The Noncompetitive Inhibitor WW781 Senses Changes in Erythrocyte Anion Exchanger (AE1) Transport Site Conformation and Substrate Binding

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abstract WW781 binds reversibly to red blood cell AE1 and inhibits anion exchange by a two-step mechanism, in which an initial complex (complex 1) is rapidly formed, and then there is a slower equilibration to form a second complex (complex 2) with a lower free energy. According to the ping-pong kinetic model, AE1 can exist in forms with the anion transport site facing either inward or outward, and the transition between these forms is greatly facilitated by binding of a transportable substrate such as Cl^-. Both the rapid initial binding of WW781 and the formation of complex 2 are strongly affected by the conformation of AE1, such that the forms with the transport site facing outward have higher affinity than those with the transport site facing inward. In addition, binding of Cl^- seems to raise the free energy of complex 2 relative to complex 1, thereby reducing the equilibrium binding affinity, but Cl^- does not compete directly with WW781. The WW781 binding site, therefore, reveals a part of the AE1 structure that is sensitive to Cl^- binding and to transport site orientation, in addition to the disulfonic stilbene binding site. The relationship of the inhibitory potency of WW781 under different conditions to the affinities for the different forms of AE1 provides information on the possible asymmetric distributions of unloaded and Cl^--loaded transport sites that are consistent with the ping-pong model, and supports the conclusion from flux and nuclear magnetic resonance data that both the unloaded and Cl^--loaded sites are very asymmetrically distributed, with far more sites facing the cytoplasm than the outside medium. This asymmetry, together with the ability of WW781 to recruit toward the forms with outward-facing sites, implies that WW781 may be useful for changing the conformation of AE1 in studies of structure–function relationships.

key words: red blood cell • oxonol • band 3 • ping-pong mechanism • obligatory exchange

INTRODUCTION

The AE1 (band 3) protein in human red blood cells catalyzes a very tightly coupled one-for-one exchange of Cl^- for HCO_3^- that functions together with the enzyme carbonic anhydrase to increase the carrying capacity of the blood for CO_2 (Knauf, 1989; Jennings, 1992b; Timmer and Gunn, 1999). Data from a wide variety of experiments, and particularly from demonstrations that each AE1 molecule can transport one and only one anion outward across the membrane under conditions where simultaneous transport of an anion inward is not possible (half-turnover experiments; Jennings, 1982; Jennings et al., 1998), strongly indicate that AE1 works by a ping-pong mechanism, as initially proposed by Gunn and Fröhlich (1979). In this model, the AE1 protein can exist in two different forms, one (E_i)[1] in which the transport site faces the inside (that is, the cyto-

plasm) and another (E_o) in which the site faces the external medium. To explain the tightly coupled one-for-one exchange of anions exhibited by this system, it is proposed that the conformational change from E_i to E_o or vice versa can only take place at a significant rate when a suitable substrate anion, such as Cl^- or bicarbonate, is bound to the transport site to create the corresponding ECl_i or ECl_o forms (see Fig. 4).

In attempting to relate this kinetic mechanism to structural changes in the AE1 protein, two questions are central. First, what changes in protein conformation take place during the transporting conformational change from ECl_i to ECl_o, or vice-versa, and second, what is there about binding of certain substrates that lowers the transition state free energy so as to make the transporting conformational change take place at an

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[1]*Abbreviations used in this paper:* diBA(5)C_4, bis-(1,3-dibutylbarbituric acid)-pentamethine oxonol; DNDS, 4,4′-dinitro-stilbene-2,2′-disulfonate; E_i, inside form of the AE1 protein; E_o, outside form of the AE1 protein; NMR, nuclear magnetic resonance; WW781, [3-methyl-1-*p*-sulfophenyl-5-pyrazolone-(4)]-[1,3-dibutylbarbituric acid]-pentamethine oxonol.

appreciable rate? This latter effect is apparent even for slowly transported substrates, such as iodide, because the rate of reorientation of the EI_i or EI_o complex is still many orders of magnitude faster than the rate of the E_i to E_o transition, which is at least $10,000\times$ slower than the rate of Cl^- exchange (Knauf et al., 1977; Knauf, 1989). Although the AE1 protein from various species has been cloned and sequenced, and although there is considerable structural information both from electron diffraction (Wang et al., 1994) and from chemical probe and mutagenesis experiments (Jennings, 1992a, 1995; Müller-Berger et al., 1995; Chernova et al., 1997; Fujinaga et al., 1999; Tang et al., 1999), there is still no information on these important points.

