'). \quad (19)$$

That is, the Dixon slope times Cl_o is inversely proportional to the Av(k') value for a given pH_o (since E_t and T are constants). If we normalize the Av(k') value by dividing by the value at a reference pH_o (with H⁺ concentration equal to [H_r⁺]), designated Av(k'_r), we obtain Eq. 24 in the text. The dependence of this ratio on pH_o can be expressed by using Eq. 19 and Eq. 17 (note that the terms in E_t/T cancel out):

$$Av(k')/Av(k'_r) = \{(1 + K_C \cdot R/[H_r^+]) / (k'_{++} + k'_+ \cdot R \cdot (K_C/[H_r^+]))\} \cdot \{(k'_{++} + k'_+ \cdot R \cdot (K_C/[H^+])) / (1 + K_C \cdot R/[H^+])\}. \quad (20)$$

If the pK value for the C residue is much higher than the reference pH_o used to define Av(k'_r), then [H_o⁺] >> K_c and so Eq. 20 can be simplified to:

$$Av(k')/Av(k'_r) = \{(k'_{++} + k'_+ \cdot R \cdot (K_C/[H^+])) / \{k'_{++} \cdot (1 + K_C \cdot R/[H^+])\}\}. \quad (21)$$

A transformation of this equation in terms of pH is shown in Eq. 25 of the text and was used to fit the data in Fig. 7.

RESULTS AND DISCUSSION

Inhibitory Effects of Citrate on Anion Transport

Citrate is often used with sucrose to replace Cl⁻ in order to maintain ionic strength and osmolality (Gunn and Fröhlich, 1979). The experiments described in this paper are designed to see how external pH affects substrate binding affinity. Therefore, it was necessary to test the effects of citrate on anion transport at different pH_o to decide if citrate would be a good Cl⁻ substitute for these experiments.

We measured the K_c of citrate at pH_o 6.8 and 8.0. In Fig. 1, A and B, the reciprocal of Cl⁻ flux is plotted against citrate concentration for two different [Cl_o] at each pH_o (Dixon plot). The K_c, determined from the x coordinate of the intersection point of the two Dixon plot lines with different Cl_o⁻, at pH_o 6.8 is ~3.8 mM (Fig. 1 A). This is much lower than the K_c value of 49.5

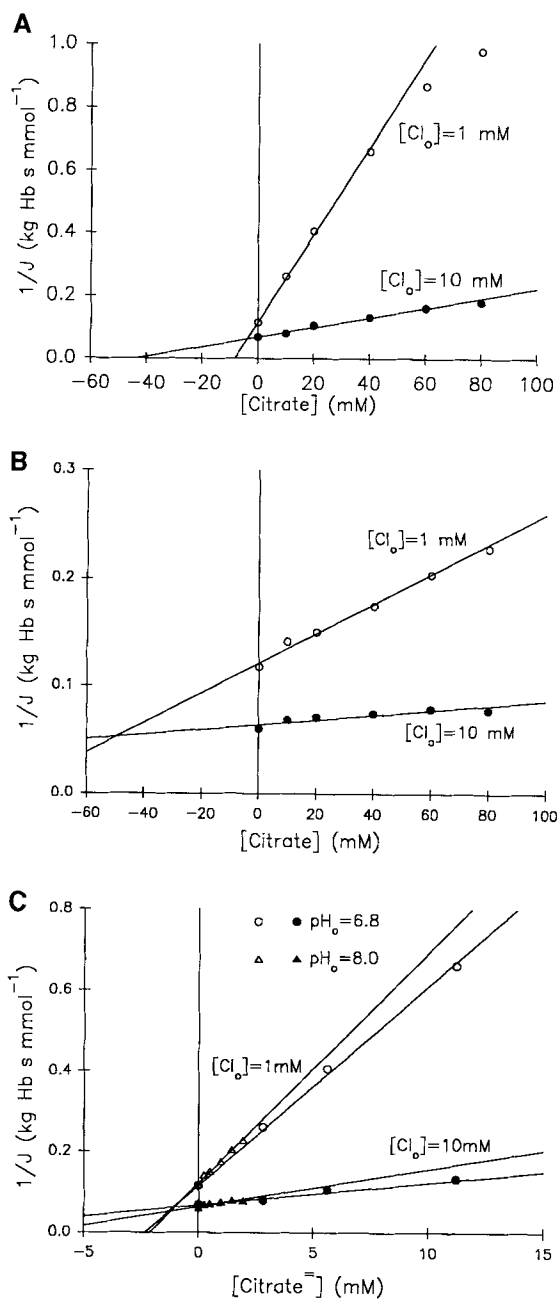


FIGURE 1. Dixon plots of the effects of citrate on Cl^- exchange flux (J) in high and low $[\text{Cl}_o]$ media at pH_o 6.8 (A) and 8.0 (B) at 0°C . The reciprocal of J is plotted against [citrate] in millimolar. The Dixon plot lines are drawn based on the values of ID_{50} and J_o , the control flux, determined from nonlinear fitting of the J vs [citrate] data (see Methods). The dissociation constant, K_c , for binding of citrate to the band 3 conformation with the transport site facing outward and without substrate bound, is given by the x coordinate of the intersection point of the Dixon plot lines with high and low Cl^- in the medium. (A) The K_c at pH_o 6.8 is 3.8 mM with a 90% confidence interval from 2.2 to 19.2 mM. (B) The K_c for citrate at pH_o 8.0 is 49.5 mM and the 90% confidence interval is 41.4 to 64.2 mM. (C) The data in Fig. 1, A and B, are replotted against divalent citrate concentrations calculated for each [citrate] at pH_o 6.8 and 8.0 at 0°C . The K_c values for citrate $^{2-}$, determined as described above, are 1.06 mM at pH_o 6.8 and 1.19 mM at pH_o 8.0.

mM measured at pH_o 8.0 (Fig. 1 B), suggesting that citrate inhibits anion transport quite strongly at pH_o 6.8 and that the inhibitory effect decreases with increasing pH_o . To see which anionic form of citrate is primarily responsible for the inhibitory effects on Cl^- exchange, we calculated the concentrations of each form of citrate at pH_o 6.8 and 8.0, and replotted $1/J$ as a function of the concentration of citrate in the divalent form (C^{2-}) at both pH_o values. As shown in Fig. 1 C, the K_c for the C^{2-} form is nearly the same at pH_o 6.8 and 8.0. This is consistent with the idea that the C^{2-} form is primarily responsible for the inhibition of Cl^- exchange (Kaufmann et al., 1986), and that the increase in pH_o from 6.8 to 8.0 reduces the C^{2-} concentration, resulting in a weaker inhibition.

If one makes the rather unlikely assumption that all of the inhibition at pH 8 is due to the trivalent form of citrate, then K_c for this form if citrate is at least 48 mM. Considering that most of the inhibition at pH 8 is probably caused by divalent citrate, the true K_c for C^{3-} is probably far higher, so trivalent citrate is a very poor inhibitor of Cl^- exchange.

To see if citrate competes with Cl^- , the Dixon slopes of citrate from Fig. 1, A and B, were plotted against $1/[\text{Cl}_o]$ at each pH_o (data not shown). Straight line fits had y intercept values of $0.00017 \text{ kg Hb}\cdot\text{s}\cdot\text{mmol}^{-1}\cdot\text{mM}^{-1}$ for pH_o 6.8 and $0.0001 \text{ kg Hb}\cdot\text{s}\cdot\text{mmol}^{-1}\cdot\text{mM}^{-1}$ for pH_o 8.0. These are close to zero, suggesting that citrate acts primarily as a competitive inhibitor (Segel, 1975; Milanick and Gunn, 1986). We also measured the Cl^- flux in media with different $[\text{Cl}_o]$ with or without 24 mM citrate at pH_o 6.8 (sucrose was used to replace Cl^-) and plotted the data on a double-reciprocal (Lineweaver-Burk) plot ($1/\text{flux}$ vs $1/[\text{Cl}_o]$) for each [citrate] (data not shown). The two Lineweaver-Burk plot lines intersect slightly to the left of the y axis, indicating mixed inhibition (Segel, 1975), in which citrate decreases both the Cl^- affinity and the maximum velocity of transport. Since the intersection point is so close to the y axis, the possibility that the inhibition is purely competitive cannot be excluded.

Because the competitive effect of citrate may change the apparent affinity for substrates such as I^- , and because this effect will vary with pH_o , we chose not to use citrate to maintain constant ionic strength in the following experiments.

Effects of pH_o on External Substrate Binding Affinity

To see how pH_o affects substrate binding, we measured K_o^1 at various pH_o values with pH_i constant at 7.2. Wieth and Bjerrum (1982) demonstrated that even at pH_o 12.4 the hydroxyl ion uptake rate is only $\sim 1\%$ of the maximum Cl^- exchange rate of human erythrocytes with pH_i of 7.1 and that pH_i changes < 0.1 pH unit per minute. When cells with high $[\text{Cl}_i]$ are suspended in

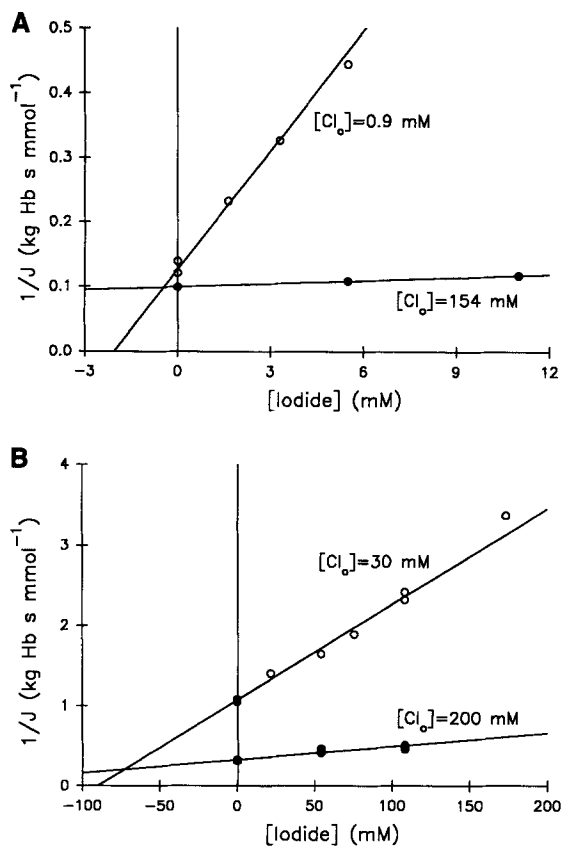


FIGURE 2. Dixon plots of I^- inhibition of Cl^- exchange flux, J , at $0^\circ C$: $1/J$ is plotted against $[I_o]$. The dissociation constant of I^- for E_o , K_o^1 , is determined as described in the legend for Fig. 1 from the x value of the intersection point of the lines calculated from nonlinear fits to the original J vs $[I_o]$ data. (A) Cells with 130 mM Cl^- in pH 8 medium. K_o^1 is 0.45 mM, with a 90% confidence interval from 0.25 to 0.99 mM. (This was the lowest value obtained in this series of experiments; see Table I). (B) Cells with 295 mM intracellular Cl^- at pH_o 12.4. The K_o^1 is 72.9 mM, with a 90% confidence interval from 43.9 to 95.7 mM.

low $[Cl_o]$ media, the rate of increase of pH_i , due to the exchange of internal Cl^- for OH^- or traces of HCO_3^- in the medium, is even smaller (Furuya et al., 1984). Since Cl^- flux can be measured in less than one minute, the effects of changes in pH_o can be observed without significant changes in pH_i .

