A Novel Method to Differentiate between Ping-pong and Simultaneous Exchange Kinetics and Its Application to the Anion Exchanger of the HL60 Cell

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ABSTRACT We have developed a new test to differentiate between ping-pong and simultaneous mechanisms for tightly coupled anion exchange. This test requires the use of a dead-end reversible noncompetitive inhibitor. As an example, we have applied the test to the anion exchanger of the HL60 cell using the salicylic acid derivative 3,5-diiodosalicylic acid (DIS), which reversibly inhibits HL60 cell Cl/C1 exchange. The concentration of DIS that causes 50% inhibition (ID50) increased only slightly as either intra- or extracellular chloride was increased, indicating that DIS inhibits HL60 anion exchange in a noncompetitive manner. In agreement with this observation, plots of the slope of the Dixon plot as a function of 1/[Cl0] or 1/[Cl1] were fit with straight lines with nonzero intercepts, indicating that DIS does not compete with either of the substrates ([Cl0] and [Cl1]). The secondary Dixon slope test is based on the fact that, for a dead-end inhibitor such as DIS, the slope of the Dixon plot slope vs. 1/[Cl1] (secondary Dixon slope or SDS) is independent of extracellular Cl when the exchange mechanism follows ping-pong kinetics. Similarly, the SDS calculated from a plot as a function of 1/[Cl0] is also independent of intracellular Cl for a ping-pong exchanger. In contrast to this prediction, we found that for DIS inhibition of Cl/C1 exchange in HL60 cells the slope of the Dixon plot slope vs. 1/[Cl1] decreased by a factor of 2.5-fold when [Cl0] was increased from 1 to 11 mM (P < 0.0001). This change in the SDS rules out ping-pong kinetics, but is consistent with a simultaneous model of Cl/C1 exchange in which there are extracellular and intracellular anion binding sites, both of which must be occupied by suitable anions in order to allow simultaneous exchange of the ions.

INTRODUCTION

One of the most fundamental questions concerning tightly coupled ion exchange systems, such as Cl/HCO3 or Na/H exchange, is whether the tight coupling of ion
influx and efflux is due to a ping-pong or a simultaneous mechanism. In the ping-pong mechanism (also called sequential) only one substrate binds to the exchanger at a given time, and substrates are translocated separately in a series of steps that result in transport of one substrate in each direction per transport cycle (Segel, 1975). (Fig. 1 A depicts a ping-pong mechanism for a Cl/HCO₃ exchanger when carrying out Cl/Cl exchange.) In the simultaneous mechanism, on the other hand, the two different substrates are bound to the protein at the same time, one at each side of the membrane, and are transported simultaneously; that is, both are translocated before either is released (Fig. 1 B).

**FIGURE 1.** Diagrams depicting inhibition by a noncompetitive inhibitor (I) for the ping-pong (A) and simultaneous (B) models of anion exchange. The simultaneous model is the random bi bi model previously used by us to explain substrate dependence (Restrepo et al., 1989) and DIDS inhibition kinetics (Restrepo et al., 1991a) of Cl/Cl exchange in HL60 cells.

In theory, these mechanisms can be distinguished on the basis of the effects of trans substrate on the parameters that describe saturation kinetics at the cis side ($J_m$, the maximum flux and $K_m$, the concentration of cis substrate that gives half-maximal flux). For a ping-pong mechanism, the ratio $J_m/K_m$ should be independent of trans substrate concentration (Segel, 1975). In practice, however, these mechanisms are often difficult to distinguish experimentally. First, scatter in the data, particularly the $K_m$ values, limits the precision with which this test can be applied. Second, since the ping-pong model is very predictive but the simultaneous model is not, it is difficult to rule out the possibility of a simultaneous mechanism. Because of the larger number
of free parameters, some form of the simultaneous mechanism can explain almost any observation. The sole exception to this is the direct demonstration of a half-turnover of the system, such that one substrate molecule per transport molecule is translocated in one direction, across the membrane, without transport of a second substrate in the reverse direction, as shown for red blood cell anion exchange by Jennings (1982). This half-turnover test, however, can only be applied in special circumstances where the number of transporters is large enough so that a flux of one ion per transporter can be observed, and this is unlikely to be the case for most transport systems.

A third problem is that discrepancies from the predictions of the ping-pong model can often be explained by postulating that there are additional “modifier” ion binding sites that can affect the transport rate (Dalmark, 1976) or that “slippage” of unloaded transport sites across the membrane can give rise to some net flow of substrate (Sachs, 1979). Such features can cause a ping-pong model to exhibit apparently simultaneous kinetics, but they make the ping-pong model more complex and hence less attractive. They also change the question of determining the mechanism of coupling from the rather simple one of distinguishing the unadorned ping-pong from the simultaneous mechanism, to the much more difficult question of whether the evidence favors a modified, usually more predictive, ping-pong model over the inherently more complex simultaneous alternative. Use of new approaches to differentiate simultaneous from ping-pong models can help to resolve this question either by eliminating certain variants of the ping-pong model or else by showing that the system indeed has more detailed properties that fit such a model.

The difficulties in differentiating between simultaneous and ping-pong exchange kinetics are clearly illustrated by studies of the kinetics of the anion exchanger of human red blood cell (band 3), which is probably the best-studied tightly coupled passive ion exchange system. In this system, because band 3 is present in red blood cells in high copy number, it has been possible to perform a half-turnover experiment in which, in the absence of extracellular substrate, the exchange protein transports a number of substrate molecules equal to the number of transport protein molecules and then stops catalyzing transport due to the absence of extracellular substrate (Jennings, 1982). In addition, various other kinetic experiments indicate that band 3 follows ping-pong kinetics (Gunn and Frohlich, 1979; Furuya, Tarshis, Law, and Knauf, 1984; Knauf, 1986). However, even though the consensus is for a ping-pong model, data from some studies apparently do not fit this model (Salhany and Rauenbuehler, 1983; Hautmann and Schnell, 1985; Benaroch and Gunn, 1992), and therefore there is still controversy about whether band 3 actually follows ping-pong kinetics.

A particular case where it is important and potentially difficult to distinguish the simultaneous and ping-pong mechanisms concerns the anion exchange system of the HL60 cell. HL60 cells are a human leukemic cell line with promyelocytic characteristics (Gallagher, Collins, Trujillo, McGredie, Ahearn, Tsai, Metzgar, Aulakh, Ting, Ruscetti, and Gallo, 1975). The Na+-independent Cl/HCO3 (anion) exchanger of the HL60 promyelocyte catalyzes a tightly coupled exchange of anions, and thereby plays an important role in maintaining steady-state pHi (Restrepo, Kozody, and Knauf, 1988a), in volume regulation (Restrepo, Kozody, and Knauf, 1987), and in recovery
from alkaline loads (Restrepo, Kozody, Spinelli, and Knauf, 1988b). Because the HL60 cell expresses mRNA for a protein (AE2) (Demuth, Showe, Ballantine, Palumbo, Fraser, Cioe, Rovera, and Curtis, 1986) whose mouse analogue has been shown to encode for a nonerythroid anion exchanger (Alper, Brosius, Garcia, Gluck, Brown, and Lodish, 1989; Lee, Gunn, and Kopito, 1991), it is probable that AE2 catalyzes CI/C1 exchange in the HL60 cell. Earlier studies in our laboratory, using substrate saturation (Restrepo, Kozody, Spinelli, and Knauf, 1989) or competitive inhibition kinetics (Restrepo, Cronise, Snyder, Spinelli, and Knauf, 1991a), indicate that HL60 CI/C1 exchange takes place by a simultaneous mechanism. These tests rule out many variants of the ping-pong model to a high degree of statistical confidence. Nevertheless, inaccuracies in measuring substrate or inhibitor affinities in HL60 cells, together with possible systematic errors to which any method is subject, limit the degree of certainty of this conclusion. An accurate answer to this question is important, since if AE2 is indeed the protein that catalyzes CI/C1 exchange in the HL60 cell, our results, which rule out ping-pong kinetics, together with studies of CI/C1 exchange in red blood cells discussed above, which indicate that band 3 follows ping-pong kinetics, indicate that the small difference in structure between AE2 and AE1 (whose membrane-spanning portions display ~81% amino acid identity (Alper, Kopito, Libresco, and Lodish, 1988; Kopito, 1990) causes a fundamental change in kinetics from simultaneous (AE2) to ping-pong (AE1). If this is true, analysis of the structural differences between AE2 and AE1 could provide clues to the structural basis for ping-pong vs. simultaneous transport mechanisms.