Oxonol dyes, such as WW781 {[3-methyl-1-p-sulfophenyl-5-pyrazolone-(4)]-[1,3-dibutylbarbituric acid]-pentamethine oxonol}, have proven to be very useful chemical probes for investigating the Cl^- exchange system (Raha et al., 1993). One oxonol, diBA(5)C_4 [bis-(1,3-dibutylbarbituric acid)-pentamethine oxonol], is the most potent known inhibitor of the transport system (Knauf et al., 1995). Although it competes with the disulfonic stilbene, 4,4′-dinitro-stilbene-2,2′-disulfonate (DNDS), whose binding is strongly affected by Cl^- (Fröhlich, 1982), Cl^- does not interfere with the binding of diBA(5)C_4. The high affinity of certain oxonols for AE1 suggests that these compounds have very specific interactions with some part of the AE1 protein. If the regions of AE1 with which the oxonols interact are involved in the transporting conformational change, it is reasonable to suppose that oxonols might act as reporters of such changes.

WW781, because of its sulfonic acid moiety, should not penetrate into the interior of red blood cells at an appreciable rate (George et al., 1988). Thus, it is particularly useful as a probe, because its actions can be assigned to external-facing sites. WW781 binds to AE1 by a two-step mechanism, in which an initial complex (complex 1) is rapidly formed, followed by much slower conversion to a second complex (complex 2) in which the WW781 is more tightly bound (Raha et al., 1993). Although this adds complexity to the kinetics of WW781 interaction with AE1, the existence of these two binding steps provides additional opportunities to see whether any of the various aspects of WW781 binding is affected by the AE1 conformation.

In the present work, we have used external WW781 binding, measured by the inhibitory effect on Cl^- exchange at 0°C, to probe possible changes in AE1 conformation caused by substrate binding and by the reorientation of the transport site from the inward- to the outward-facing form. Brief reports of some of these experiments have been presented in abstract or summary form (Knauf et al., 1990, 1992; Mendoza et al., 1990).

Nomenclature

Because of the complicated nature of the ping-pong model and the need to specify the various forms of AE1 with WW781 bound (see Figs. 4 and 5), we will use the following standardized nomenclature.

Forms of AE1 and other molecules, such as WW781 and Cl^-, are indicated in roman type, while dissociation constants and rate constants are in italic.

Dissociation constants for binding equilibria are indicated by $K_{a,b}$, where a is the molecule bound (e.g., Cl^-) and b is the form of AE1 to which it is bound; e.g., E_o. For simplicity, in the subscripts, anions are shown without a $-$ superscript, and WW781 is designated as W. For example, the dissociation constant for binding of Cl^- to E_o is designated $K_{Cl,Eo}$.

Rate constants for conversion of one form of AE1 to another are designated $k_{a,b}$, where a is the initial form of AE1 and b is the form resulting from the conversion.

The total of both inward- and outward-facing forms (e.g., E_o and E_i) is designated without a subscript; e.g., E. Dissociation constants for binding to both forms are indicated in a similar manner (e.g., $K_{Cl,E}$). The total of all forms of the AE1 protein is simply designated AE1.

The complex (complex 1) formed by the initial rapid binding of WW781 to an AE1 conformation is designated by • between W and the form of AE1; e.g., W•E_o. The second complex, which is formed more slowly, complex 2, is designated by a -; e.g., W-E_o. The total of the two complexes (e.g., W•E_o + W-E_o) is indicated with no intervening symbol; e.g., WE_o. Dissociation constants for WW781 binding after equilibration with both complex 1 and complex 2 are indicated by an eqW subscript; e.g., $K_{eqW,Eo}$. The dissociation constant for WW781 binding to form complex 1 is designated with a 1W subscript; e.g., $K_{1W,Eo}$. For general cases, where a dissociation constant can be taken to mean either that for the formation of complex 1 or that at equilibrium, a plain W subscript is used; e.g., $K_{W,Eo}$. The constant describing the equilibrium ratio between complex 2 and complex 1 for a particular form of AE1 (e.g., E_o) is defined as $K_{2/1W,Eo}$. Apparent dissociation constants for binding to all forms of AE1 are designated without a second subscript; e.g., K_{1W} or K_{eqW}.