To determine K_o^1 , the ^{36}Cl exchange rate constant of red blood cells with ~ 150 mM $[Cl_i]$ was measured in media with two different $[Cl_o]$ and various $[I_o]$. The results were plotted on a Dixon plot ($1/\text{flux}$ vs $[I_o]$). The negative of the x value of the intersection point of the Dixon plot lines for the two different $[Cl_o]$ gives K_o^1 . For example, Fig. 2 shows the Dixon plot lines at pH_o 8 (A) and 12.4 (B). At pH 8, K_o^1 is 0.45 mM, with a 90% confidence interval from 0.25 to 0.99 mM. At pH_o 12.4, K_o^1 rises to 73 mM, with a 90% confidence interval of 44 to 96 mM.

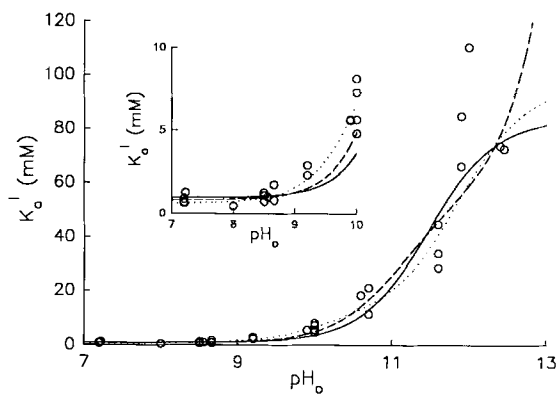


FIGURE 3. Effects of pH_o on K_o^1 . K_o^1 values from Table I (open circles) are plotted against pH_o . The inset shows that there is a pronounced increase in K_o^1 at pH_o 9.2 and 10, compared with lower pH_o . The solid line shows the best fit for a substrate binding site (SBS) model with one titratable site (C) as described by Eq. 22. The dashed line is for a SBS model with two titratable sites (C and D) described by Eq. 23, but the completely deprotonated form is assumed to have no affinity for I^- , that is, the dissociation constant is infinite, as in Bjerrum's (1992) model. The dotted line shows the best fit obtained with a two-site model if the data point at pH 12, in the absence of buffer, was excluded. Best fits were calculated by nonlinear least squares using Sigmaplot (Jandel Scientific), giving each point a weight equal to the inverse of its value.¹ See text for parameter values.

Table I shows that K_o^1 increases dramatically with pH_o , indicating a decrease in I^- binding affinity as pH_o increases. The significant increase of K_o^1 at $pH_o \leq 10.8$ means that titration takes place at pH_o values well below the apparent pK (~ 12) of Arg-a (Wieth and Bjerrum, 1982), suggesting that amino acids other than this arginine affect K_o^1 .

The K_o^1 values (Table I) for each pH_o from 7.2 to 12.4 are plotted in Fig. 3. At pH_o values < 8.5 , K_o^1 is relatively constant. K_o^1 then steadily increases with pH_o up to pH_o 12.4. At pH_o values > 12.4 , the extent of I^- inhibition of Cl^- flux was not consistent, so we could not measure K_o^1 .

Factors Influencing the Accuracy of K_o^1 values

Iodide influx rate. In the derivation of the method for measuring K_o^1 , it is assumed that Cl^- influx is equal to Cl^- efflux (Milanick and Gunn, 1986; Knauf and Brahm, 1989). In fact, since I^- is a substrate for band 3, there will be some I^- influx. Since the Cl^- efflux is equal to the sum of Cl^- influx and I^- influx, if I^- influx is significant in comparison to Cl^- efflux, then Cl^- influx and efflux will not be nearly equal, as required for the application of this method.

Although I^- is transported at a much slower rate than Cl^- (Dalmark and Wieth, 1972) at neutral pH, the conditions of these experiments, with very low $[Cl_o]$ and relatively high $[I_o]$, should maximize I^- influx. Also, at

high pH_o the relative rates of I^- and Cl^- fluxes may not be the same as at neutral pH. Therefore, it was necessary to measure I^- influx by using ^{125}I as described in Methods, for comparison with Cl^- efflux measured under the same conditions.

Previous measurements at pH 7.2 at 0°C (Knauf et al., 1986) in media with citrate replacing Cl^- demonstrate that the I^- influx is $\leq 12\%$ of the Cl^- efflux, even with higher $[I_o]$ than $[Cl_o]$. Under the conditions used in the present experiments, with no citrate and with $[Cl_o] = 0.28$ mM and $[I_o] = 2.2$ mM, the I^- influx was still less than 6% of the Cl^- efflux. At pH_o 10.6, with 1.65 mM $[Cl_o]$ and up to 15 mM $[I_o]$ (as in the K_o^1 experiments at this pH), I^- influx was always less than 10% of the Cl^- efflux.

At pH 11.5, it was more difficult to measure I^- influx accurately, because the higher $[I_o]$ required to mimic the conditions of the Cl^- exchange experiments resulted in lower specific activities for I_o^- . Nevertheless, even with 53 mM $[I_o]$ and 16.5 mM $[Cl_o]$, the I^- influx was only 16% of the Cl^- efflux. I^- influx in DIDS-treated cells was always less than 10% of the value in control cells, except at pH 11.5 where very scattered values for the DIDS-insensitive flux ranged up to 25% of the control flux.

Thus, it appears that at least over the range of pH_o from 7.2 to 11.5, it is correct to assume that I^- influx is small compared to Cl^- efflux, so Cl^- influx and efflux are nearly equal, as required for the validity of the method for measuring K_o^1 .

Further evidence that I_o^- acts as a nontransported inhibitor under these conditions comes from the Dixon plot data. If there were significant I^- influx, the Dixon plot would curve downward at high $[I_o]$, due to the additional Cl^- efflux occurring in exchange for external I^- influx (Milanick and Gunn, 1986). In contrast to this prediction, the Dixon plots were nearly perfectly linear, even at pH_o values as high as 12.4 (see, e.g., Fig. 2 B). Thus, it seems very unlikely that significant I^- influx occurs even at this extreme of the pH range examined.

Competitive vs noncompetitive inhibition by iodide. Dalmark (1976) discovered that, in addition to competing with Cl^- for the transport site, I^- (with $[I_i] = [I_o]$) noncompetitively inhibits Cl^- exchange, with a dissociation constant ~ 60 mM. Bjerrum (1992) recently found an even higher dissociation constant (lower affinity) for I^- binding to the noncompetitive site, ~ 120 mM, when I^- was only present in the external medium, as in our experiments. Both Jennings et al. (1985) and we (Knauf and Spinelli, 1995; G. Harnadek, E. Ries, and P. Knauf, unpublished data) have also observed noncompetitive inhibition by I_o^- .

If the values of K_o^1 obtained by the Dixon plot intersection technique are to be relevant to the affinity of I^- for the transport site, rather than some other external

inhibitory site, it is necessary to demonstrate that the inhibition of Cl^- exchange by I_o^- is largely competitive at the comparatively low $[I_o]$ used in these experiments. One way to do this is to compare the apparent affinities of I_o^- as a substrate and as an inhibitor of Cl^- transport under the same conditions. For a competitive inhibitor, the affinities should be identical. At pH 10.6, for example, the apparent ID_{50} for I^- as an inhibitor of Cl^- exchange (with 1.65 mM $[Cl_o]$) was 23.6 ± 2.1 mM. The half-saturation concentration for I_o^- activation of

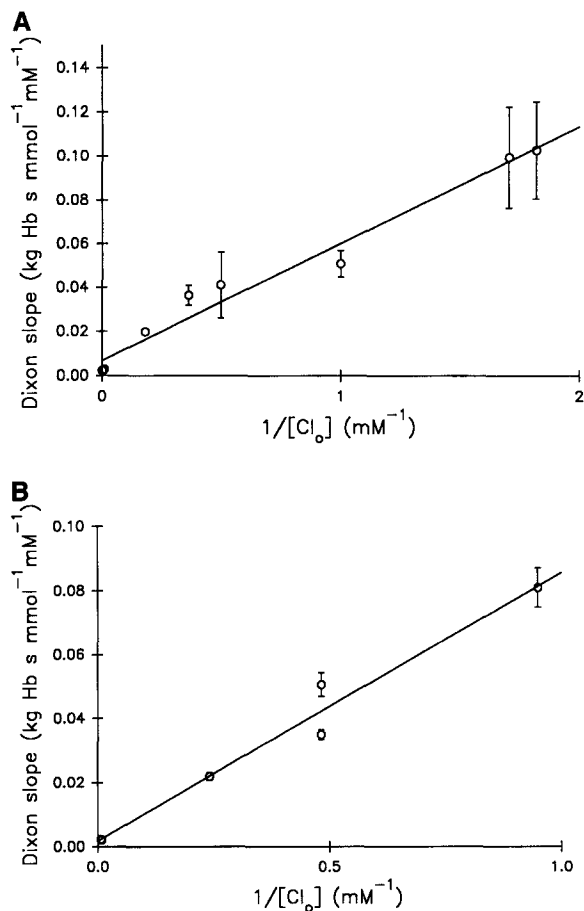


FIGURE 4. Dixon slopes for I_o^- inhibition of Cl^- efflux at pH_o 7.2 vs $1/[Cl_o]$. Bars indicate SE if larger than the size of the points. (A) Sucrose was used to replace $[Cl_o]$. The linear regression line intersects the y-axis at 0.006 kg Hb \cdot s \cdot mmol⁻¹ \cdot mM⁻¹, which is not significantly different from zero ($P = 0.064$). Only data at $[Cl_o]$ from 0.5 to 150 mM were included; one data point with $[Cl_o] = 0.275$ mM was excluded because statistical analysis showed that it had a large standard residual as well as a large influence on the fit. Even with this point included, the y intercept was still not significantly different from zero. (B) Cl^- was replaced by a mixture of 25 mM K_3 citrate plus sucrose to maintain ionic strength constant. The y intercept is 0.0017 kg Hb \cdot s \cdot mmol⁻¹ \cdot mM⁻¹, which is not significantly different from zero ($P = 0.633$). Thus, both sets of data (A and B) are consistent with mainly competitive inhibition of Cl^- exchange by I^- .

I⁻ influx under identical conditions was 26.4 ± 1.8 mM, in good agreement with the hypothesis that under these conditions I⁻ acts like a competitive inhibitor of Cl⁻ exchange.

A further test for competitive inhibition is to assess whether or not the plot of the slope of the Dixon plot vs $1/[Cl_o]$, at constant $[Cl_i]$, gives a straight line passing through the origin (Milanick and Gunn, 1986). At pH 7.2, with sucrose replacing Cl_o⁻ (Fig. 4 A), the data scatter along a straight line which intersects the y-axis near the origin. Similar results are also obtained in media with citrate replacing Cl⁻ (Fig. 4 B). In both Figs. 4, A and B, as well as in the data of Milanick and Gunn (1986), the best-fit straight lines do not pass exactly through the origin, although the y intercepts are not significantly different from zero. This suggests the presence of a small amount of noncompetitive inhibition, which would be most noticeable at high $[Cl_o]$. The fact that similar results are obtained, regardless of whether or not citrate was used to maintain constant ionic strength, suggests that the effects of ionic strength on the Dixon slope, while perhaps not negligible, are not of major importance.

If inhibition by I_o⁻ were strictly competitive, the product of the Dixon plot slope, DS, times $[Cl_o]$ would be a constant for all $[Cl_o]$ (because for competitive inhibition, $DS = \text{const} \cdot 1/[Cl_o]$). As Table II shows, at pH_o 7.2 either with or without citrate to maintain ionic strength, this product increases at high $[Cl_o]$, as would be expected if there is some noncompetitive inhibition. At pH_o 10.7, the increase in DS· $[Cl_o]$ at high $[Cl_o]$ is not as great, and at pH_o values higher than 11.6, essentially no increase is seen. Because the noncompetitive inhibition by I_o⁻ also decreases markedly at high pH_o (G. Harnadek, E. Ries, and P. Knauf, unpublished data), this is consistent with the hypothesis that the increase in DS· $[Cl_o]$ results from a small amount of noncompetitive inhibition.