To help address such questions in this and other exchange transport systems, we present here a new method for distinguishing simultaneous from ping-pong mechanisms. Unlike other methods, the secondary Dixon slope method is based on the discovery that, even for an inhibitor that can bind with different affinities to each form (with the transport site facing inward or outward, and unloaded or loaded with substrate) of the ping-pong exchanger (Fig. 1), the model still makes a very restrictive prediction about the kinetics of inhibition. Specifically, Dixon plots (1/flux, vs. inhibitor concentration, [I]) are constructed under conditions with different internal and external substrate concentrations (e.g., [Clᵢ] and [Clₒ] for a Cl/HCO₃ exchanger operating in the Cl/Cl exchange mode). If the slope of such Dixon plots is then plotted as a function of 1/[Clᵢ] (or 1/[Clₒ]), the ping-pong model predicts that the slope of such a plot (SDS) should be the same regardless of the Cl concentration on the other side of the membrane ([Clₒ] or [Clᵢ], respectively). In general, this is not true for a simultaneous model. Such a test can be used for any transport system for which a reversible, dead-end inhibitor exists.

We have applied this method to the sodium-independent Cl/HCO₃ exchange system in HL60 cells, using the reversible inhibitor 3,5-diiodosalicylic acid (DIS) (Motais and Cousin, 1978), and have found that the SDS increases significantly (P < 0.0001) when [Clᵢ] is lowered from 11 to 1 mM. The results agree with our earlier studies using other methods (Restrepo et al., 1989, 1991a), and thus strongly suggest that the mechanism of anion exchange is simultaneous in these cells.

Portions of this work have previously appeared in abstract form (Restrepo, Kozody, Spinelli, Diefenbach, Snyder, and Knauf, 1991b).
Methods

Cell Culture and Media

Experiments were performed with exponentially growing HL60 cells (passages 20–75; American Type Culture Collection, Rockville, MD). Cell growth conditions were as described previously (Restrepo et al., 1989). The (nominally) bicarbonate-free solution (solution 1) used was (mM): 145 NaCl, 5 KCl, 0.5 CaCl$_2$, 0.5 MgCl$_2$, and 20 HEPES titrated to pH 7.4 at 37°C by adding tetramethylammonium hydroxide or N-methyl-D-glucamine hydroxide in powder form. The chloride-free solution (solution 2) was made by replacing all salts in solution 1 with gluconate salts. All solutions used for Cl/Cl exchange measurements were bubbled with humidified nitrogen for at least 1 h before the experiment began. DIS (sodium salt) was from Fluka Chemie AG (Buchs, Switzerland) and all other chemicals were of reagent grade. $^{36}$Cl (sodium salt) was from ICN Biomedicals, Inc. (Costa Mesa, CA).

Manipulation of [Cl$^-$]

[Cl$^-$] was altered by preincubation for 1–2 h in media with varying concentrations of chloride (mixtures of solutions 1 and 2 supplemented with 5 mM D-glucose). As shown in Fig. 2 A, using this method it is possible to set the [Cl$^-$] to desired values in the range from 2 to 60 mM. Steady-state intracellular chloride was assayed in each experiment by centrifugation of cells through oil using a triple label technique ($^{36}$Cl to assay total chloride, $[^3$H]water for total volume, and $[^{14}$C]sucrose for extracellular volume; see Restrepo et al., 1988b). The energy windows used for counting the triple label samples were 0–25 keV for $[^3$H], 26–156 keV for $[^{14}$C], and 157–2,000 keV for $^{36}$Cl. In a typical experiment, the distribution of counts in each window was: for $[^3$H], 100% 0–25 keV; for $[^{14}$C], 37% 0–25 keV and 63% 26–156 keV; and for $^{36}$Cl, 4% 0–25 keV, 23% 26–156 keV, and 73% 157–200 keV. These values were measured in each experiment using appropriate standards and the spillover values were used to determine the total counts per minute for the three isotopes. No quench correction was necessary because the amount of quench was the same for all samples and standards as monitored by using an external standard.

Manipulation of intracellular chloride can alter other parameters that may affect the activity of the Cl/Cl exchanger. In particular, Cl/Cl exchange in the HL60 cell is highly dependent on intracellular pH (Restrepo et al., 1988a). However, in earlier work we found that our method produces changes in steady-state intracellular chloride without altering internal pH (Restrepo et al., 1988b). Similarly, the Cl/Cl exchange system could be affected directly or indirectly by changes in cell volume. Fig. 2 B shows that there is only a small change in cell volume when intracellular Cl is changed by preincubation in media with different [Cl$^-$]. These data are not significantly different from our earlier measurements of cell volume at two different [Cl$^-$] (10 and 53 mM, filled circles in Fig. 2 B) (Restrepo et al., 1989). The cell volume data indicate that the intracellular Cl lost is replaced to a large extent by another anion, which could be either gluconate or an intracellularly synthesized anion. Previous measurements of Cl influx into HL60 cells depleted of intracellular Cl have shown that the replacement anion is not transported by the Cl/Cl exchange system (Restrepo et al., 1991a). Therefore, the manipulation of intracellular Cl does not appear to alter other parameters that could modify the activity of the exchanger.

Measurement of Cl/Cl Exchange Using $^{36}$Cl Efflux

$^{36}$Cl efflux into media with varying concentrations of unlabeled chloride was assayed by using a centrifugation technique described previously (Restrepo et al., 1989). As shown in Results, and
in agreement with earlier measurements, intracellular $^{36}$Cl decreases exponentially as a function of time. The efflux of $^{36}$Cl in millimoles Cl per liter cell water $^{-1}$ per minute $^{-1}$ was calculated as the product of the efflux rate constant in minutes $^{-1}$ times the internal chloride concentration in millimolar. This efflux expressed in units of millimoles Cl per liter cell water $^{-1}$ per minute $^{-1}$ would be directly proportional to the rate calculated in units of femtomoles Cl per cell $^{-1}$ per minute $^{-1}$ if cell volume were constant as a function of intracellular Cl. This is due to the fact that it is necessary to multiply the rate in millimoles Cl per liter cell water $^{-1}$ per
minute⁻¹ times the cell volume in picoliters per cell⁻¹ to convert it to femtoliters Cl⁻ per cell⁻¹ per minute⁻¹. As shown in Fig. 2 B, cell volume appears to increase slightly as a function of [Cl⁻]. As described in detail in Results, this slight change in cell volume is too small to affect our conclusions about the kinetics of DIDS inhibition. We routinely measure efflux in units of millimoles Cl⁻ per liter cell water⁻¹ per minute⁻¹ because, as can be seen by comparing the standard errors in Fig. 2, A and C, the determination of [Cl⁻], which is used to calculate efflux in units of millimoles Cl⁻ per liter cell water⁻¹ per minute⁻¹ is more precise than the determination of Cl⁻ content per cell, which is used to calculate efflux in units of femtomoles Cl⁻ per cell⁻¹ per minute⁻¹. The larger standard errors for the determination of Cl⁻ content per cell are probably due to errors inherent in the determination of cell number.