Materials

All chemicals were reagent grade, except for WW781, which was either supplied by Dr. J.C. Freedman (SUNY Health Science Center at Syracuse, Syracuse, NY), or else purchased from Molecular Probes. WW781 stocks were dissolved in ethanol. The total ethanol concentration in all flux and pretreatment media was maintained at 1% vol/vol.

Cell Preparation

Blood was obtained, with heparin as anticoagulant, from apparently healthy volunteers, with informed consent. The blood was washed three times in 150 KH (150 mM KCl, 20 mM HEPES, 24 mM sucrose, pH 6.9 at room temperature with KOH), and the white cell layer was removed during the washes. Cells were made up to 50% hematocrit.

Treatment with WW781 and Flux Measurement

For some experiments, cells were pretreated at a hematocrit of 0.25% with WW781 in 10-ml syringes with 400-μl Eppendorf microfuge tubes attached, as described previously (Raha et al.,

1993). Cells were then loaded with ^{36}Cl by incubation in 2–10 μCi/ml ^{36}Cl (ICN Chemical and Radioisotope Division) for 10 min at 0°C. For other experiments, cells were loaded with isotope without pretreatment. ^{36}Cl exchange was begun by resuspending the packed, isotope-loaded cells in 30 ml of ice-cold medium containing various concentrations of WW781 that, for the pretreated cells, were the same as those used for pretreatment. Timed samples were taken by a rapid filtration method, and the rate constant for Cl$^-$ exchange, k, was determined from the rate of appearance of ^{36}Cl in the medium with time (Knauf and Brahm, 1989), by fitting a straight line to the plot of $\ln[P_\infty - P_t]$ versus time, where P_t is the cpm in an aliquot of cell-free medium at a particular time, and P_∞ represents the cpm in a similar aliquot after complete isotope equilibration. Intracellular Cl$^-$ content was determined as previously described (Knauf and Mann, 1986), and the flux was calculated as the product of the rate constant (s^{-1}) times the intracellular Cl$^-$ content (mmol/kg dry solids).

When extracellular [Cl$^-$] was lower than intracellular [Cl$^-$], Cl$^-$ was replaced by sucrose or a mixture of sucrose and K$_3$citrate (with 200 mM sucrose and 25 mM citrate taken as equivalent to 150 mM KCl), as indicated. For experiments in which the external [Cl$^-$] was much lower than the [Cl$^-$] in the cells, appropriate corrections for the ratio of the amount of Cl$^-$ in the internal and external compartments were made, as described in detail elsewhere (Knauf et al., 1989). For some experiments, cells were loaded with various [Cl$^-$] by using nystatin, as described (Raha et al., 1993). The final washes and fluxes for nystatin-treated cells were in solutions analogous to 150 KH, except with the [Cl$^-$] indicated.

Determination of Parameters

The strategies for determining dissociation constants for various conformations of AE1 were as previously described and used in our laboratory (Knauf and Brahm, 1989; Knauf et al., 1989). The concentrations of WW781 required to half-inhibit Cl$^-$ exchange either initially (K_{1W}) or after completion of the binding reactions (K_{eqW}) were determined from fits of the flux (J) versus [WW781] data to the equation for single-site inhibition, which for K_{eqW} is:

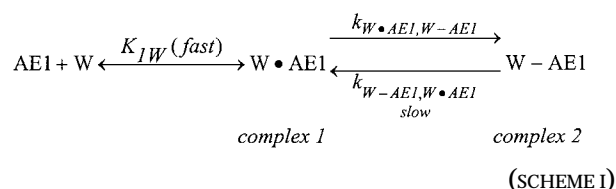$$J = J_u/(1 + [\text{WW781}]/K_{eqW}), \tag{1}$$

where J_u is the flux with no inhibitor present. Nonlinear least-squares fits were done either with Enzfitter (Elsevier Biosoft) or with Origin (Microcal) software.