The major result of this noncompetitive effect is to increase the slope of the Dixon plot for high $[Cl_o]$ slightly. The true Dixon slope in the absence of any noncompetitive inhibition would lie between the observed value and zero. As is evident from Fig. 2, even if the slope of the Dixon plots for high $[Cl_o]$ were zero, this would have little effect on the intersection point with the low $[Cl_o]$ line and therefore little effect on the K_o^I value. If the only effect of the noncompetitive inhibition were to cause the same increase in Dixon slope at low and high $[Cl_o]$, there would be no effect whatsoever on the value of K_o^I .

Ionic strength effects. To avoid the effects of citrate (see above), and since we were unable to find a substituent anion which did not interact with the transport system, we elected to use sucrose as a substitute for Cl⁻, or I⁻, even though this meant that the external ionic

TABLE II
Dixon Slope (DS) Times $[Cl_o]$ for Selected pH_o and $[Cl_o]$ Values

Experiment	pH _o	Cl _o	DS	DS·Cl _o	High/low*
		<i>mM</i>			
60	7.2	150	0.00257	0.386	
	sucrose	2	0.0412	0.0823	4.69
60a		150	0.00226	0.339	
		1	0.0509	0.0509	6.67
70		110	0.00293	0.322	
		0.59	0.0996	0.0584	5.52
N223	7.2	145	0.00233	0.338	
	citrate	1.05	0.081	0.0854	3.96
		2.07	0.0506	0.1048	3.22
N225		130	0.00204	0.2652	
		2.07	0.0349	0.0724	3.66
		4.14	0.0219	0.0907	2.92
60	10.7	150	0.00140	0.2096	
	sucrose	2	0.0584	0.1169	1.79
60a		150	0.00153	0.2300	
		2	0.0539	0.1078	2.13
61	11.6	150	0.00179	0.2682	
	sucrose	50	0.00572	0.2858	0.94
61a		110	0.00147	0.1613	
		27.5	0.00760	0.2089	0.77
86		200	0.00118	0.2354	
		30	0.00699	0.2098	1.12
86	12.4	200	0.001959	0.3918	
		30	0.01206	0.3617	1.08
87		200	0.001412	0.2824	
		30	0.01164	0.3492	0.81

*Value of DS·Cl_o at the highest $[Cl_o]$ value divided by that at the given value of $[Cl_o]$.

strength would vary. Thus, the reported affinities for ions, as well as the pH_o dependence of these affinities, may be affected by changes in ionic strength and must be regarded as apparent affinities which are strictly relevant only to the conditions defined in these experiments.

In general, the effects of ionic strength on the transport system appear to be small (Gunn and Fröhlich, 1979). Working at high pH_o values, where citrate effects should be minor, Bjerrum (1992) found a small decrease in the Cl⁻ transport rate in low ionic strength media as compared with high ionic strength conditions. These data suggest that the effects of ionic

strength are not entirely negligible, and that the pH dependencies of K_o^1 and other parameters in this study may have to be adjusted to take into account the effects of ionic strength. As discussed below, however, comparison of our values of K_o^1 with those of Bjerrum (1992) at pH_o 8–9 suggest that ionic strength effects on this parameter, if present, are probably small.

Membrane potential effects. When cells with high $[\text{Cl}_i]$ are suspended in media with low $[\text{Cl}_o]$, the membrane potential (E_m) tends to become more positive (inside with respect to outside), since the permeability of the membrane to net flow of Cl^- is greater than for other ions. Based on the assumption that Cl^- is at electrochemical equilibrium, E_m can be estimated to fall between 0 mV and +143 mV. Such changes in E_m might be expected to have some effects, especially since Grygorczyk et al. (1987) have reported that a change in E_m from -10 to -100 mV increases the rate of Cl^- exchange by mouse band 3 inserted into *Xenopus* oocytes. Working with human red blood cells, Jennings et al. (1990) found no effects of E_m on either Cl-Cl or Cl- HCO_3 exchange, in agreement with several earlier reports (Gunn and Fröhlich, 1979; Wieth et al., 1980; Fröhlich et al., 1983; Furuya et al., 1984). Jennings (1989a,b), however, found that E_m affects the binding affinity of the external-facing transport site for sulfate. The effect is small, suggesting that the potential at the external transport site is only about 10% of the total E_m (Jennings et al., 1990). Preliminary experiments in our laboratory, in which valinomycin or gramicidin were used to alter E_m , have shown no significant effect of E_m on the affinity of I^- (P. A. Knauf, L. J. Spinelli, and D. Restrepo, unpublished data). Because the experiments reported here involve competition between two monovalent ions, Cl^- and I^- , according to the data of Jennings et al. (1990), E_m should have no effect on the ID_{50} for K_o^1 and therefore on the x intercept of the Dixon plot. The positive E_m at low $[\text{Cl}_o]$ might, however, increase the Cl^- exchange flux and thereby lower the y intercept on the Dixon plot. This would cause K_o^1 to be slightly lower than the value which would be obtained if all measurements were carried out at zero E_m . On the basis of Jennings et al.'s (1990) experiments with sulfate, however, such effects should be small and should not greatly influence the K_o^1 values.

Comparison with Other Values of K_o^1

Milanick and Gunn (1986) reported a value of ~ 3 mM for K_o^1 at pH_o 7.8, somewhat higher than the values (0.45–1.0) for similar pH_o reported in Table I, possibly due to competition between I^- and gluconate, which was used to replace Cl^- and which appears to compete with Cl^- for the external transport site (Knauf and Mann, 1986). We have earlier reported K_o^1 values of 2.5–2.8 mM (Knauf et al., 1986). Again, the reason for

the higher values is probably competition of the Cl^- replacement ion, citrate, with I^- . Bjerrum (1992) has reported values of 0.95–1.19 for K_o^1 from measurements at pH 8–9 with citrate replacing Cl^- , in good agreement with the data in Table I. When Bjerrum (1992) included the effects of uncertainty about the degree of modifier site inhibition, the range of the mean value was 0.96–1.4 mM. The surprisingly good agreement with Table I, despite the presence of citrate in Bjerrum's experiments and its absence in ours, may reflect the much less potent inhibition by citrate at higher pH (Fig. 1 B). This agreement also suggests that the effects of ionic strength on K_o^1 are not very strong, because otherwise one would expect different K_o^1 values in our experiments at low ionic strength in comparison to Bjerrum's at constant ionic strength. It is, however, possible that significant effects of ionic strength on the pK value for the titration (see below) and on K_o^1 happen to cancel each other out at this pH_o , so these data do not rule out important effects of ionic strength at other pH_o values.

Mechanism of Changes in External Substrate Affinity

The simplest explanation for the decrease of external I^- binding affinity with increasing pH_o is that some residue(s) C, with an apparent pK value < 12 , participate in external substrate binding (substrate binding site (SBS) model). Titration of these positive groups to their neutral form would reduce the favorable electrostatic interactions with external anions and thereby decrease the substrate affinity. To determine the number and nature of these groups, we fit this model to the experimental data for the dependence of K_o^1 on pH_o . Because in the SBS model the concentration of protons in the local region near the transport site, like the concentration of anions, is similar to the concentration in the bulk external medium, such calculations permit estimation of the pK values of the titratable groups, which in turn should provide clues to their chemical nature.

One site model. We first consider the simplest model in which only one titratable residue, C, participates in the substrate binding site. If one assumes that K_+^1 is the dissociation constant for I^- binding to E_o at the pH at which all of the C residues are protonated and that K^1 is the corresponding value when all of the C residues are deprotonated, the following equation describes the pH_o dependence of K_o^1 (see Theory, Eq. 5):

$$1/K_o^1 = (1/K^1) \cdot \{1/(1 + [\text{H}^+]/K_C)\} + (1/K_+^1) \cdot \{1/(1 + K_C/[\text{H}^+])\}, \quad (22)$$

where K_C is the proton dissociation constant for residue C and $[\text{H}^+]$ is the external proton concentration. This equation predicts a titration curve with plateaus in the high and low external pH regions. K_o^1 does reach a plateau at low pH_o (Fig. 3), but because we could not ob-

tain reliable K_o^I measurements at pH_o values higher than 12.4, a plateau at high pH_o was not observed. Despite the absence of data at very high external pH values, the data can be fitted to Eq. 22 (solid line in Fig. 3), with a pK_C value of 9.5 (± 0.2 SE), K_+^I of 0.94 (± 0.35) mM and K^I value of 84 (± 10) mM.¹ The line is far below some of the experimentally determined K_o^I values at pH_o 9–10 or higher than 11.6, but in general the fit is not too bad.

Two site model. Since Bjerrum (1992) has recently proposed a model for the effects of pH_o on external Cl^- binding which postulates two titratable sites, we tried to see whether or not a slightly more complex model, with two titratable groups, C and D, would fit the data better than the one-site model. By assuming that there are no significant interactions between C and D, the following equation was derived (see Theory, Eq. 9) and was used to fit the experimental data:

$$1/K_o^I = \{ [H^+]^2 / (K_C \cdot K_D \cdot K_{++}^I) + [H^+] / (K_+^I \cdot K_D) + (1/K^I) \} / \{ 1 + [H^+] / K_D + [H^+]^2 / (K_C \cdot K_D) \} \quad (23)$$

where K_{++}^I is the external iodide dissociation constant when both C and D are protonated to form C^+ and D^+ , K_+^I is the iodide dissociation constant when D is protonated but C is not, and K^I is the iodide dissociation constant when both C and D are in the deprotonated, uncharged form. K_C and K_D are the proton dissociation constants for C and D, respectively. A nonlinear least squares fit to this equation gives a pK_C value of 9.3 (± 0.3) and pK_D value of 12.7 (± 0.3), with $K_{++}^I = 0.81$ (± 0.36) mM, $K_+^I = 56$ (± 12) mM and with K^I very large and poorly determined (Fig. 3, dashed line). (Exclusion of the data point without buffer at pH 12 has some effect on this fit (Fig. 3, dotted line): pK_C is 8.8 ± 0.4 and pK_D is 11.0 ± 0.4 , while $K_{++}^I = 0.63$ (± 0.27) mM, $K_+^I = 13$ (± 8) mM and $K^I = 97$ (± 14) mM. The sensitivity of the parameters to small changes in the data reflects the interactions among the parameters, such that nearly the same fit can be achieved when one parameter is raised if another is simultaneously lowered.) The two-site model gives a slightly better fit to the experimental data at pH_o values ~ 9 –10, and the normalized χ^2 is slightly smaller (7.84 vs 7.98 for the

one-site model), although the parameters K_+^I , K^I , and pK_D are very imprecisely determined.

Because the parameter K^I is so poorly determined, we also considered the alternate possibility that after titration of the D residue, the transport site has no significant affinity for I^- . Bjerrum (1992) has proposed a similar model to explain the effects of external pH on external Cl^- affinity. Indeed, as expected in view of the very large value of K^I obtained from the fit to the complete two-site model, this alternate model gives almost exactly the same fit as does the model with a finite K^I (dashed line in Fig. 3). The parameters are exactly the same: pK_C is 9.3 ± 0.3 , pK_D is 12.7 ± 0.3 , K_{++}^I is 0.81 ± 0.36 mM and K_+^I is 56 ± 12 mM. Very similar values were obtained by Bjerrum from measurements of Cl^- flux as a function of pH_o at three different Cl^-/I^- ratios: K_{++}^I ranged from 0.95 to 1.19 mM and K_+^I was 35 to 41 mM (with very large standard errors), when fits were performed with pK_C set at 9.4 and pK_D at 11.35, values obtained from a model used to fit Cl^- transport data. The small differences may in part be due to the fact that we allowed ionic strength to vary, whereas Bjerrum used citrate to maintain a constant ionic strength.