Although all solutions were thoroughly bubbled with nitrogen, in ~ 20% of the experiments a small 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS)-sensitive Cl⁻ efflux was detected when cells were suspended in Cl⁻-free medium (solution 2). The increase in the efflux rate constant, presumably caused by a small amount of bicarbonate in the Cl⁻-free medium, was never larger than 0.02 min⁻¹, and was always smaller than the increase in efflux caused by addition of 0.1 mM Cl⁻. When this small effect was detected, the Cl⁻/Cl⁻ exchange rate was estimated by subtracting the rate of efflux of ⁵²Cl⁻ in the presence of 50 μM DIDS in Cl⁻-free medium from the rate in the presence of Cl⁻. In all other cases, the Cl⁻/Cl⁻ exchange rate was calculated by subtracting the rate of efflux of ⁵²Cl⁻ in the absence of extracellular Cl⁻ from the efflux rate in the presence of Cl⁻.

Nonlinear Least-Squares Fits

The equation describing inhibition of anion exchange by an inhibitor interacting at a single site is:

\[
J = J_o / \left( 1 + \left( [I] / ID_{50} \right) \right) \]

where \(J\) is Cl⁻/Cl⁻ exchange flux at a given inhibitor concentration ([I]), \(J_o\) is flux in the absence of inhibitor, and \(ID_{50}\) is the concentration of inhibitor that reduces the flux to one-half of control. Eq. 1 can be rearranged in the form:

\[
J = 1 / \left( 1 / J_o + \left( [I] / (J_o ID_{50}) \right) \right)
\]

where \(1/J_o ID_{50}\) is the slope of the Dixon plot (1/J vs. [I]). Experimental data (J vs. [I]) were fit with Eqs. 1 and 2 using the nonlinear least-squares fitting program ENZFITTER (Elsevier-Biosoft, Amsterdam, The Netherlands). Whenever least-squares fit parameters are shown, estimates of the standard error calculated by the method of matrix inversion are included. The significance of differences between estimates was assessed by using t tests. Unless otherwise specified, the significance level used was \(P < 0.05\).

For simultaneous fits of Eqs. 27 and 28 to the data in Figs. 5, 6, and 7 A (ID₅₀ vs. [Clo] and [Cl⁻] and Dixon slope vs. [Cl_o] and [Cl⁻]) (see Discussion) we used a program of Cacceci and Cacheris (1984) based on the simplex algorithm. We modified this program to allow input of two independent variables ([Cl_o] and [Cl⁻]) and use of two different equations (Eqs. 27 and 28). Because the ID₅₀ values and the Dixon slope values have different units, we minimized the sum of the squares of the percent deviations from the theoretical line.

THEORY

Derivation of Equations Describing Inhibition of Cl⁻/Cl⁻ Exchange Flux (J) by a Noncompetitive Inhibitor (I)

For ease of presentation, the test is discussed in terms of a Cl⁻/Cl⁻ exchange protein that catalyzes the one-for-one exchange of [Cl⁻] for extracellular chloride ([Cl_o]). Fig.
1 shows the schemes for ping-pong kinetics (Fig. 1 A) and simultaneous kinetics (Fig. 1 B) for such a transport system. Although the results are discussed in terms of a Cl/C1 exchange mechanism, they are applicable to any tightly coupled exchange transport system whose transport models can be drawn in the form shown in Fig. 1.

All derivations in this section are based on the assumption that all forms of the enzyme and the substrates are at equilibrium. The derivations were performed utilizing standard methods for enzyme kinetics (Segel, 1975). Other theoretical aspects of substrate and inhibitor kinetics for the ping-pong and simultaneous models of Cl/C1 exchange are discussed in Frohlich and Gunn (1986), Knauf and Brahms (1989), and Restrepo et al. (1989, 1991a).

**Ping-pong Model (Fig. 1 A)**

A noncompetitive inhibitor (I) can bind to all forms of the exchanger with different affinities. The Cl/C1 exchange flux ($J$) is given by the equation

$$J = k[E_{Cl}]$$  (3)

where $k$ is the rate constant for conversion of the ECl$_i$ form to the ECl$_o$ form. The following equations can be stated using the law of mass action (see Fig. 1 A for nomenclature):

- $K_o = [E_o][Cl_o]/[ECl_o]$  (4)
- $K_i = [E_i][Cl_i]/[ECl_i]$  (5)
- $K_j = [I][E_o]/[E_{oI}]$  (6)
- $K_t = [I][ECl_o]/[ECl_oI]$  (7)
- $K_g = [I][ECl_i]/[ECl_iI]$  (8)
- $K_n = [I][E_i]/[E_{iI}]$  (9)

In addition, Eq. 10 follows from the assumption that the exchange of Cl$_o$ for Cl$_i$ is tightly coupled:

$$k[E_{Cl}] = k'[ECl_o]$$  (10)

Furthermore, because the total amount of exchange protein [Et] is constant,

$$[E_t] = [E_i] + [E_o] + [ECl_i] + [ECl_o] + [E_{oI}] + [E_{oI}'] + [E_{iI}] + [E_{iI}']$$  (11)

Substituting Eqs. 4–10 into Eq. 11 and rearranging it is possible to express [ECl$_i$] in terms of the equilibrium constants, the substrate concentrations, and the concentration of inhibitor. Substituting this expression into Eq. 3 yields the equation for the reciprocal of the Cl/Cl exchange flux as a function of inhibitor concentration (Dixon plot):

$$1/J = 1/J_u + DS[I]$$  (12)

where the uninhibited flux $J_u$ is given by:

$$J_u = \frac{k[E_i]}{(K_i/[Cl_i]) + [kK_o/(k'[Cl_o])] + 1 + (k/k')}$$  (13)
and the slope of the Dixon plot (DS) by:

$$DS = \left( \frac{K_i}{[C_i]K_n} + \frac{kK_o}{k'[C_o]K_i} + \frac{1}{K_g} + \frac{k}{k'[K_o]/k_E} \right)$$  \hspace{1cm} (14)

In addition, the ID$_{50}$, the concentration of I that gives 50% inhibition, is given by the expression

$$ID_{50} = \frac{1}{(J_oDS)}$$  \hspace{1cm} (15)

Depending on the affinity of the inhibitor for each of the loaded and unloaded forms of the exchanger, the ID$_{50}$ will decrease or increase as intra- or extracellular chloride is increased. On the other hand, the slope of the Dixon plot (DS) will increase linearly as a function of either 1/[C$_o$] or 1/[C$_i$]. Note, however, that because there are no terms involving the product of [C$_o$] times [C$_i$] in Eq. 14, the slope of the plot of DS vs. 1/[C$_o$] (SDS) is constant as a function of [C$_i$]. Similarly, SDS calculated from a plot of DS vs. 1/[C$_i$] is constant as a function of [C$_o$]. This result is the basis for the secondary Dixon slope test proposed in this manuscript.