A problem, particularly when WW781 is allowed to equilibrate with cells, is that the inhibitory potency is so large that the concentration required for half inhibition is comparable to the concentration of AE1 molecules in the cell suspension, even at the very low hematocrits used. As noted previously (Raha et al., 1993), depletion of the WW781 in the medium because of binding to AE1 can be minimized by keeping the concentration of WW781 high and the hematocrit low. There is, however, a region of WW781 concentrations near the K_{eqW} at which it is difficult or impossible to control the free concentration of WW781 in the medium. Thus, we were forced to take data at higher concentrations, where inhibition is very large, as well as with no inhibitor present (control) (see Fig. 11 for example). To avoid artifacts in fitting such data to Eq. 1, because of the gap in the data at low [WW781], we made several measurements of the control flux, J_u, averaged these, made this a fixed parameter in Eq. 1, and then fitted the equation with only one variable parameter, K_{eqW} (or K_{1W} for initial inhibition conditions), with each point weighted equally. For consistency, this was done for all data presented here, even for conditions where experiments could be done at concentrations near that which causes half inhibition.

Determination of Dissociation Constant for Complex 1 (K_{1W})

We have previously shown that WW781 (W) binds to AE1 by a two-step mechanism (Raha et al., 1993), which can be described by Scheme I,

$$\text{AE1} + \text{W} \xleftrightarrow{K_{1W}\,(fast)} \text{W} \cdot \text{AE1} \underset{\substack{k_{W-AE1,W\cdot AE1}\\ slow}}{\overset{k_{W\cdot AE1,W-AE1}}{\rightleftarrows}} \text{W} - \text{AE1}$$

$$\quad complex\ 1 \qquad\qquad complex\ 2$$

(SCHEME I)

where K_{1W} is the dissociation constant for formation of the initial, rapidly formed complex (complex 1), $k_{W\cdot AE1,W-AE1}$ is the forward rate constant for formation of the second complex, and $k_{W-AE1,W\cdot AE1}$ is the backward rate constant for conversion of complex 2 back to complex 1. We have previously shown that, for cells with 150 mM Cl$^-$ inside and outside, $k_{W\cdot AE1,W-AE1}$ has a value of ~1.1 min^{-1} and $k_{W-AE1,W\cdot AE1}$ is 0.15 min^{-1}. These values give a half-time for formation of complex 2, at saturating concentrations of WW781, of $0.693/(k_{W\cdot AE1,W-AE1} + k_{W-AE1,W\cdot