It may seem somewhat surprising that the pK_C value calculated from the models is so low, since substantial increases in K_o^I do not occur until the pH rises well above 9. Also, the inflection point of the solid line in Fig. 3 for the one-site model is located above pH 11, whereas the calculated pK_C is only 9.5. The reason for this is that the apparent pK is strongly influenced by the K_o^I values corresponding to the protonated and unprotonated forms, because the form with the lowest K_o^I value has a stronger effect on the average K_o^I than do the other forms (see Eq. 23 for the two-site model and Eq. 22 for the one-site model). In the one-site model, for example, the apparent pK is the pK value for titration of the iodide-loaded forms, which is equal to $pK_C + \log (K^I / K_+^I)$ (Milanick and Gunn, 1982). For the fit shown by the solid line in Fig. 3, the inflection point is therefore $\sim pH$ 11.5.

Alternate Model: Fixed Charge Model

Although the SBS model provides a good explanation of the experimental results, other possible models must be considered. In particular, it is possible that the positively charged residues, instead of directly participating in substrate binding, could act as fixed charges that attract anions to a local region near the transport site. This would raise the local substrate (I^-) concentration, and therefore increase the apparent affinity of the transport site for I^- . In this model, when the C residues are titrated to the uncharged form at high pH, the $[I^-]$ in the local region would decrease to that in the bulk extracellular solution, and so the apparent affinity for

¹If equal weights are assigned to each point, nonlinear least squares fits to the data in Fig. 3 give results which fit the low pH_o data very poorly, because small absolute (but large percentage) deviations of the best-fit line from these low points tend to be disregarded by the least-squares criterion. To avoid this problem, and because we observed that the deviations are in general proportional to the K_o^I values, the points were assigned weights equal to the inverse of the K_o^I value. Such weighted fits, together with the corresponding standard errors for the parameters, were obtained by using Sigmaplot 4.1 (Jandel Scientific, Corte Madera, CA).

I^- would decrease. A particular reason for considering this model is that Wieth and Bjerrum (1982) previously interpreted the effects of $[Cl_o]$ on the pK value for the sites (Arg-a) that are essential for transport as being due to changes in a positive interfacial (fixed charge) potential between the transport site region and the bulk medium.

In this model, the changes in external substrate affinity are determined by the anion concentration in the local region relative to that in the bulk external medium. Unfortunately, precise calculations of the local anion concentrations require that we know the exact position of the charges and the dielectric constant of the medium surrounding them. As this information is not available, such detailed calculations are impossible.

As a first-order approximation, however, the system can be modeled (for complete description, see Liu, 1990) in terms of a simple Donnan distribution of ions between the bulk external medium and a local region near the transport site, containing the positive charges at some effective concentration $[C^+]$. This harkens back to the earlier fixed charge models of Passow (1969) which were useful in explaining the Cl^-/SO_4^- selectivity of the membrane. As in any positive charge model, the anion concentration in the region near the charges will be higher than in the bulk medium, and the total anion concentration will tend to equal the total cation concentration plus the concentration of the positive fixed charges, so as to maintain electroneutrality.

Either at high pH_o when most of the C residues are deprotonated or in media with anion concentrations much larger than the fixed charge concentration, the local concentration near the charges approaches that in the bulk external medium. Thus, the K_o^1 determined at very high pH_o can be considered as the true local dissociation constant for I^- . However at low pH, when the C groups are in the C^+ form, and at low external anion concentrations, the sum of the total local anion concentrations is equal to the effective concentration of fixed charge.

Based on this constraint, the effects of changes in $[Cl_o]$ on the $[I_o]$ which causes half inhibition of the Cl^- exchange flux (ID_{50}) can be calculated for the case where the fixed charge concentration is greater than the sum of Cl^- and I^- concentrations in the medium (Liu, 1990). Despite the complex interactions of the counterions Cl^- and I^- , Dixon plots such as those in Fig. 2 are still predicted to be linear by this simplified treatment, so ID_{50} measurements are meaningful. Because of the competition of Cl^- and I^- for the fixed positive charges, this model predicts a very strong effect of $[Cl_o]$ on the ID_{50} for I^- , such that $ID_{50} = a \cdot [Cl_o]$, where a is a constant. That is, when the $[Cl_o]$ and $[I_o]$ are low, the ID_{50} for I^- should be a linear function of $[Cl_o]$ and should pass through the origin. This predic-

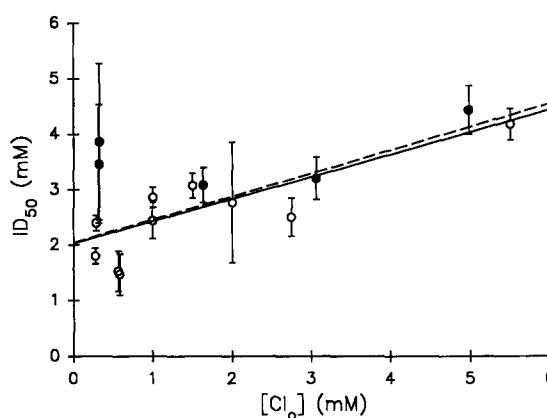


FIGURE 5. ID_{50} for I^- inhibition of Cl^- exchange vs $[Cl_o]$. Cl^- exchange fluxes were measured as described in Methods in cells with $[Cl_i] \sim 150$ mM and with pH_i and pH_o 7.2 at $0^\circ C$. (Open circles) Media with 5 mM Hepes; (solid circles) media with 0.5 mM Hepes. (Bars) SE estimates from nonlinear least squares fits to the flux vs $[I_o]$ data. The solid line shows the best fit, with the points weighted according to their standard errors, for the data with 5 mM Hepes. The y intercept is 2.0 ± 0.2 mM and is significantly different from zero at the $P < 0.001$ level. The dashed line is the best fit to all of the data, with y intercept of 2.0 ± 0.2 mM ($P < 0.001$).

tion holds not only for a pure fixed charge model, but also for a hybrid model in which there are fixed charges as well as titratable binding sites, so long as the fixed charge concentration exceeds the sum of $[Cl_o]$ and $[I_o]$ (Liu, 1990).

Dependence of ID_{50} for Iodide Inhibition on $[Cl_o]$

To test this prediction, we measured the ID_{50} for I^- at different low $[Cl_o]$ at pH_o 7.2. The results (Fig. 5) show that the intersection point with the ordinate is at 2.0 mM (± 0.2), which is significantly different from zero ($P < 0.001$).

Jennings (personal communication) has pointed out a possible difficulty with these experiments, arising from the fact that the buffer used, 5 mM Hepes, exists partly as an anion at neutral pH. The Hepes anion might partition into the local region near the transport site and might obscure the effects of changes in $[Cl_o]$. Thus, the effective $[Cl_o]$ for the calculations shown in Fig. 5 would be the sum of $[Cl_o]$ and $[HEPES^-]$.

To test for such an effect of Hepes, we repeated some of these experiments with the buffer concentration reduced to 0.5 mM. The results, shown by the solid circles in Fig. 5, were not significantly different from those with the higher [Hepes], except that there was greater scatter at the very low $[Cl_o]$, perhaps due to cell stickiness in the very low ionic strength medium. Thus, both the data with high and low [Hepes] are contrary to the prediction of the fixed charge model.

In contrast, the data fit well to the predictions of the substrate binding site model, in which the anion con-

centrations in the local region are the same as those in the external solution. In this model, at very low $[\text{Cl}_o^-]$ the ID_{50} approaches a limiting value which is equal to the dissociation constant for I^- binding to the transport site, K_o^1 .² As $[\text{Cl}_o^-]$ increases, the ID_{50} increases because of competition of Cl^- with I^- for the external transport site, so $\text{ID}_{50} = a \cdot [\text{Cl}_o^-] + K_o^1$ (Fröhlich, 1982).

The nonzero intercept for ID_{50} as $[\text{Cl}_o^-]$ approaches zero argues strongly against the fixed charge model. The confidence with which this model can be rejected, however, is limited by the approximate nature of the Donnan ratio calculations of its predicted behavior. In particular, the assumption of electroneutrality does not hold at anion concentrations very near zero, because at very low ionic strength the counterions will not completely neutralize the positive charge in a region of the size to be expected in a membrane protein (e.g., at 1 mM, the Debye length increases to $\sim 90 \text{ \AA}$ [Harned and Owen, 1950]). Thus, the counterion (anion) concentration will be less than that expected for full positive charge neutralization, so the anion concentration in the local region will decrease with decreasing $[\text{Cl}_o^-]$.

The failure of the counterions to completely neutralize the positive fixed charge at low ionic strength should have the effect of modifying the simple Donnan model such that the effective fixed charge concentration is lower and depends on the external ion concentration in a complex way. Without further detailed information on the location of the charges relative to the transport site, more precise calculations are impossible. It seems unlikely, however, that these complicated effects would be compatible with the linear Dixon plots observed (e.g., Fig. 2) or with the apparently linear dependence of ID_{50} on $[\text{Cl}_o^-]$ (Fig. 5). Both behaviors are predicted by the much simpler and more predictive SBS model, which therefore seems preferable to the fixed charge model.

Alternate Model: Allosteric Effects

So far, the increase in dissociation constant for external substrate with pH_o has been interpreted as being due

²The y -intercept value from Fig. 5, which should give K_o^1 (Fröhlich, 1982), is somewhat higher than the values of K_o^1 determined from the intersection points of Dixon plots at low and high $[\text{Cl}_o^-]$ (Table I). The range of the latter values at the same pH_o is 0.65 to 1.24 mM, with a mean of 0.83 mM (see also Fig. 2 A). The lower value from the intersection points might in part be due to a slight external Cl^- inhibition, noted by Gasbjerg and Brahm (1991), which would raise the y intercept of the high Cl^- Dixon plot and therefore move the intersection point for the two lines nearer to the y axis, to smaller values of K_o^1 . It seems unlikely, however, that this would account for all of the discrepancy. The rest is probably due to the different statistical effects of scatter in the Dixon plot data on the ID_{50} as compared to the intersection point of the high and low $[\text{Cl}_o^-]$ lines, as well as possible effects of the increased ionic strength at the high $[\text{Cl}_o^-]$ used in Table I.

to the direct titration of the substrate binding site. An alternative explanation is that a conformational change is induced at high pH_o . For example, the tertiary structure of the external substrate binding site of band 3 might be altered at high pH or the titration of some residues other than substrate binding site residues might allosterically affect the substrate binding site. Although Wieth and Bjerrum (1982) have shown that the alkaline inhibition of Cl^- exchange in intact red cells at 0°C is completely reversible when the pH_o is changed back from 13 to 7.7, making it unlikely that the external Cl^- binding site is denatured at high pH_o , the allosteric alternative cannot be ruled out from the available evidence. In the absence of more complete information about the tertiary structure, however, the hypothesis that the titratable groups participate directly in substrate binding seems to be the simplest model which can explain the data.