As stated in Methods, we replace intracellular Cl by gluconate. We know that gluconate is not transported by the anion exchanger of the HL60 cell (Restrepo et al., 1991); however, internal gluconate could inhibit the exchange system by competing with internal Cl. If gluconate were an internal competitive inhibitor, the only term in Eqs. 13-15 that would be modified would be the term ($K_i/[C_i]$) in the denominator of Eqs. 13 and 14, which would be multiplied by the factor $[1 + ([G]/K_{glu})]$, where [G] is the concentration of intracellular gluconate and $K_{glu}$ is the dissociation constant for binding of gluconate to the E$_i$ form. The form [C$_i$] and [C$_o$] dependence of the Dixon slope equation (Eq. 14) would not be modified. Therefore, the SDS is constant as a function of trans substrate for a ping-pong model, regardless of the presence or absence of an internal competitive inhibitor.

**Simultaneous Model (Fig. 1 B)**

The Cl/Cl exchange flux in this case is given by:

$$J = k_p[C_oE][C_i]$$  \hspace{1cm} (16)

The following equations can be stated using the law of mass action:

$$K_o = [E][C_o]/[C_oE]$$  \hspace{1cm} (17)

$$K_i = [E][C_i]/[ECl_i]$$  \hspace{1cm} (18)

$$\alpha K_i = [C_i][E]/[Cl_oECl_i]$$  \hspace{1cm} (19)

$$K_j = [E][I]/[EI]$$  \hspace{1cm} (20)

$$K_n = [Cl_oE][I]/[Cl_oEI]$$  \hspace{1cm} (21)

$$K_s = [ECl_i][I]/[ECl_iI]$$  \hspace{1cm} (22)

$$K_f = [Cl_oECl_i][I]/[Cl_oECl_iI]$$  \hspace{1cm} (23)

In addition, because the total amount of exchange protein [E$_t$] is constant,

$$[E_t] = [E] + [C_oE] + [ECl_o] + [Cl_oECl_i] + [EI] + [Cl_oEI] + [ECl_iI] + [Cl_oECl_iI]$$  \hspace{1cm} (24)
By substituting Eqs. 17–23 into Eq. 24 it is possible to write an expression for \([\text{Cl}_0,\text{Cl}_i]\) in terms of the substrate concentrations, the concentration of inhibitor, and the dissociation constants. Substituting this expression into Eq. 16 yields the following equation for the reciprocal of the flux \((1/J)\) as a function of inhibitor concentration (Dixon plot):

\[
1/J = 1/J_u + DS[I]
\]

(25)

where

\[
J_u = \frac{k_p[E_t][\text{Cl}_0][\text{Cl}_i]/(\alpha K_o K_i)}{1 + ([\text{Cl}_0]/K_o) + ([\text{Cl}_i]/K_i) + ([\text{Cl}_0][\text{Cl}_i]/(\alpha K_o K_i))}
\]

(26)

DS = \left[\frac{\alpha K_o K_i}{k_p[E_t]}\right]\left[\frac{1}{K_i[\text{Cl}_0][\text{Cl}_i]} + \frac{1}{(K_o K_i)[\text{Cl}_0][\text{Cl}_i]}\right] + \frac{1}{(\alpha K_o K_i)}

(27)

and the ID_{50} (Eq. 15) is given by the expression:

\[
\text{ID}_{50} = \frac{1 + ([\text{Cl}_0]/K_o) + ([\text{Cl}_i]/K_i) + ([\text{Cl}_0][\text{Cl}_i]/(\alpha K_o K_i))}{(1/K_i) + ([\text{Cl}_0]/(K_o K_i)) + ([\text{Cl}_i]/(K_o K_i)) + ([\text{Cl}_0][\text{Cl}_i]/(K_o K_i))}
\]

(28)

Depending on the values of the dissociation constants for inhibitor binding, the value of ID_{50} will decrease or increase as \([\text{Cl}_0]\) or \([\text{Cl}_i]\) is increased. The slope of the Dixon plot (DS) increases linearly as a function of either \(1/\text{Cl}_0\) or \(1/\text{Cl}_i\). However, in contrast to the ping-pong model (Eq. 14), there is a term in Eq. 27 that involves the product of \([\text{Cl}_0]\) times \([\text{Cl}_i]\). Thus, the slope of the plot of DS vs. \(1/\text{Cl}_0\) decreases as \([\text{Cl}_0]\) is increased, provided that the term with \([\text{Cl}_0][\text{Cl}_i]\) contributes significantly to DS, which takes place provided that \(K_i\) is sufficiently low with respect to the other inhibitor dissociation constants. From Eq. 28 it is evident that the ID_{50} approaches \(K_i\) in the limit when \([\text{Cl}_0]\) and \([\text{Cl}_i]\) approach zero. If \(K_i\) is sufficiently low the ID_{50} should not increase appreciably when both \([\text{Cl}_0]\) and \([\text{Cl}_i]\) are decreased.

For an ideal noncompetitive inhibitor \((K_j = K_k = K_g = K_i)\), Eq. 27 becomes

\[
DS = \left[\frac{\alpha K_o K_i}{k_p[E_t]}\right]\left[\frac{1}{(1/K_i) + ([\text{Cl}_0]/(K_o K_i)) + ([\text{Cl}_i]/(K_o K_i)) + ([\text{Cl}_0][\text{Cl}_i]/(K_o K_i))}\right]
\]

(29)

The x intercept of the plot of DS vs. \(1/\text{Cl}_i\) for this special case is equal to:

\[
x\text{-int-DS} = -\frac{1/[\text{Cl}_0] + [1/(\alpha K_o)]}{K_i(1/[\text{Cl}_i]) + (1/K_o)}
\]

(30)

The quotient on the right-hand side of the equation is identical to the reciprocal of the expression for the apparent dissociation constant for intracellular chloride \((K_{\text{app}})\) (Eq. 3 in Restrepo et al., 1989). Therefore, for an ideal noncompetitive inhibitor,

\[
x\text{-int-DS} = -1/K_{\text{app}}
\]

(31)

Inhibitor Binds to Two Different Sites

If inhibition is due to binding of the inhibitor molecule at two separate sites on the transport molecule, it is still possible to utilize a modified secondary Dixon slope test. In this case, the inhibitor will bind to the two sites with different affinities, and the affinities may be conformation dependent. In addition, one of the inhibitor sites
is occupied, the inhibitor may bind to the second site. For example, for the unloaded form of the transporter (E) for the simultaneous model (Fig. 1 B), the inhibitor can bind to the first site with a binding constant $K_{j1}$ to form the complex EI, or to the second site with a binding constant $K_{j2}$ to form the complex IE. Once one of these enzyme–inhibitor complexes forms, the inhibitor may bind to the other site to form the secondary complex IEI.