AE1})$, or 33 s, and the rate should be much slower at WW781 concentrations below K_{1W}. To measure K_{1W}, therefore, we exposed cells to WW781 at 0°C and measured the ^{36}Cl efflux for short times. Under these conditions, there should be little of complex 2 formed, and so the extent of inhibition should largely reflect the amount of complex 1, and hence should depend on the WW781 concentration and the value of K_{1W}. We have previously observed (Raha et al., 1993) that as complex 2 is formed the rate constant for ^{36}Cl efflux gradually decreases, and this is reflected in a decrease in slope of the logarithmic plot of appearance of ^{36}Cl in the medium versus time. We have previously shown (Raha et al., 1993) that, for cells with 5 mM Cl$^-$ inside and outside exposed to WW781 at the start of the flux measurement, if the flux samples are taken at short times, then there is no substantial deviation of the logarithmic plot from a straight line, indicating that K_{1W} can be measured from inhibition under these conditions without much effect from the formation of complex 2. Fig. 1 shows that this is also true for cells with 150 mM Cl$^-$ inside and outside. The plots were very linear, with correlation coefficients (r^2) usually >0.98. Note, however, that the data at the highest WW781 concentration, shown in Fig. 1, ●, does show some systematic deviation toward a concave-downward curve, as expected if some of complex 2 is being formed. The data thus indicate that measurements at short times can give a very good approximation for K_{1W}, but that the apparent values of K_{1W} obtained by this method may be underestimated because of the un-
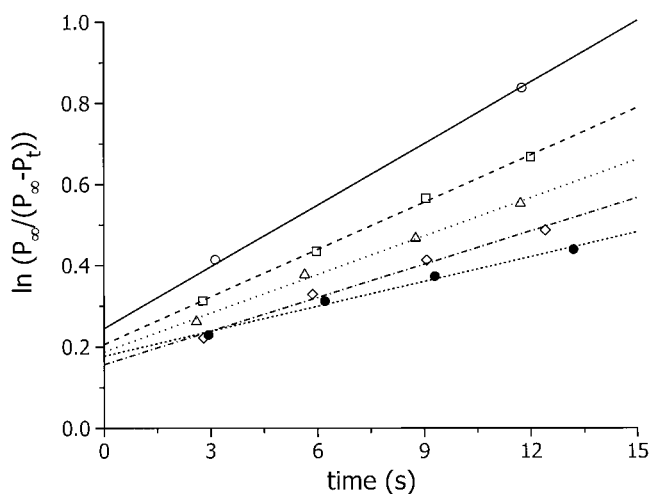
Figure 1. Logarithmic plot of ^{36}Cl efflux from cells exposed to various concentrations of WW781 at time zero. Cells were loaded with ^{36}Cl and efflux was measured in 150 mM Cl^- medium, as described in materials and methods. The slope of the line gives the rate constant for ^{36}Cl exchange, k. $\bigcirc$, control cells (no WW781, some points at later times not shown), $k = 0.0507$ s^{-1}, $r^2 = 0.9997$; $\square$, 0.25 μM WW781, $k = 0.0389$ s^{-1}, $r^2 = 0.9988$; $\triangle$, 0.5 μM WW781, $k = 0.0316$ s^{-1}, $r^2 = 0.9954$; $\diamond$, 1 μM WW781, $k = 0.0274$ s^{-1}, $r^2 = 0.9887$; $\bullet$, 1.5 μM WW781, $k = 0.0203$ s^{-1}, $r^2 = 0.9903$.
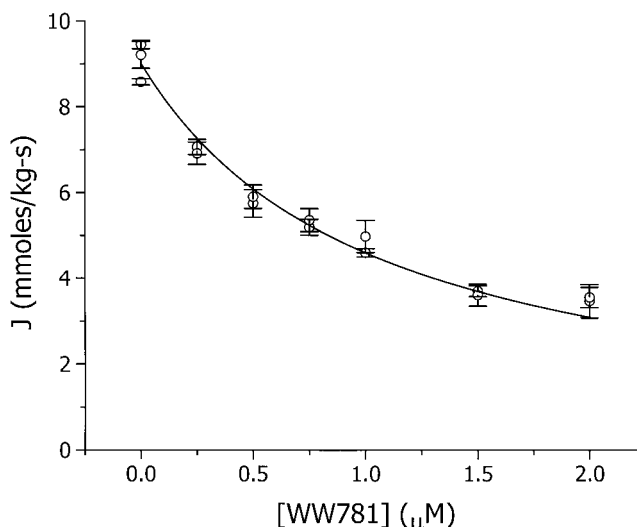