Relationship to Wieth and Bjerrum's (1982) and Bjerrum's (1992) Models

Wieth and Bjerrum (1982) found that the Cl^- exchange rate increased around $\text{pH}_o = 11$ and then decreased with a pK around 12. They interpreted the rise around $\text{pH} 11$ as being due to inactivation of an inhibitory modifier site by titration. Recently, Bjerrum (1992) has presented additional evidence and has reinterpreted some of the data of Wieth and Bjerrum (1982) in terms of a new model for the external pH dependence of Cl^- exchange. According to this new model, there is no external inhibitory "modifier" site which is affected by increasing pH_o . On the contrary, the increase in Cl^- exchange at pH values around 11, particularly in high $[\text{Cl}]$ media, is explained on the basis that titration of an external group converts the transport system to an "alkaline state." This alkaline state has a much higher maximum flux, J_m , and a higher $K_{1/2o}$ (external half-saturation concentration) for Cl^- than does the neutral state. As pH_o is increased further, a second group is titrated. This reduces the affinity of the system for Cl^- and/or the translocation rate to negligible levels, so transport stops. The concept that the high pK group is a Cl^- binding site comes from a reanalysis of Wieth and Bjerrum's (1982) data on the effects of $[\text{Cl}_o^-]$ on the apparent pK of the site that is essential for transport, pK_{ts} . The apparent plateau of pK_{ts} at high $[\text{Cl}_o^-]$ was due to corrections for the effects of pH_o on the modifier site inhibition. When the modifier site is removed from the model, as in Bjerrum's (1992) new analysis, the high pK , which corresponds to Wieth and Bjerrum's (1982) pK_{ts} , increases linearly with $\log[\text{Cl}_o^-]$, as expected if the titratable site is involved in the binding of Cl^- .

Although Bjerrum's model provides an excellent fit to the Cl^- exchange data under a variety of conditions,

there are no direct data concerning the effect of pH_o on the external Cl^- dissociation constant, K_o . The data for Cl^- fluxes in the presence of external I^- indicate that K_o^1 increases with increasing pH_o , but only if it is assumed that the pK values for the groups that affect I^- binding are identical to those that affect Cl^- binding. In contrast, the present results (Fig. 3) provide more direct evidence that increasing pH_o increases K_o^1 .

The SBS model presented here is completely compatible with Bjerrum's new model for the effects of pH_o . In media with citrate used to maintain ionic strength at 150 mM, Bjerrum finds that the pK for conversion from the neutral state to the alkaline state is ~ 9.4 . The apparent pK was reduced at lower ionic strength. Taking this effect into account, Bjerrum estimates that under our conditions the pK could be as low as 8.7 to 8.8. Either value is in good agreement with our values of pK_C (9.3 ± 0.3 [two-site] or 9.5 ± 0.2 [one-site]) for the titration effect on K_o^1 .

Bjerrum gives a value for the pK for conversion from the alkaline state to the inhibited state of 11.3. Here it is less certain what corrections to apply for ionic strength, but it seems that the correction should be smaller, since our experiments at high pH were conducted with higher $[\text{Cl}_o^-]$ and $[\text{I}_o^-]$, and therefore a higher total ionic strength (Table I). Our data, when interpreted according to a similar two-site model, do not give a very precise value for pK_D , but they do indicate that pK_D is >11 , in good agreement with Bjerrum's estimate.

Despite this remarkable agreement, we differ from Bjerrum (1992) somewhat in the interpretation of the nature of the titratable groups. He notes that the pK values suggest that one group is lysine and the other arginine, but goes on to argue that a second model with two arginines cannot be ruled out. This latter model proposes that the arginine residues are in a moderately hydrophobic region, and that electrostatic interactions between them are responsible for the very low pK of the first titration.

We prefer the first model in which the lower pK group corresponds to a lysine. (Tyrosine would be another possibility, but there is no evidence at present for tyrosine involvement in anion exchange, whereas potentially important lysine residues have been identified.) This would have the advantage that the lysine and arginine would be nearer to their normal pK values; that is, strong electrostatic interactions would not have to be postulated to explain their apparent pK values. Furthermore, the pH dependence in low $[\text{Cl}_o^-]$ media for reaction with phenylglyoxal (PG), an arginine reagent which reacts preferentially with the uncharged (basic) form of the guanidino group (Wieth et al., 1982), indicates a pK value of ~ 10.2 at 25°C . (See Appendix for a discussion of the effects of $[\text{Cl}_o^-]$ on PG reaction.) Taking into account the ionization enthalpy

for the guanidino group of arginine of 12.4 kcal/mol (Greenstein, 1933), this gives a pK value of 11.04 at 0°C for the PG modified arginine, Arg-b. Therefore, it seems very likely that the titratable group with apparent pK value >11 at the external substrate binding site is the PG modified arginine(s), Arg-b.

Thus, the PG data fit well to a model with a single arginine (together with a lysine) near the external transport site. On the other hand, since PG reacts with the alkaline, uncharged form of the guanidino group, if both residues are arginines as in Bjerrum's (1992) second model it would be difficult to understand why the high pK arginine, rather than the low pK arginine, is more reactive with PG. This conclusion is rendered less certain, however, by the fact that PG also combines reversibly with band 3 at a site separate from the covalent reaction site (Wieth et al., 1982), and this reversible binding might alter the conformation of band 3 and the covalent reaction kinetics of PG.

An objection to the model in which one group is Lys and the other Arg is that, if the two residues are located in the substrate binding site, they would be so close that the electrostatic interactions would decrease the pK of the Lys to a level below that observed. To see if this is true, we can make a very rough calculation for a situation where Lys and Arg are located on opposite sides of the transported Cl^- ion in the binding site. If we assume that the effects of the Lys positive charge on binding are due to an electrostatic attraction for Cl^- , and that the effects on Cl^- and on I^- should be similar, we can calculate that lowering the K_o^1 value from 56 mM to 0.81 mM requires an electrostatic free energy of 2.30 kcal/mol. Assuming that the dielectric constant is 30, the minimum value used by Bjerrum (1992), this corresponds to a distance of 4.7 Å (Gabler, 1978). The minimum distance between the Lys and Arg, assuming that they are on opposite sides of the Cl^- ion, can be determined by adding this to the sum of the radii for the Cl^- and the guanidinium group, which gives a total of ~ 7.8 Å. This gives an electrostatic interaction energy of 1.38 kcal/mol, which corresponds to about a 1.1 pH unit decrease in the pK value. The pK of the ϵ -amino group of Lys at 0°C and low ionic strength is 11.31 (Greenstein, 1933) (for comparison, the ϵ -amino groups in insulin have a pK of 11.83 at 0°C at 2 M ionic strength [Gruen et al., 1959]), so the apparent pK would be lowered to ~ 10.2 , well above that observed (9.3–9.5). The further decrease could be attributed to location of the lysine in a somewhat hydrophobic region (Bjerrum, 1992).

Effects of External pH on the Inward Translocation Rate Constant, k'

According to the ping-pong model, the substrate-loaded band 3 molecules can undergo a spontaneous confor-

mational change which translocates the transport site to the other side of the membrane (Fig. 6). In this section, we describe a method for estimating the ratio of the inward translocation rate constant, k' , at various pH_o relative to its value, k'_r , at some reference pH_o . This method is used to test the hypothesis that deprotonation of the C residue, that according to Bjerrum's (1992) model (Fig. 6) converts band 3 to the alkaline state, also results in a change in the inward translocation rate constant.

Iodide, as a substrate for band 3, competes with Cl^- for the external-facing transport site. The inhibitory effects of I_o^- on Cl^- transport can be measured by a Dixon plot ($1/J$ vs $[\text{I}_o^-]$). For a model in which band 3 exists in two states, that differ by the protonation of a single residue, the Dixon plot slope for I_o^- is a function of k'_{++} and k'_+ (where subscripts ++ and + indicate the protonated (neutral) and deprotonated (alkaline) states, respectively, see Fig. 6), E_t (total number of band

3 molecules), K_C , K_o^I for each state, K_o (the dissociation constant for Cl^- binding to E_o) for each state, and $[\text{Cl}_o^-]$ (see Theory Eq. 16). $[\text{Cl}_o^-]$ is known and E_t is constant, although it may vary somewhat from donor to donor. If one assumes that K_o/K_o^I is a constant, T , that is, that changes in pH_o affect Cl^- and I^- affinity to the same extent, one obtains the following equation (see Theory):

$$\text{DS}_r [\text{Cl}_o^-]_r / (\text{DS} \cdot \text{Cl}_o^-) = \text{Av}(k') / \text{Av}(k'_r) \quad (24)$$

where DS is the Dixon slope and $\text{Av}(k')$ is the average of inward translocation rate constants for the neutral and alkaline forms of band 3 (with the C residue protonated or deprotonated), weighted according to the prevalence of the two forms at a given pH_o (see Theory Eq. 18). DS_r and $[\text{Cl}_o^-]_r$ are the Dixon slope and its corresponding $[\text{Cl}_o^-]$ at some standard pH_o , which for our measurements (Fig. 7) is taken to be pH_o 8.5. Note that the $\text{Av}(k')$ value is inversely proportional to the $\text{DS} \cdot [\text{Cl}_o^-]$ product.

Because it is not possible to measure K_o directly, the assumption that K_o/K_o^I is constant cannot be tested at present, so Eq. 24 only gives an estimate of the behavior of k' with respect to pH_o . This assumption seems reasonable, however, because the principal contribution of a titratable positively charged amino acid side chain to the free energy for binding of an ion in its vicinity should be due to the electrostatic interaction, which ought to be similar for Cl^- and I^- , since they are both monovalent anions.³

The results in Fig. 7 are calculated according to Eq. 24, using the Dixon slopes for the lowest $[\text{Cl}_o^-]$ at a given pH_o . This was done because it appears that a small noncompetitive inhibitory component is present (see Fig. 4 and Table II), which would artificially inflate the values of $\text{DS} \cdot [\text{Cl}_o^-]$ at high $[\text{Cl}_o^-]$. For example, if the true y intercept of Fig. 4 A is assumed to be 0.0015 (instead of 0 for purely competitive inhibition) and if the slope is 0.06, at 150 mM $[\text{Cl}_o^-]$ the $\text{DS} \cdot [\text{Cl}_o^-]$ will be overestimated by over fourfold, whereas at $[\text{Cl}_o^-] \leq 2$ mM, the value is overestimated by <5%. Thus, low $[\text{Cl}_o^-]$ data are likely to provide the best estimates for $\text{DS} \cdot [\text{Cl}_o^-]$, at least for the pH_o range <11, where noncompetitive inhibition is noticeable. The data show that $\text{Av}(k')$ decreases with increasing pH_o , suggesting that deprotonation of some residue reduces the inward translocation rate. The simplest explanation for this is that titration of the C residue, besides reducing the affinity of the outward-facing transport site for substrate anions, also reduces k' .