In terms of the derivation of the kinetic equations for the simultaneous or ping-pong models, the result of including a second site is to replace each of the equilibrium equations for the interaction of the inhibitor with one conformation of the transport molecule with three equations, two for the complexes with one inhibitor bound and one for the complex with two inhibitors bound. Thus, in the case of the interaction of E with I in Fig. 1 B, Eq. 20 will be replaced by the following three equations:

\[
\begin{align*}
K_{j1} &= [E][I]/[EI] \\
K_{j2} &= [E][I]/[IE] \\
K_{j3} &= [IE][I]/[IEI]
\end{align*}
\]

In addition, the new enzyme–inhibitor complexes must be included in Eqs. 11 and 24. As a result, the equation for the reciprocal of the Cl/Cl exchange flux as a function of inhibitor concentration (Dixon plot) will contain a quadratic term:

\[
1/J = 1/J_o + DS_1[I] + DS_2[I]^2
\]

$J_o$ is the uninhibited flux and $DS_1$ and $DS_2$ are coefficients independent of [I] containing the [Cl\textsubscript{1}] and [Cl\textsubscript{2}] dependencies.

Utilizing the procedure outlined above for the derivation of the expression for the slope of the Dixon plot it is possible to derive expressions for the linear coefficient in Eq. 35 ($DS_1$) for both the ping-pong and simultaneous models. The expression for the ping-pong model is:

\[
DS_1 = \frac{(K_i/[Cl\textsubscript{1}]K_o)}{((k/K_{j1})(k'/K_{j2})) + (1/K_{j1}) + (1/(k'/K_{j2}))}
\]

where the inhibitor binding constants $K_i$, $K_0$, $K_{j1}$, and $K_{j2}$ are composite constants given by:

\[
\begin{align*}
(1/K_{j1}) &= (1/K_{j1}) + (1/K_{j2}) \\
(1/K_{j2}) &= (1/K_{j1}) + (1/K_{j2}) \\
(1/K_{j3}) &= (1/K_{j3}) + (1/K_{j2}) \\
(1/K_{j4}) &= (1/K_{j3}) + (1/K_{j2})
\end{align*}
\]

where $K_{j1}$, $K_{j2}$, $K_{j3}$, and $K_{j4}$ are the binding constants for binding of the inhibitor to the first site and $K_{j2}$, $K_{j3}$, $K_{j4}$, and $K_{j5}$ are the binding constants for the second site.

Similarly, for the simultaneous model the expression for $DS_1$ is:

\[
DS_1 = [aK_oK_i/(k_i[E_o])]\left[1/(K_{j1}[Cl\textsubscript{1}])([Cl\textsubscript{1}]) + 1/(K_{j2}[Cl\textsubscript{2}])([Cl\textsubscript{2}])\right] + [1/(K_{j1}K_{j2}[Cl\textsubscript{1}])]
\]

\[
+ [1/(K_{j2}K_{j3}[Cl\textsubscript{2}]) + 1/(K_{j4}K_{j5}[Cl\textsubscript{3}])]
\]
Again, the inhibitor binding constants $K_i$, $K_o$, $K_e$, and $K_n$ are composite constants given by Eqs. 37–40.

As can be seen by comparison of Eq. 36 with Eq. 14 and Eq. 41 with Eq. 27, for both the ping-pong and simultaneous models the term $\Delta S_i$ in Eq. 35 has the same $[Cl_o]$ and $[Cl_i]$ dependence that the slope of the Dixon plot displays in Eqs. 14 and 27 (for the ping-pong and simultaneous models, respectively). This is an important result because it indicates that the secondary Dixon slope test can be performed even if the inhibitor interacts with the transporter at two different sites, provided that either the slope of the Dixon plot is measured in a concentration range where the Dixon plot is linear, or that the linear coefficient $\Delta S_i$ is determined by performing a nonlinear least-squares fit of Eq. 35 to the Dixon plot data.

RESULTS

Mechanistic Basis for the Secondary Dixon Slope Plot

In mechanistic terms, the fundamental difference between the ping-pong and simultaneous models of exchange is that in the simultaneous model the two substrates ($Cl_o$ and $Cl_i$) bind simultaneously to the exchange protein, whereas in the ping-pong model only one of the two substrates is bound to the exchanger at any given time. Because in the ping-pong model the two substrates never bind to the transport system simultaneously (i.e., there is no $Cl_o EC_i$ form in the diagram in Fig. 1A), there are no terms in the equation for the Dixon slope (Eq. 14) that involve the product of $[Cl_o]$ times $[Cl_i]$. Because of this, for the ping-pong model the slope of the plot of the Dixon slope vs. $1/[Cl_i]$ (SDS) is independent of extracellular $Cl$. The slope of the plot of the Dixon slope vs. $1/[Cl_o]$ is also independent of $[Cl_i]$ for the ping-pong model. On the other hand, for the simultaneous model there is a form of the enzyme with both substrates bound (form $Cl_o EC_i$ in Fig. 1B). Therefore, the equation for the Dixon slope (Eq. 27) has a term involving the product of $[Cl_i]$ times $[Cl_o]$. It follows that, for the simultaneous model, SDS changes as a function of trans $Cl$ concentration. This characteristic behavior, which is the basis for the secondary Dixon slope test, holds for any dead-end reversible inhibitor in a tightly coupled exchange transport system.

Use of the Secondary Dixon Slope Test to Investigate the Kinetic Mechanism for HL60 Cell Anion Exchange

Inhibition of HL60 Cl/Cl exchange by 3’5’-diiodosalicylic acid. As shown in Fig. 3, 3’5’-diiodosalicylic acid (DIS) inhibits HL60 cell Cl/Cl exchange in a reversible manner. The flux for DIS-pretreated cells after washing (squares) was nearly identical to that for control cells (filled circles). Fig. 4 shows the concentration dependence of DIS inhibition of Cl/Cl exchange flux ($J$) in the range from 0 to 500 $\mu$M DIS. The data in the range from 0 to 100 $\mu$M are adequately fit by Eq. 1, which describes inhibition of anion exchange by the interaction of one DIS molecule per anion exchange protein (solid line in Fig. 4). In agreement with this, a Dixon plot of the data ($1/J$ vs. [DIS]) (shown in the inset in Fig. 4) is adequately fit by a straight line in the range from 0 to 100 $\mu$M DIS (the slope is $0.00173 \pm 0.00002$ mmol$^{-1}$/min-liter-$\mu$M$^{-1}$, the $y$ intercept is $0.033 \pm 0.001$ mmol$^{-1}$/min-liter, and the square of
the regression coefficient, $R^2$, is 0.9994). The linearity of the Dixon plot shown in Fig. 4, which is representative of results of 25 experiments performed at different intra- and extracellular chloride concentrations, indicates that DIS interacts with the HL60 Cl/Cl exchanger at a single site when the DIS concentration is < 100 μM. To test this statement more stringently, we fit the Dixon plot data in the inset in Fig. 4 using a quadratic equation (Eq. 35), which would model the data if DIS interacted with the exchanger at two sites (see Theory). We found that the coefficient of the quadratic term (DS₂) is not significantly different from zero ($P < 0.3$), while the coefficient for the linear term (DS₁) is different from zero ($P < 0.005$), confirming that DIS interacts with only one site in this concentration range (the best-fit parameters for Eq. 35 are $1/f_0$, $0.034 \pm 0.001$ mmol⁻¹/min⁻⁻¹; DS₁, $0.00160 \pm 0.00008$

\[ \text{mmol}^{-1}/\text{min} \cdot \text{liter} \cdot \mu\text{M}^{-1} \] and DS₂, $1.3 \times 10^{-6} \pm 7 \times 10^{-7}$ mmol⁻¹/min⁻⁻¹/\text{liter} \cdot \mu\text{M}^{-2}$). Because DIS is a somewhat hydrophobic molecule and probably crosses the membrane, the DIS binding site could be either on the extracellular or the cytoplasmic side of the transport protein, or maybe even at a site within the lipid bilayer.