Figure 2. Effect of short exposure to WW781 on chloride exchange flux. Fluxes were measured for times up to 15 s, as described in materials and methods and as shown in Fig. 1. Bars indicate SEM of each measurement of the rate constant, which was converted to flux by multiplying by the Cl^- content of the cells in mmol/kg dry solids. The line represents the least squares best fit to the equation for single-site inhibition, $J = J_u/(1 + [WW781]/K_{1W})$, with $J_u = 9.01$ mmol/kg-s (the mean of the fluxes without WW781) and $K_{1W} = 1.04 \pm 0.03$ μM.

avoidable formation of complex 2 during the time required for sufficient radioactivity to appear in the medium for an accurate measurement of the flux.

Fig. 2 shows the unidirectional Cl^- exchange flux, J, as a function of WW781 concentration under these conditions for cells suspended in 150 mM Cl^- medium. Note that the decrease in flux as a function of WW781 concentration fits very well to the equation for single-site inhibition, as indicated by the least squares best fit line drawn through the data. The half-inhibition constant from such data should be a good approximation of K_{1W} and certainly gives an upper limit for K_{1W}, because any formation of complex 2 during the flux measurement will cause additional inhibition of transport, corresponding to a higher inhibitory potency and hence a lower value of K_{1W}.

Fig. 3 shows K_{1W} values calculated from several similar experiments in cells that had been equilibrated with media with different Cl^- concentrations by the nystatin technique (except for those in 150 mM Cl^-). If WW781 inhibits anion exchange by competing with Cl^- for the transport (substrate) site, then K_{1W} should increase with increasing $[Cl^-]$. In contrast to this prediction, if anything there is a decrease in K_{1W} with increasing $[Cl^-]$, although the slope of a best-fit straight line (solid line) relating K_{1W} to $[Cl^-]$ is not significantly different from zero ($P = 0.14$). The mean values of K_{1W} at various $[Cl^-]$ are shown in Table I, together with SEM and number of experiments. In no case are the K_{1W} values for different $[Cl^-]$ significantly different ($P \geq 0.26$).

Effect of AE1 Conformation on K_{1W}

Because WW781 is not a competitive inhibitor, it may in principle bind to any of the different forms of the anion exchange protein, with the transport site facing inward or outward and unloaded or loaded with Cl^- (Fig. 4), and each of these forms may bind WW781 by a two-step process, as shown in Scheme I and Fig. 4. If these transport-related changes in AE1 conformation affect the binding of WW781, the affinities of WW781 for the various conformations may be different.

To see whether this is the case for the initial binding step (Fig. 5 A), we measured the effect of short exposure to WW781 on the Cl^- exchange flux and used Dixon plot intersection techniques (Knauf and Brahm, 1989; Knauf et al., 1989) to determine the affinity of WW781 for certain forms of AE1. As previously shown (Fröhlich and Gunn, 1986; Knauf and Brahm, 1989), the dissociation constant for the binding of an inhibitor such as WW781 to E_o is given by the negative of the intersection point of Dixon plot ($1/J$ versus [WW781]) lines for cells with the same $[Cl_i]$ and different external Cl^- concentrations, $[Cl_o]$. If measurements are made at early times, the intersection point will largely reflect the dissociation constant for WW781 binding to E_o to form the first complex, complex 1, which is designated $K_{1W,Eo}$ (Fig. 5 A).

Fig. 6 shows data for a typical experiment. The line for fluxes measured in 150 mM Cl^- medium ($\triangle$) intersects

$[Cl_{i=o}]$	$K_{1W} \pm$ SEM (n)	$K_{eqW} \pm$ SEM (n)	K_{1W}/K_{eqW}	$P*$
mM	μM	μM		
5	0.83 ± 0.14 (3)	0.053 ± 0.004 (2)	15.5	0.031
20	0.90 ± 0.21			
150	0.59 ± 0.05 (16)	0.066 ± 0.012 (4)	9.0	<0.001
600	0.54 ± 0.10 (3)	0.143 ± 0.029 (3)	3.8	0.060
$[Cl_i] = 150$, $[Cl_o] = 2$	0.14 ± 0.01 (4)	0.0055 ± 0.0003 (8)	25.2	0.0009
$P‡$ vs. $[Cl_{i=o}] = 150$	<0.0001	0.014		

*P from unpaired t test for K_{1W} versus K_{eqW} values. ‡P for unpaired t test for 150 mM $[Cl_i]$, 2 mM $[Cl_o]$ values versus 150 mM $[Cl_i] = [Cl_o]$.

that for fluxes in 4 mM Cl⁻ medium (○) at a point whose x value is equal to $-K_{1W,Eo}$, the dissociation constant for binding of WW781 to E_o. It can be seen that this value is much smaller than the concentration required for half-inhibition of the Cl⁻ flux in 150 mM Cl⁻ medium under similar conditions (K_{1W}), given by the negative of the x intercept for the 150 mM Cl⁻ line, which lies far to the left of the y axis in Fig. 6. As previously shown (Knauf et al., 1992), the K_{1W} in 150 mM Cl⁻ reflects an average of the dissociation constants for binding of WW781 by all of the forms of AE1, weighted according
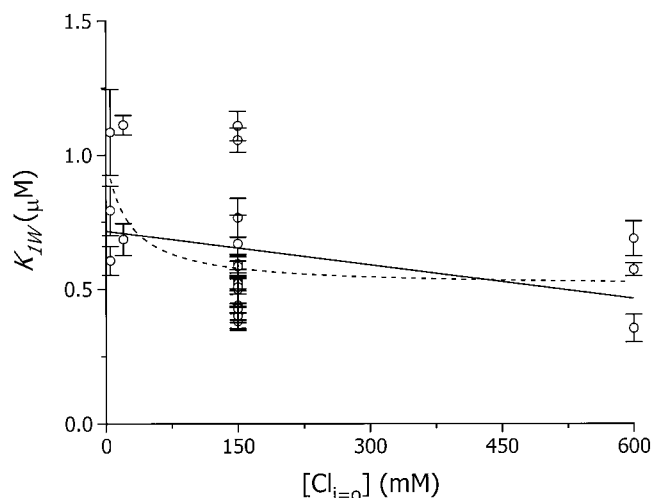