To test this hypothesis, we fit the data in Fig. 7 to the form of Eq. 21 shown below:

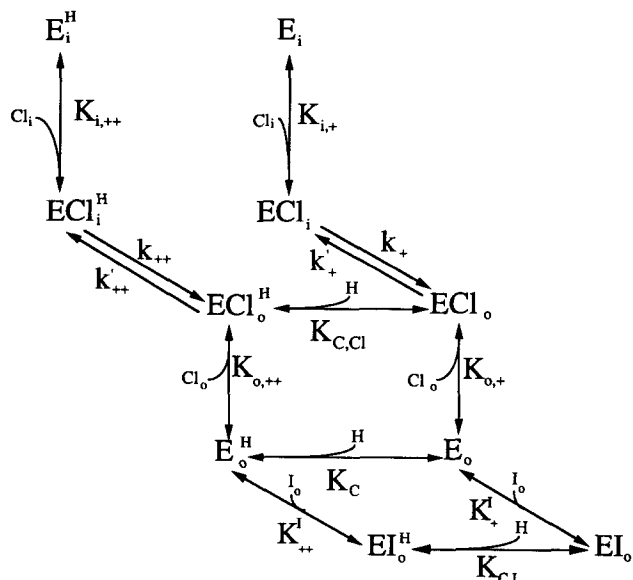


FIGURE 6. Diagram of band 3 molecule in different conformational states, according to Bjerrum's (1992) version of the ping-pong model showing two different titration states (depending on whether the C residue is protonated or not), neutral (left), indicated by superscript H or subscript ++ and alkaline (right), indicated by subscript +. $E\text{Cl}_i$ (or E_i) is the conformation with the transport site facing the cytoplasmic side with (or without) Cl^- bound. $E\text{Cl}_o$ and E_o are the corresponding forms with the transport site facing outward. k_+ and k'_+ are the outward and inward translocation rate constant respectively for band 3 in the deprotonated (alkaline) form. k_{++} and k'_{++} are the corresponding translocation rate constants for the protonated forms. K 's are the dissociation constants for protons with subscript C, for iodide with superscript I, and for chloride with no superscript. The subscripts i and o stand for intracellular and extracellular side. For simplicity the second deprotonation step that converts the alkaline form into the inactivated form is not shown.

³Bjerrum (1992) has made a similar argument that the effects of bound Cl^- should be the same as that of I^- on the pK values of titratable residues at the external anion binding site.

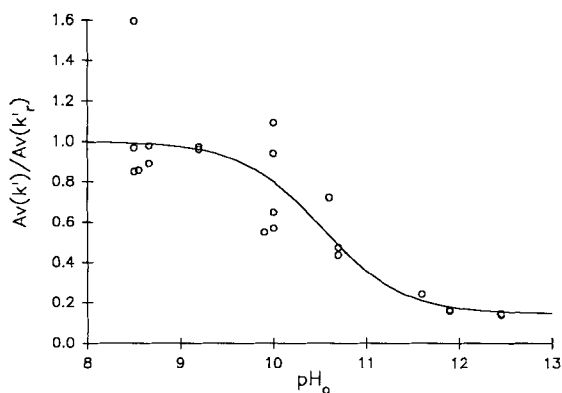


FIGURE 7. External pH titration of inward translocation rate constant, k' . The ratio of inward translocation rate relative to the average k' at pH_o 8.5 (k'_r), $\text{Av}(k)/\text{Av}(k'_r)$, was calculated from Eq. 24, based on the assumption that the ratio of the external dissociation constants for Cl^- and for I^- is a constant at various pH_o . To avoid unwanted interference from the small noncompetitive inhibitory component, $\text{DS}\cdot[\text{Cl}_o]$ data were obtained at low $[\text{Cl}_o]$ (see text for details). The data were fitted to a model in which the same low-pK (C) titration that decreases the affinity for I^- also affects k' , as shown in Fig. 6, using Eq. 25. For the best fit, $p(K_C R)$ (which equals $-\log_{10}(K_C R)$, where R is the ratio of K_{++}^1 to K_+^1) is $10.5 (\pm 0.2)$ and the ratio of k'_+ to k'_{++} is 0.14 ± 0.10 .

$$\text{Av}(k')/\text{Av}(k'_r) = \frac{(1 + 10^{\text{pH}_o} \cdot 10^{-p(K_C R)}) \cdot (k'_+/k'_{++})}{(1 + (10^{\text{pH}_o} \cdot 10^{-p(K_C R)})} \quad (25)$$

where R equals K_{++}^1/K_+^1 , the ratio of I^- dissociation constants for the neutral and alkaline forms, respectively. Because the terms K_C and R always appear as a product, they were combined into a single term and expressed as $p(K_C R)$, defined as $-\log_{10}(K_C R)$. The result, shown by the solid line in Fig. 7, fits the data well. The ratio of k'_+ to k'_{++} , designated G , is $0.14 \pm .10$, indicating a substantial decrease in k' at high pH_o . The apparent pK for the titration, $p(K_C R)$, is 10.5 ± 0.2 . The fit is not significantly affected if the more complete Eq. 20 is used instead of 21, because the best-fit parameters give a value of 0.9985 for the additional factor in Eq. 20, close enough to 1 so that it can be ignored. Because the measurements of $\text{DS}\cdot[\text{Cl}_o]$ at $\text{pH}_o < 11.6$ were obtained in media with very low $[\text{Cl}_o]$, the G value might include unwanted effects of changes in ionic strength. However, even if the data at pH_o 7.2 with citrate used to maintain ionic strength constant (Table II) were used as the reference value for $\text{DS}\cdot[\text{Cl}_o]$ instead of the data without citrate at pH_o 8.5, the G value would only increase to 0.19. Thus, the fact that most measurements were done at low $[\text{Cl}_o]$ does not seem to have a very strong influence on the G value.

According to the two-titratable-site fit to the K_o^1 data in Fig. 3, pK_C is 9.3 ± 0.3 and $R = 0.0145$. Using this R value, the calculated value for pK_C from the data in Fig.

7 would be 8.7, 0.6 units lower than the result expected for a perfect fit to the model where titration of the C residue affects both K_o^1 and k' . Similarly, if we use Bjerrum's (1992) data, pK_C is 9.4 ± 0.1 and R is 0.0279, yielding a calculated pK_C from the k' data of 8.9, ~ 0.5 units too low. If, however, one considers the errors involved in the determinations of pK_C and R , the fit to the model seems acceptable. Unless the model fails some future test, therefore, it seems most reasonable to conclude that a single site controls both K_o^1 and k' .

It is also possible to fit the data in Fig. 7 to a model in which the titration that affects k' is completely separate from and is not affected by the titration that changes the external substrate affinity (Liu, 1990). This model gives an equally good fit to the data with the same apparent pK value of 10.5 ± 0.2 and with k'_+/k'_{++} of 0.15 ± 0.02 . Because the transporting conformational change takes place only when band 3 is in the Cl^- -loaded forms, this pK should be compared with the $\text{pK}_{C,\text{Cl}}$ value for those forms (Fig. 6). Assuming that titration of the C residue affects the binding of Cl^- and I^- to an equal extent, this can be estimated as $\text{pK}_C - \log_{10} R$ which, from Bjerrum's (1992) data is 10.95 and from our data is 11.1. Once again the result (pK 10.5) is somewhat lower than that (~ 11) expected if the C residue titration also decreases k' , but the discrepancy is probably not sufficient to reject this model.

Implications of the Decrease in k' for the Two-state Titration Model

Bjerrum (1992) has shown that titration of the low-pK site, here designated as C, besides reducing the affinity for external substrates, also causes a 1.6 ± 0.2 -fold increase in J_m , the maximum velocity with saturating Cl^- at both sides of the membrane. In a ping-pong model, J_m is given by:

$$J_m = kE_i/(1 + k/k') \quad (26)$$

Thus, if there is a substantial decrease in k' in the alkaline state, such that $k'_+/k'_{++} = G = 0.14$, the increase in k required to give a 1.6-fold increase in J_m will be affected. If we write Eq. 26 for both the neutral and alkaline states, and solve for the ratio of the maximum fluxes in the alkaline and neutral states, $J_{m,+}/J_{m,++}$, we obtain:

$$\frac{J_{m,+}/J_{m,++}}{\{k_{++}[1 + FL/(k'_+/k'_{++})]\}} = \frac{k_+(1 + L)}{\{k_{++}[1 + FL/(k'_+/k'_{++})]\}} \quad (27)$$

where $L = k_{++}/k'_+$, the ratio of rate constants for outward and inward translocation in the neutral state, and $F = k_+/k_{++}$, the factor by which k increases in the alkaline state.

Bjerrum (1992) has argued on the basis of calculations of $K_{o,+}/K_{o,++}$ as a function of $K_{i,+}/K_{i,++}$ and $1/L$ (which he calls K_{ex}^a), that $K_{i,+}/K_{i,++}$ must be > 1 and

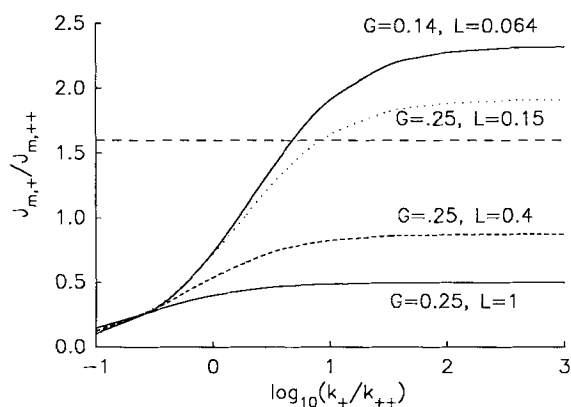


FIGURE 8. Calculated values for the ratio of maximum flux in the alkaline state to that in the neutral state, $J_{m,+}/J_{m,++}$, as a function of the log of the ratio of the constant for outward Cl^- translocation in the alkaline state to that in the neutral state, k_+/k_{++} . The horizontal dashed line shows the experimental value of $J_{m,+}/J_{m,++}$ obtained by Bjerrum (1992), 1.6. The curves represent calculations for various values of L for the neutral state ($= \text{ECI}_0^{\text{H}} / \text{ECI}_0^{\text{H}}$) and for G (k_+/k_{++}). Note that for some values of L , the experimental result cannot be achieved, regardless of the value chosen for k_+/k_{++} .

that K_{ex}^a must be < 2.5 . This argument is based on the idea that the system should not function in a “metastable” state, where the $K_{o,+}/K_{o,++}$ ratio is very sensitive to the $K_{i,+}/K_{i,++}$ and K_{ex}^a parameters. Bjerrum’s (1992) conclusion, however, that $K_{\text{ex}}^a < 2.5$ implies that L ($= 1/K_{\text{ex}}^a$) > 0.4 . Fig. 8 shows a calculation based on Eq. 27 of $J_{m,+}/J_{m,++}$ as a function of $\log_{10}F$ for $L = 0.4$ (short dashed line). Even if k_+/k_{++} (G) is assigned the very high value of 0.25, it is impossible to get $J_{m,+}$ to be larger than $J_{m,++}$ with this value of L , regardless of how large we make k_+ relative to k_{++} . For higher values of L , the problem is even worse (lower solid line). Thus, if $G \leq 0.25$, the hypothesis that $L \geq 0.4$ is incompatible with Bjerrum’s (1992) observations concerning J_m . It is important to realize that the value of k_+/k_{++} that gives rise to this problem comes from a calculation based on precisely the same flux equation used by Bjerrum (1992; see Theory), using exactly the same assumptions.

If L is much lower, as suggested by our earlier data on flufenamic acid inhibition (Knauf et al., 1989), for example, 0.064, then it is possible to get the observed ratio of $J_{m,+}/J_{m,++}$, even if k_+/k_{++} is assigned the measured value of 0.14, for about a fivefold increase in k (Fig. 8, upper solid line). The observed J_m increase can also be calculated if L is as large as 0.15, providing that k_+/k_{++} is taken as 0.25 and that there is a 10-fold increase in k (dotted line). Thus, it seems that in order to interpret the data consistently in terms of the ping-pong model, the L value must be $\leq \sim 0.15$, so the Cl^- -loaded transport sites are very asymmetrically distributed in favor of the inward-facing form. (With $G = 0.25$, the highest L that will give $J_{m,+}/J_{m,++} = 1.6$ is 0.19.)