At concentrations of DIS > 100 μM, inhibition by DIS is greater than predicted by Eq. 1. This is due to the fact that at high concentrations DIS causes a decrease in pH due at least in part to inhibition of Na/H exchange (Restrepo, D., and R. B. Snyder, unpublished data). This decrease in pH is of the order of 0.55 pH units after 5 min of exposure to 500 μM DIS. Such a change in pH would inhibit Cl/Cl exchange in the HL60 cell, which decreases as intracellular pH is decreased (Restrepo et al., 1988a).
FIGURE 4. Dependence of inhibition of CI/Cl exchange on DIS concentration. Cells were loaded with $^{36}$Cl and Cl$\textsubscript{i}$-dependent $^{36}$Cl efflux was measured after resuspension in medium without radioactive Cl$\textsuperscript{-}$ as detailed in Methods. Results shown are means ± SEM ($n = 3$). [Cl$\textsubscript{i}$] and [Cl$\textsubscript{o}$] for these experiments were 4 and 57 mM, respectively. The CI/Cl exchange efflux rate ($J$), calculated as described in Methods, decreases monotonically as DIS concentration [I] is increased. The data are adequately fit by Eq. 1 with $J_u = 28.6 ± 0.6$ mmol/min$^{-1}$-liter$^{-1}$ and $ID_{50} = 21.9 ± 1.5$ μM. A Dixon plot (1/$J$ vs. [DIS]) is shown in the inset. The solid line shown in the inset was calculated by rearranging Eq. 1 in the form $1/J = 1/J_u + [I]/(ID_{50}J_u)$.

To avoid this problem we always used concentrations of DIS < 100 μM. Under the conditions of our experiments we estimate that the drop in pH caused by DIS causes an underestimation by 25% of the $ID_{50}$ for DIS. However, this percent decrease in $ID_{50}$ is roughly the same at different intra- and extracellular Cl concentrations. Because of this, and because the uninhibited flux ($J_u$) is not affected, this pH correction does not affect the relative changes in $ID_{50}$ or Dixon slope ($1/J_uID_{50}$) as a function of Cl concentrations in the kinetic analysis that follows.

Evidence for noncompetitive inhibition. To determine whether DIS inhibits the HL60 cell anion exchanger by competing for binding with either of the substrates (Cl$\textsubscript{i}$ or Cl$\textsubscript{o}$), we measured the inhibition of the exchanger by DIS at various intra- and extracellular Cl concentrations. As shown in Fig. 5, the concentration of DIS

FIGURE 5 (opposite). The concentration of DIS that produces 50% inhibition of CI/Cl exchange ($ID_{50}$) was determined in experiments with various [Cl$\textsubscript{i}$] and [Cl$\textsubscript{o}$]. (A) [Cl$\textsubscript{o}$] concentration dependence. Solid line shown is the result of a linear regression with a slope of 0.07 ± 0.04 μM/mM and a y intercept of 20.9 ± 2.3 μM ($n = 16$). The mean [Cl$\textsubscript{i}$] for the cells used in these experiments was 59.6 ± 1 mM (mean ± SEM, $n = 16$). The slope is not significantly different from zero at $P < 0.05$. (B) [Cl$\textsubscript{i}$] dependence of $ID_{50}$ at [Cl$\textsubscript{o}$] = 11 mM. Solid line is straight line regression with a slope of 0.13 ± 0.04 μM/mM and a y intercept of 17.6 ± 0.7 μM ($n = 7$). Slope is different from zero with $P < 0.05$. (C) $ID_{50}$ vs. [Cl$\textsubscript{i}$] at a [Cl$\textsubscript{o}$] of 1 mM. Solid line is straight line regression with a slope of 0.22 ± 0.09 μM/mM and a y intercept of 14.7 ± 2.6 μM ($n = 8$). Slope is different from zero with $P < 0.05$. Dashed and dotted curves represent fits of Eq. 28 to data with kinetic parameters stated in the legend to Fig. 7.
Figure 5.
necessary to cause 50% inhibition (ID$_{50}$) increases slightly as either intra- or extracellular chloride is increased, with a fixed chloride concentration on the other side of the membrane. A much steeper linear increase in the ID$_{50}$ would have been expected for competition between DIS and either Cl$\text{in}$ or Cl$\text{out}$. For instance, at a fixed [Cl$\text{i}$], if DIS competes with extracellular chloride, the slope of the line describing the increase in ID$_{50}$ as a function of [Cl$\text{o}$] is equal to $y$-int/$K_{\text{app}}$ (where $y$-int is the $y$ intercept of the plot of ID$_{50}$ vs. [Cl$\text{o}$]) (Restrepo et al., 1989). For the data in Fig. 5A (at a [Cl$\text{i}$] of 59.5 ± 1 mM, $n = 16$), the $K_{\text{app}}$ is 4.5 mM (Restrepo et al., 1989) and the $y$-int is 20.9 ± 2.3 μM. The slope expected for competitive inhibition ($y$-int/$K_{\text{app}} = 4.6$ μM/mM) is two orders of magnitude larger than the observed slope ($0.07 ± 0.04$ μM/mM), indicating that DIS does not compete with [Cl$\text{o}$]. A similar calculation for Fig. 5, B and C yields slopes expected for competition between DIS and Cl$\text{i}$ ($y$-int/$K_{\text{app}}$) of 0.79 and 1.08 μM/mM (for [Cl$\text{o}$] of 11 and 1 mM, respectively), which are significantly larger than the measured slopes of 0.13 ± 0.04 and 0.22 ± 0.09 μM/mM, respectively. These results suggest that DIS is a noncompetitive inhibitor of anion exchange in the HL60 cell.

**Figure 6.** Slope of the Dixon plot (1/(f$_\text{i}$ID$_{50}$)) vs. 1/[Cl$\text{o}$] at a [Cl$\text{i}$] of 59.5 ± 1 mM ($n = 16$). Solid line is weighted straight line regression with a slope of 0.0025 ± 0.0004 mmol$^{-1}$/min-liter$^{-1}$μM$^{-1}$mM and an intercept of 5.2 ± 0.7 $10^{-5}$ mmol$^{-1}$/min-liter$^{-1}$μM$^{-1}$. Dashed and dotted curves represent fits of Eq. 27 to data with kinetic parameters stated in the legend to Fig. 7.

Because the slope of the Dixon plot is equal to 1/(f$_\text{i}$ID$_{50}$), this parameter conveys information about the relationship between the uninhibited flux ($f_\text{i}$) and the concentration of inhibitor that causes 50% inhibition (ID$_{50}$). As shown below, some kinetic models make fairly restrictive predictions about the nature of changes in the slope of the Dixon plot as a function of substrate concentrations.
Figure 7. Slope of the Dixon plot $1/(f_{ID_{50}})$ vs. $1/\left[\text{Cl}_i\right]$ (DS vs. $1/\left[\text{Cl}_i\right]$ plot) at either 1 mM (filled circles) or 11 mM (open circles) $\left[\text{Cl}_i\right]$. (A) DS vs. $1/\left[\text{Cl}_i\right]$ plot with efflux expressed in units of millimoles Cl per liter cell water$^{-1}$ per minute$^{-1}$. The solid lines shown are weighted straight line regressions with slope and intercept of 0.084 ± 0.008 mmol$^{-1}$/min-liter $\mu$M$^{-1}$mM and 0.0014 ± 0.0003 mmol$^{-1}$/min-liter $\mu$M$^{-1}$mM and 0.0008 ± 0.0001 mmol$^{-1}$/min-liter $\mu$M$^{-1}$mM for 1 mM $[\text{Cl}_i]$ and 0.033 ± 0.002 mmol$^{-1}$/min-liter $\mu$M$^{-1}$mM and 0.0008 ± 0.0001 mmol$^{-1}$/min-liter $\mu$M$^{-1}$mM for 11 mM $[\text{Cl}_i]$. Dashed and dotted lines represent fits of Eq. 27 to data with $K_f$ fixed. The kinetic parameters for these fits are $K_i = 11.4 \mu$M, $K_o = 9.7 \mu$M, $K_a = 21 \mu$M, and $K_t = 300 \mu$M (dashed line) or with $K_a = 7.3 \mu$M, $K_o = 30.7 \mu$M, $K_a = 32 \mu$M, and $K_t = 20 \mu$M (dotted line) (see Discussion). (B) DS vs. $1/\left[\text{Cl}_i\right]$ plot with efflux expressed in units of femtomoles Cl per cell$^{-1}$ per minute$^{-1}$. Solid lines are weighted straight line regressions with slope and intercept of 0.179 ± 0.017 fmol$^{-1}$/cell-min $\mu$M$^{-1}$mM and 0.0011 ± 0.0005 fmol$^{-1}$/cell-min $\mu$M$^{-1}$mM for 1 mM $[\text{Cl}_i]$ and 0.072 ± 0.004 fmol$^{-1}$/cell-min $\mu$M$^{-1}$mM and 0.00085 ± 0.00025 fmol$^{-1}$/cell-min $\mu$M$^{-1}$mM for 11 mM $[\text{Cl}_i]$. 

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If DIS were competing with Cl\textsubscript{o} as [Cl\textsubscript{o}] increased the uninhibited flux \((J_0)\) would saturate and approach \(J_{\text{max}}\), while the concentration of DIS that produced 50% inhibition \((I_{D,50})\) would increase linearly because of the competition with Cl\textsubscript{o}. Thus, as [Cl\textsubscript{o}] approaches infinity (and \(1/[Cl_o]\) approaches zero), the product of \(J_0\) times \(I_{D,50}\) increases linearly with [Cl\textsubscript{o}] and so the slope of the Dixon plot \([1/(J_0I_{D,50})]\) approaches zero. Fig. 6 shows that, contrary to what is expected for a competitive inhibitor, the y intercept of a straight line regression of a plot of the slope of the Dixon plot as a function of \(1/[Cl_o]\) is different from zero. In addition, Fig. 7 A shows that the y intercept of a straight line regression is different from zero for the dependence of the slope of the Dixon plot on \(1/[Cl_i]\). These data indicate that DIS does not compete with either Cl\textsubscript{i} or Cl\textsubscript{o}, lending further support to the conclusion that DIS is a noncompetitive inhibitor of CI/Cl exchange in the HL60 cell.

A plot of the slope of the Dixon plot as a function of \(1/[Cl_i]\) at two different \([Cl_i]\) rules out ping-pong kinetics. To carry out the secondary Dixon slope test we studied the dependence of the Dixon slope on \(1/[Cl_i]\) at two different extracellular Cl concentrations (1 and 11 mM). As stated earlier, although we chose to study DS vs. \(1/[Cl_i]\), the test could also have been carried out using the dependence of DS on \(1/[Cl_o]\) at different [Cl\textsubscript{o}]. As shown in Fig. 7 A, the slope of the plot of the Dixon slope as a function of \(1/[Cl_i]\) decreases by 2.5-fold when Cl\textsubscript{i} is increased from 1 to 11 mM. The change in slope is statistically significant with \(P < 0.0001\). As discussed above, this observation is inconsistent with the ping-pong kinetic scheme shown in Fig. 1 A.

As discussed in Methods, we have chosen to express efflux in units of millimoles Cl per liter cell water\(^{-1}\) per minute\(^{-1}\) because the values are more precise than when efflux is measured in units of femtomoles Cl per cell\(^{-1}\) per minute\(^{-1}\). The kinetics for Cl efflux should be equivalent for efflux values expressed in these two units, provided that cell volume is constant as a function of [Cl\textsubscript{i}]. Because Fig. 2 B shows a slight, but statistically significant, increase in cell volume as [Cl\textsubscript{i}] is increased, we expressed the data for the plot of the Dixon slope vs. \(1/[Cl_i]\) with fluxes measured both in units of millimoles Cl per liter cell water\(^{-1}\) per minute\(^{-1}\) (Fig. 7 A) and in units of femtomoles Cl per cell\(^{-1}\) per minute\(^{-1}\) (Fig. 7 B). As shown in Fig. 7, there is a substantial increase in the slope of the best fit lines when extracellular Cl is changed from 11 to 1 mM, regardless of the units used for Cl efflux (there is a 2.5-fold increase in slope regardless of the units used).

**DISCUSSION**

We have developed a new test, the secondary Dixon slope test, for differentiating between ping-pong and simultaneous kinetic schemes, and we have used this method to show that the Cl/Cl exchanger of the HL60 cell does not follow ping-pong kinetics. The secondary Dixon slope test takes advantage of the fact that for any dead-end reversible inhibitor of Cl/Cl exchange that inhibits by binding at a single site, the slope of the plot of the Dixon plot slope as a function of either \(1/[Cl_i]\) or \(1/[Cl_o]\) is independent of \textit{trans} substrate concentration for a ping-pong kinetic scheme. This is a surprisingly strong prediction if one considers that no assumptions need be made about the affinity of the inhibitor for the different forms of the transporter. Although the kinetics of inhibition by competitive inhibitors have been used to differentiate between these two kinetic schemes (Furuya et al., 1984;
Restrepo et al., 1991a), the new test can be used with any dead-end reversible inhibitor interacting with the transport system at a single site. In addition, as shown in Theory, the secondary Dixon slope test can also be used if the inhibitor binds to more than one site, provided either that the slope of the Dixon plot is measured in a concentration range where the Dixon plot is linear, or that the linear coefficient DS is determined by performing a nonlinear least-squares fit of a quadratic equation (Eq. 35) to the Dixon plot data.

Because it can be performed with noncompetitive inhibitors, the secondary Dixon slope test is particularly advantageous for cases where no competitive inhibitors are available. However, even in cases where other tests have already been done it is important to perform independent tests to differentiate between ping-pong and simultaneous kinetics. This is because for each particular test it is generally possible to find a variation of the simultaneous model that fits the data. Because these variations of the simultaneous model are such that specific kinetic parameters are set to fit a particular set of data, it is generally possible to disprove them by using an independent test for simultaneous kinetics.