If Bjerrum’s (1992) theoretical approach is used, the values of L (for the neutral state) that fit the J_m data are consistent with the data for $K_{o,+}/K_{o,++}$. If $K_{i,+}/K_{i,++}$ is taken as 0.7, for a value of the asymmetry factor, A ($= E_o/E_i$ with $\text{Cl}_i = \text{Cl}_o$), in the neutral state of 0.093 (Bjerrum, 1992),⁴ and with $L = 0.064$, the ratio of $K_{o,+}/K_{o,++}$ is predicted to be 31, close to Bjerrum’s (1992) observed value of 35.8 ($1/R$) and not far from our value of 69, when the scatter in the data is considered along with the rather weak dependence of the calculated values on this ratio.

There is, however, one remaining problem. The calculations of $K_{o,+}/K_{o,++}$ above imply that in the alkaline state, the L value is close to that in the neutral state. This is inconsistent with the idea that k increases and k' decreases in the alkaline state (Fig. 8), which should cause L for the alkaline state to be much higher. If we calculate the L value for the alkaline state from k_+ and k'_+ , however, then the values of $K_{o,+}/K_{o,++}$ are inconsistent with Bjerrum’s (1992) data. Thus, it seems that the experimentally observed decrease in k' in the alkaline state is basically incompatible with the simple two-state model, which Bjerrum has shown provides an elegantly simple explanation of all of the rest of the flux versus pH_o data.

One way out of this dilemma is to question the extent of the decrease in k' at alkaline pH_o . If G were 0.5, the observed increase in J_m could be reconciled with an L value of 0.4, but even then a consistent value for L in the alkaline state does not seem to exist. If G were 0.6, a consistent solution would be possible, but only if A for the alkaline state were higher than that observed by Bjerrum (1992). Some support for a higher G value comes from the fact that i' , the rate constant for inward translocation of the EI_o form, as measured by I^- influx in exchange for Cl^- , at high pH_o only falls to 0.42 ± 0.02 of its value at neutral pH_o . Even if G were 0.42, however, a self-consistent two-state model would not be possible. Also, such high values of G seem highly unlikely in view of the decrease in $\text{Av}(k')$ at pH_o values above 11 (Fig. 7). The observed G value might be affected by the different ionic strengths used to measure $\text{DS} \cdot [\text{Cl}_o]$ at different pH_o , but experiments in which citrate was used to maintain ionic strength (Fig. 4 B, Table II) argue that such effects, while not negligible, would only raise G slightly (see above).

Thus, it seems that the observations in Fig. 7 create considerable problems for the two-state pH_o titration model, and perhaps for the ping-pong model itself. Further experiments with competitive inhibitors other

⁴Bjerrum (1992) actually defines A as E_i/E_o . We have used the alternate definition, E_o/E_i , for consistency with our previous publications on this subject.

than I^- are required, however, to see whether or not the pronounced decrease in k' at alkaline pH_o is also seen by other techniques. If it is, then modifications may have to be made in the two-state model. Another possibility is that the assumption used by Bjerrum (1992) and by us that titration of the C residue has identical effects on Cl^- and I^- affinities is invalid. In that case, calculations of changes in k and k' in the alkaline state would be impossible by our method, and Bjerrum's (1992) arguments about the value of L would be invalid, so there would be no inconsistencies with the two-state model. Indirect estimates of the effects of pH_o on K_o in the following paper (Liu et al., 1994) suggest that this assumption is at least approximately correct. At present, however, there is no direct method available to measure K_o (for Cl^-), so the question remains open.

If our measurements are correct, in the alkaline state k' decreases to ~ 0.14 times its value in the neutral state. Bjerrum's data on J_m , together with the analysis in Fig. 8, imply that k increases by about fivefold in the alkaline state. In terms of transition state theory, the increase in k means that the free energy difference between the ECl_i form and the ECl^* transition state must be reduced. Similarly the decrease in k' means that the free energy difference between the ECl_o form and ECl^* is increased. This in turn implies that in the alkaline state the free energy of ECl_i is large relative to ECl^* , while that of ECl_o is smaller relative to ECl^* . Thus, in the alkaline state, the free energy of ECl_i increases relative to ECl_o . It may seem surprising that titration of an external site would affect the free energy of the ECl_i form, with the transport site facing inward, as well as that of the ECl_o form, but this is perhaps to be expected in view of the evidence from inhibitor binding for transmembrane effects of band 3 conformational changes (Knauf et al., 1989).

Role of Positive Charges in Transport

Both from our data and that of Bjerrum (1992), it seems very likely that positively-charged amino acid side chains play an important role in binding of external an-

ions to the transport site. The concept that positive charges are located near the external transport site was first proposed by Passow (1986) to explain substrate competition. The role of such charges in substrate binding is thus fairly well-established, but their possible function in the anion translocation process is less clear. The high-pK residue, which is very likely Arg, could form a neutral complex with the substrate anion, which is then transported across the permeability barrier during the conformational change from ECl_o to ECl_i . This seems an attractive model, but it is important to emphasize that there are no data at present which prove that the Arg crosses the permeability barrier with the transported anion.

On the other hand, as Bjerrum (1992) has pointed out, there is strong evidence that the low-pK residue, which we think is probably Lys, does not cross the permeability barrier with the anion substrate. If it were to do so under conditions where the external pH is high, when it arrived at the inside it would gain a proton because of the much lower intracellular pH, and thus there would be a proton flow from inside to outside roughly equal to the anion exchange rate. In contrast, Wieth and Bjerrum (1982) have shown that the rate of H^+ (or OH^-) efflux at pH_o 12.4 was only 3% of the Cl^- exchange rate. Thus, it seems that the function of the C (low pK) residue is to increase the affinity of the external transport site for anions, but that this residue does not cross the membrane with the anion.

A similar argument does not rule out the possibility that the high-pK residue (D) crosses the membrane, as it is assumed in Bjerrum's model that the form of the protein with this residue deprotonated does not transport anions or does so at a negligible rate. This would also be compatible with our data. Because the D residue does not cross the membrane in the deprotonated form, it cannot mediate net proton efflux on the return trip. Thus, movement of the charged form of this residue with Cl^- across the permeability barrier is compatible with the observation of a very low H^+ flux at high pH_o .

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REFERENCES

- Bjerrum, P. J. 1992. The human erythrocyte anion transport protein, band 3. Characterization of exofacial alkaline titratable groups involved in anion binding/translocation. *J. Gen. Physiol.* 100:301-339.
- Bjerrum, P. J., J. O. Wieth, and C. L. Borders, Jr. 1983. Selective phenylglyoxalation of functionally essential arginyl residues in the erythrocyte anion transport protein. *J. Gen. Physiol.* 81:453-483.
- Dalmark, M. 1975. Chloride transport in human red cells. *J. Physiol. (Lond.)* 250:39-64.
- Dalmark, M. 1976. Effect of halides and bicarbonate on chloride transport in human red blood cells. *J. Gen. Physiol.* 67:223-234.
- Dalmark, M., and J. O. Wieth. 1972. Temperature dependence of chloride, bromide, iodide, thiocyanate and salicylate transport in human red cells. *J. Physiol. (Lond.)* 224:583-610.
- Falke, J. J., and S. I. Chan. 1986. Molecular mechanisms of band 3 inhibitors. 1. Transport site inhibitors. *Biochemistry.* 25:7888-7894.
- Fröhlich, O. 1982. The external anion binding site of the human erythrocyte anion transporter: DNDS binding and competition with chloride. *J. Membr. Biol.* 65:111-123.
- Fröhlich, O., and R. B. Gunn. 1986. Erythrocyte anion transport: the kinetics of a single-site obligatory exchange system. *Biochim. Biophys. Acta.* 864:169-194.
- Fröhlich, O., C. Leibson, and R. B. Gunn. 1983. Chloride net efflux from intact erythrocytes under slippage conditions. Evidence for a positive charge on the anion binding/transport site. *J. Gen. Physiol.* 81:127-152.
- Furuya, W., T. Tarshis, F.-Y. Law, and P. A. Knauf. 1984. Transmembrane effects of intracellular chloride on the inhibitory potency of extracellular H₂DIDS. Evidence for two conformations of the transport site of human erythrocyte anion exchange protein. *J. Gen. Physiol.* 83:657-681.
- Gabler, R. 1978. *Electrical Interactions in Molecular Biophysics: An Introduction.* Academic Press, New York, p. 149.
- Gasbjerg, P. K., and J. Brahm. 1991. Kinetics of bicarbonate and chloride transport in human red cell membranes. *J. Gen. Physiol.* 97:321-350.
- Greenstein, J. P. 1933. Studies of the peptides of trivalent amino acids. III. The apparent dissociation constants, free energy changes, and heats of ionization of peptides involving arginine, histidine, lysine, tyrosine, and aspartic and glutamic acids, and the behavior of lysine peptides toward nitrous acid. *J. Biol. Chem.* 101:603-621.
- Gruen, L., M. Laskowski, Jr., and H. A. Scheraga. 1959. Thermodynamics of the ionization of the lysyl residue of insulin. *J. Am. Chem. Soc.* 81:3891-3901.
- Grygorczyk, R., W. Schwarz, and H. Passow. 1987. Potential dependence of the "electrically silent" anion exchange across the plasma membrane of *Xenopus* oocytes mediated by the band-3 protein of mouse red blood cells. *J. Gen. Physiol.* 99:127-136.
- Gunn, R. B., and O. Fröhlich. 1979. Asymmetry in the mechanism for anion exchange in human red blood cell membranes. Evidence for reciprocating sites that react with one transported anion at a time. *J. Gen. Physiol.* 74:351-374.
- Gunn, R. B., and O. Fröhlich. 1989. Methods and analysis of erythrocyte anion fluxes. *Methods Enzymol.* 173:54-80.
- Harned, H. S., and B. B. Owen. 1950. *The physical chemistry of electrolytic solutions.* Reinhold Publishing Corporation, New York.
- Jay, D., and L. Cantley. 1986. Structural aspects of the red cell anion exchange protein. *Annu. Rev. Biochem.* 55:511-538.
- Jennings, M. L. 1989a. Characteristics of the binding site for extracellular substrate anions in human red blood cell band 3. *Ann. NY Acad. Sci.* 574:84-95.
- Jennings, M. L. 1989b. Evidence for an access channel leading to the outward-facing substrate site in human red blood cell band 3. In *Anion Transport Protein of the Red Blood Cell Membrane.* N. Hamasaki and M. L. Jennings, editors. Elsevier Science Publishers B. V. (Biomedical Division), Amsterdam, 59-72.
- Jennings, M. L. 1992. Cellular anion transport. In *The Kidney: Physiology and Pathophysiology.* D. W. Seldin and G. Giebisch, editors. Raven Press, New York. 113-145.
- Jennings, M. L., R. Monaghan, S. M. Douglas, and J. S. Nicknisch. 1985. Functions of extracellular lysine residues in the human erythrocyte anion transport protein. *J. Gen. Physiol.* 86:653-669.
- Jennings, M. L., R. K. Schulz, and M. Allen. 1990. Effects of membrane potential on electrically silent transport: Potential-independent translocation and asymmetric potential-dependent substrate binding to the red blood cell anion exchange protein. *J. Gen. Physiol.* 96:991-1021.
- Kaufmann, E., G. Eberl, and K. F. Schnell. 1986. Characterization of the band 3 substrate site in human red cell ghosts by NDS-TEMPO, a disulfonatosilbene spin probe: the function of protons in NDS-TEMPO and substrate-anion binding in relation to anion transport. *J. Membr. Biol.* 91:129-146.
- Knauf, P. A. 1989. Kinetics of anion transport. In *The Red Cell Membrane: A Model for Solute Transport.* B. U. Raess, and G. Tunncliffe, editors. Humana Press, Clifton, NJ. 171-200.
- Knauf, P. A., and J. Brahm. 1989. Functional asymmetry of the anion-exchange protein, capnophorin: effects on substrate and inhibitor binding. *Methods Enzymol.* 173:432-452.
- Knauf, P. A., F. Law, T. Tarshis, and W. Furuya. 1984. Effects of the transport site conformation on the binding of external NAP-taurine to the human erythrocyte anion exchange system. *J. Gen. Physiol.* 83:683-701.
- Knauf, P. A., and N. A. Mann. 1984. Use of niflumic acid to determine the nature of the asymmetry of the human erythrocyte anion exchange system. *J. Gen. Physiol.* 83:703-725.
- Knauf, P. A., and N. A. Mann. 1986. Location of the chloride self-inhibitory site of the human erythrocyte anion exchange system. *Am. J. Physiol.* 251 (*Cell Physiology* 20):C1-C9.
- Knauf, P. A., N. Mann, J. Brahm, and P. Bjerrum. 1986. Asymmetry in iodide affinities of external and internal-facing red cell anion transport sites. *Fed. Proc.* 45:1005 (Abstr.).
- Knauf, P. A., N. A. Mann, J. E. Kalwas, L. J. Spinelli, and M. Ramjeesingh. 1987. Interactions of NIP-taurine, NAP-taurine, and Cl⁻ with the human erythrocyte anion exchange system. *Am. J. Physiol.* 251 (*Cell Physiology* 22):C652-C661.
- Knauf, P. A., and L. J. Spinelli. 1995. NIP- and NAP-taurine bind to external modifier site of AE1 (band 3), at which iodide inhibits anion exchange. *Am. J. Physiol.* 269 (*Cell Physiology* 38):C410-C416.
- Knauf, P. A., L. J. Spinelli, and N. A. Mann. 1989. Flufenamic acid senses conformation and asymmetry of human erythrocyte band 3 anion transport protein. *Am. J. Physiol.* 257 (*Cell Physiology* 26):C277-C289.
- Liu, S. J. 1990. Kinetic analysis of factors which affect external substrate binding and of the mechanism whereby eosin derivatives interact with the human erythrocyte anion transport protein, band 3. Ph.D. thesis, University of Rochester, Rochester, NY.
- Liu, S., and P. A. Knauf. 1990. Evidence from pH effects that amino acids other than arginine affect substrate affinity of the external-facing transport site of human erythrocyte band 3. *Biophys. J.* 57: 89a (Abstr.).
- Liu, S. J., E. Ries, and P. A. Knauf. 1996. 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. *J. Gen. Physiol.* 107:293–306.
- Lux, S. E., K. M. John, R. R. Kopito, and H. F. Lodish. 1989. Cloning and characterization of band 3, the human erythrocyte anion-exchange protein (AE1). *Proc. Natl. Acad. Sci. USA.* 86:9089–9093.
- Milanick, M. A., and R. B. Gunn. 1982. Proton-sulfate co-transport. Mechanism of H⁺ and sulfate addition to the chloride transporter of human red blood cells. *J. Gen. Physiol.* 79:87–113.
- Milanick, M. A., and R. B. Gunn. 1986. Proton inhibition of chloride exchange: asynchrony of band 3 proton and anion transport sites? *Am. J. Physiol.* 250 (*Cell Physiology* 19):C955–C969.
- Passow, H. 1969. Passive ion permeability of the erythrocyte membrane. An assessment of the scope and limitations of the fixed charge hypothesis. *Prog. Biophys. Mol. Biol.* 19:425–467.
- Passow, H. 1986. Molecular aspects of band 3 protein-mediated anion transport across the red blood cell membrane. *Rev. Physiol. Biochem. Pharmacol.* 103:61–202.
- Reithmeier, R. A. F., D. M. Lieberman, J. R. Casey, S. W. Pimpliker, P. K. Werner, H. See, and C. A. Pirraglia. 1989. Structure and function of band 3 Cl⁻/HCO₃⁻ transporter. *Ann. NY Acad. Sci.* 574:75–83.
- Segel, I. H. 1975. *Enzyme Kinetics*. John Wiley and Sons, Inc., New York.
- Walker, H. M., and J. Lev. 1953. *Statistical Inference*. Holt, Rinehart, and Winston. New York. 398–404.
- Wieth, J. O., J. Brahm, and J. Funder. 1980. Transport and interactions of anions and protons in the red blood cell membrane. *Ann. NY Acad. Sci.* 314:394–418.
- Wieth, J. O., and P. J. Bjerrum. 1982. Titration of transport and modifier sites in the red cell anion transport system. *J. Gen. Physiol.* 79:253–282.
- Wieth, J. O., P. J. Bjerrum, and C. L. Borders, Jr. 1982. Irreversible inactivation of red cell chloride exchange with phenylglyoxal, an arginine-specific reagent. *J. Gen. Physiol.* 79:283–312.
- Wood, P. G. 1992. The anion exchange proteins: homology and secondary structure. In *Progress in Cell Research*. Vol. 2. E. Bamberg and H. Passow, editors. Elsevier Science Publishers, Amsterdam. 325–352.
- Zaki, L., and T. Julien. 1985. Anion transport in red blood cells and arginine-specific reagents. Interaction between the substrate-binding site and the binding site of arginine-specific reagents. *Biochim. Biophys. Acta.* 818:325–332.