As shown in Fig. 7, for the Cl/Cl exchanger of the HL60 cell, the slope of the plot of the slope of the Dixon plot vs. 1/[Cl] decreases by 2.5-fold when [Cl] is increased from 1 to 11 mM. As discussed above, this observation rules out the ping-pong kinetic scheme shown in Fig. 1A, in agreement with earlier studies of the substrate kinetics (Restrepo et al., 1989) and of the competitive inhibition of the exchanger by the disulfonic stilbene DIDS (Restrepo et al., 1991a).

Another possible interpretation for the data in Fig. 7 must be considered, however. In principle, a modifier site for Cl, which when occupied had little effect on anion flux itself, but affected DIS binding, could bring about the 2.5-fold increase in slope observed in Fig. 7 when [Cl] is changed from 11 to 1 mM. This is because, as shown by the equation below (which was derived from Eq. 14), the slope of the plot of the Dixon plot slope as a function of 1/[Cl] (SDS) is inversely proportional to the dissociation constant for binding of DIS to Ei (Kn; see Fig. 1A):

\[ SDS = \frac{1}{k(E_i)} \frac{K_n}{K_n} \] (42)

However, to obtain the 2.5-fold increase in SDS measured for the data in Fig. 7 for a change in [Cl] from 11 to 1 mM, the value of Kn would have to increase by 2.5-fold when [Cl] was increased from 1 to 11 mM. This follows from the fact that SDS is inversely proportional to Kn (Eq. 42). However, this would mean that at low [Cl], when the value of the ID50 approaches Kn, the ID50 would have to increase by 2.5-fold when [Cl] was increased from 1 to 11 mM, which contradicts the data in Fig. 5, B and C, where it is shown that ID50 at low [Cl] is roughly the same at 1 and 11 mM [Cl]. Therefore, the DIS inhibition data cannot be interpreted in terms of a ping-pong model with a Cl modifier site.

As discussed above, the data in Fig. 7 rule out ping-pong kinetics. However, do they agree with the simultaneous scheme in Fig. 1B? The simplest case would be if DIS were an ideal noncompetitive inhibitor whose binding affinity is not affected by binding of either of the substrates (Cl or Cl). Two observations rule out this simple inhibition scheme: (1) For ideal noncompetitive inhibition, the concentration of DIS that causes 50% inhibition (ID50) must be independent of [Cl], in disagreement with
the data in Fig. 5, B and C (slope of straight line regressions is different from zero at
P < 0.05). (2) Because for an ideal noncompetitive inhibitor the ID$_{50}$ is constant, for
such an inhibitor the plot of the slope of the Dixon plot (DS = 1/(J$_{u}$ID$_{90}$)) vs. 1/[Cl$_{i}$]
is equivalent to a Lineweaver-Burk plot (1/J$_{u}$ vs. 1/[Cl$_{i}$]). Therefore, for an ideal
noncompetitive inhibitor, as expected for a Lineweaver-Burk plot (see Eq. 31 in
Theory), the x intercept of the Dixon slope vs. the 1/[Cl$_{i}$] plot is equal to minus the
reciprocal of the apparent affinity for intracellular chloride (−1/K$_{iapp}$). However,
contrary to this prediction, the x intercepts for the least-squares best fit (solid) lines
shown in Fig. 7 are −0.017 ± 0.005 and −0.025 ± 0.005 mM$^{-1}$ (for 1 and 11 mM
[Cl$_{o}$], respectively), which are substantially smaller in absolute magnitude than the
respective values for −1/K$_{iapp}$ of −0.073 and −0.045 mM$^{-1}$ (from the data in
Restrepo et al., 1989). Because of these two facts, DIS cannot act as an ideal
noncompetitive inhibitor in a simultaneous model of exchange.

To find out if the general model of noncompetitive inhibition for a simultaneous
exchanger (Fig. 1 B) could explain the observed behavior, we used the simplex
algorithm (Caceci and Cacheris, 1984; see Methods) to determine the best fit of the
data for the dependence of the Dixon plot slope and the ID$_{50}$ on [Cl$_{i}$] and [Cl$_{o}$] (Figs.
5–7) to Eqs. 27 and 28. [Cl$_{o}$] and [Cl$_{i}$] were independent variables and the
equilibrium binding constants for DIS, K$_{p}$, K$_{m}$, K$_{g}$, and K$_{f}$ were the best-fit parameters.
Previously determined values (Restrepo et al., 1989) were used for the constants
describing substrate binding (K$_{o}$ = 2.2 mM, K$_{i}$ = 11 mM, and α = 2.55) and for the
maximum velocity ($k_{p}[E_{i}] = 104$ mmol Cl/liter of cell water$^{-1}$min$^{-1}$). An adequate fit
of Eqs. 27 and 28 to the data was obtained with K$_{j}$ = 11.4 μM, K$_{m}$ = 9.7 μM, K$_{g}$ = 21
μM, and K$_{f}$ = 300 μM (dashed lines in Figs. 5, 6, and 7A), demonstrating that the
simultaneous model in Fig. 1 B can account for the observations.

The fit of Eqs. 27 and 28 to the data in Figs. 5, 6, and 7A yields a value for the
equilibrium binding constant for DIS (K$_{f}$) to the fully loaded form of the exchanger
(Cl$_{o}$ECl$_{i}$) of 300 μM, which is substantially higher than the values for the other DIS
binding constants (K$_{j}$ = 11.4 μM, K$_{m}$ = 9.7 μM, and K$_{g}$ = 21 μM). Forced fits with
higher values of K$_{f}$ (> 300 μM) yielded curves that were essentially the same as those
for the fit with 300 μM (dashed lines in Figs. 5, 6, and 7A). However, forced fits with
lower values of K$_{f}$ greatly underestimated the slope of the dependence of the Dixon
plot slope on 1/[Cl$_{i}$] at 11 mM [Cl$_{o}$] (Fig. 7A; cf. open circles with the lower dotted
straight line with K$_{f}$ = 20 μM). Therefore, it appears that the dissociation constant
(K$_{f}$) for binding of DIS to the fully loaded form of the transport molecule, Cl$_{o}$ECl$_{i}$, is
substantially larger than the dissociation constants for binding of DIS to the other
forms.

The linearity of Dixon plots at different [Cl$_{o}$] and [Cl$_{i}$] implies that DIS binds at a
single site, which could be facing the extracellular side or the cytoplasm, but not both
sides. However, the fact that the constant for binding of DIS to the fully loaded form
of the transporter K$_{f}$ is larger than the other DIS binding constants means that the
affinity for DIS binding decreases substantially upon binding of both Cl$_{i}$ and Cl$_{o}$. Since
DIS binds at a single site this must imply that the interaction of DIS with its site is
modified by binding of substrate at either side of the membrane (Cl$_{o}$ or Cl$_{i}$) and thus
implies that there is a transmembrane effect on DIS binding. In other words, DIS
binding affinity is decreased allosterically by a conformational change that takes place
only when both substrates (Cl\textsubscript{o} and Cl\textsubscript{i}) are bound. This observation is interesting, because this change in DIS affinity may reflect a conformational change that occurs upon formation of the Cl\textsubscript{o}ECl\textsubscript{i} complex and that may be related to the decrease in transition state free energy which allows this form of the protein to undergo the ion-exchanging conformational change. Further experiments are necessary to obtain a precise estimate of $K_f$.

We acknowledge the technical assistance of David J. Kozody.

This work was supported by NIH grants GM-4295, HL-18208, and DK-27495.

Original version received 26 June 1991 and accepted version received 20 June 1992.

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