APPENDIX

Effects of pH_o on Inhibition of Cl⁻ Exchange by Phenylglyoxal

A piece of evidence which seemed to fit well with the fixed charge or positive interfacial potential (Wieth and Bjerrum, 1982; Wieth et al., 1982) model concerns the effects of [Cl_o] on the rate of reaction of phenylglyoxal (PG) with a putative arginine residue (here designated Arg-b) which is essential for anion transport (Wieth et al., 1982). Interactions of PG with band 3 are very complex: there is a reversible binding which causes inhibition of Cl⁻ exchange, but which is unrelated to the irreversible inhibition caused by a second-order covalent reaction; the labeling pattern of PG is strongly affected by the pH_o at which the reaction is carried out; and thus the reaction site may be different depending on the pH (Wieth et al., 1982). Here we will

only be concerned with the covalent reaction which occurs at high pH_o (typically above 10). The rate of PG reaction increases with increasing pH_o, since PG reacts with the deprotonated form of Arg-b (Wieth et al., 1982). The reaction rate also decreases with increasing [Cl_o]. Since the same effects were seen with [Cl_i] constant or equal to [Cl_o] (Fig. 5 of Wieth et al., 1982), Wieth et al. (1982) proposed that [Cl_o] was affecting a positive interfacial potential near the reactive site.

These data can be reinterpreted in terms of the substrate site titration model if one assumes that PG can only react with band 3 (or does so much more rapidly) when band 3 is in the E_o conformation, with the transport site facing outward and unloaded with substrate. Since the ratio of E_o to E_i (the form of band 3 with the transport site unloaded and facing the cytoplasm) is given by A([Cl_i]/[Cl_o]), where $A = k \cdot K_o / (k' \cdot K_i)$, increasing [Cl_o] will decrease the fraction of band 3 molecules in the E_o form⁵ and hence will decrease the rate of PG reaction, as observed. Wieth et al. (1982) argued against this model, however, because they concluded that it would predict greater effects of changing [Cl_o] with constant [Cl_i], because of the change in the [Cl_i]/[Cl_o] ratio, than when [Cl_i] = [Cl_o], contrary to their observations.

From Bjerrum's (1992) and our data it seems that A may be much larger at pH_o = 10.3, at which Wieth et al.'s (1982) experiments were done, than the value at pH 7.2 (0.064–0.1, Knauf and Brahm, 1989), although the precise value of A is unknown at the temperature of their experiments, 25°C. Under these conditions, the effects of changes in [Cl_o] on the E_o/E_i ratio are reduced, and so the effects of [Cl_o] on the PG reaction rate are similar with [Cl_i] constant or with [Cl_i] = [Cl_o]. For example, if K_o is assumed to be 12 times higher at pH 10.3 than at pH 7.2, and if k/k' is twice as high, A would be 1.54. Further, we assumed a value for K_{Cl}, the half-saturation concentration for Cl⁻ with [Cl_i] = [Cl_o], of 30 mM, which gives a K_{1/2o} (apparent half-saturation concentration for external Cl⁻ with 165 mM [Cl_i]) of 17 mM, in good agreement with observed values for the similar pH_o at 0°C (Bjerrum, 1992; Liu et al., 1994). Under these conditions, E_o/E_t (where E_t is the total amount of band 3) would be .459, .326, .207 and .131 for 20, 35, 65, and 112 mM [Cl_o] with [Cl_i]

⁵Note that in this analysis E_o refers to the total of outward-facing, unloaded transport sites, without regard to their degree of protonation. E_o/E_t is calculated from the apparent transport parameters at a single pH_o (10.3). No attempt is made here to derive an expression for the amount of unprotonated E_o (designated S_o by Bjerrum, 1992), although in terms of the model this should be the form that actually reacts with PG. Thus, our calculations should predict the Cl⁻ dependence of the PG reaction rate, but they do not include the dependence on pH_o.

constant. These are very similar to the calculated reaction rates for PG relative to the extrapolated rate in zero $[Cl_o]$, when $E_o/E_i = 1$ (Fig. 5 and Table II of Bjerrum, 1992). With $[Cl_o] = [Cl_i]$, the corresponding values of E_o/E_i are .363, .280, .191, and .128. The predicted differences in PG reaction rate with $[Cl_i]$ constant or equal to $[Cl_o]$ might therefore be within the limits of experimental error, and would even be smaller if the value of A were larger or if the reversible binding of PG were to recruit band 3 toward the E_o form. Thus, Wieth et al.'s (1982) data do not necessarily disprove the hypothesis that PG reacts preferentially with E_o .

Wieth et al. (1982) also found that increasing external $[SO_4^-]$ had no significant effect on PG reaction rate. This might be explained even if PG only reacts with E_o , however, if the SO_4^- affinity is very low at pH 10.3, as suggested by our data (Table I of Liu et al., 1994). Indeed, Zaki and Julien (1985) found that effects of SO_4^- on the PG reaction rate were much weaker at pH 8 than at pH 7.4. The fact that external I^- and Cl^- have similar effects on PG reaction rate (Wieth et al., 1982) could also be explained if the apparent affinities for these two ions are similar at 25°C. The protective effect of a competitive disulfonic stilbene inhibitor (Fröhlich, 1982), DNDS (4,4'-dinitrostilbene-2,2'-disulfonate), against PG reaction, which is reduced at high

$[Cl_o]$ (which competes with DNDS and reduces its apparent affinity for band 3), fits well with the concept that PG reacts with E_o , as does the fact that PG reaction reduces the reaction rate of another disulfonic stilbene, DIDS (4,4'-diisothiocyano-stilbene-2,2'-disulfonate). Furthermore, Falke and Chan (1986) have found that PG reaction abolishes the binding of Cl^- to the external-facing transport site, as measured by ^{35}Cl NMR.

Although it is by no means proven that PG reacts exclusively with E_o , this remains a strong possibility. If so, the effects of $[Cl_o]$ on the pK_{app} for PG reaction could easily be explained in terms of the changes in apparent external Cl^- affinity with pH_o : at lower pH_o Cl^- is a more effective inhibitor of PG reaction rate than at high pH_o , leading to a shift of the inactivation rate versus pH_o curve toward the right at high $[Cl_o]$, as observed (Wieth et al., 1982). This hypothesis would require reinterpretation of the true pK of the Arg-b residue: if Cl^- interferes with the reaction, the true pK would be best estimated from data at low $[Cl_o]$, where pK is least affected, so the true pK would be around 10.2 at 25°C or ~ 11 at 0°C. This pK value suggests that the PG reactive residue is identical to the higher- pK titratable D residue, which has a pK of >11 from our data or 11.3 from Bjerrum's (1992) data.