Figure 3.   Dependence of K_{1W} on $[Cl_{i=o}]$. Cells in media other than 150 mM Cl⁻ medium were treated with nystatin to equalize the concentrations of Cl⁻ inside and outside the cells. Cells were exposed to WW781 at time zero, and unidirectional Cl⁻ exchange fluxes were measured as described in materials and methods and Fig. 2. For most experiments, the last flux sample used to calculate the rate constant was taken before 30 s and in no experiment was the last sample later than 45 s. Data were fit to the equation for single-site inhibition (Eq. 1) by the method of least squares to obtain K_{1W} values at each Cl⁻ concentration, as described in materials and methods and as shown in Fig. 2. Each point shows the result of a single experiment, with SEM indicated by the bars. The solid line shows a linear fit through the data, which has a y intercept of 0.72 ± 0.07 μM and a slope that is not significantly different from zero ($P = 0.14$). The dashed line shows a fit to Eq. A6, with $K_{Cl,E} = 50$ mM (fixed value, taken from Gasbjerg and Brahm, 1991), $K_{1W,E} = 0.99 \pm 0.05$ μM, and $K_{1W,ECl} = 0.51 \pm 0.01$ μM.

to their prevalence in 150 mM Cl⁻ medium. The fact that this value is much larger than the value of $K_{1W,Eo}$ indicates that the affinity of WW781 for E_o is greater than for the mixture of the other forms of AE1 present in 150 mM Cl⁻ medium. This conclusion is further reinforced by the significant difference ($P = 0.003$) between the K_{1W} value in 150 mM Cl⁻ (Table I) and the average $K_{1W,Eo}$ value (Table II), as well as by the fact that the K_{1W} value for 2 mM $[Cl_o]$ medium (Table I), in which the relative amount of E_o is increased, is significantly smaller than the K_{1W} value for 150 mM $[Cl_o]$ ($P < 0.0001$).

Because of the rapid equilibration of ECl_i and ECl_o, and because the ratio of ECl_o to ECl_i is fixed by the ratio of $k_{ECli,EClo}$ to $k_{EClo,ECli}$ (see Figs. 4 and 5 A), it is not possible to determine the value of $K_{1W,EClo}$ for WW781 binding to the ECl_o form by any flux technique. This can be done, however, for the E_o form that is associated with a slowly transported anion, such as iodide. As shown in Fig. 6, the dissociation constant for EI_o, designated $K_{1W,EIo}$, is given by the negative of the x value of the intersection point for the data with 4 mM Cl⁻ media either without (○) or with (●) iodide (6 mM) present (Knauf and Brahm, 1989). This value is only about twice as large as $K_{1W,Eo}$, suggesting that binding of an external anion to E_o, even a large anion such as I⁻, has a relatively small effect on the binding affinity for WW781.

Similar techniques can be used to measure $K_{1W,Ei}$ and $K_{1W,EIi}$, as shown in Fig. 7, except that in this case the external Cl⁻ concentration is maintained constant at 20 mM. Note that the dissociation constant for initial WW781 binding to E_i, $K_{1W,Ei}$, given by the negative of the x coordinate of the intersection point of the line for 20 mM $[Cl_i]$ (○) and that for 50 mM $[Cl_i]$ (△), is much larger than $K_{1W,Eo}$ (compare with Fig. 6; note difference in abscissa scale). The corresponding value for $K_{1W,EIi}$, given by the intersection point of the lines for cells with 20 mM $[Cl_i]$ and either no (○) or 30 mM (●) $[I_i]$, is similar to $K_{1W,Ei}$, suggesting little effect of binding of even a large anion on the WW781 affinity for E_i.

Table II shows the average K_{1W} values for various forms of AE1 obtained from a number of experiments similar to those shown in Figs. 6 and 7. The only statistically significant difference in the K_{1W} values is that $K_{1W,Ei}$
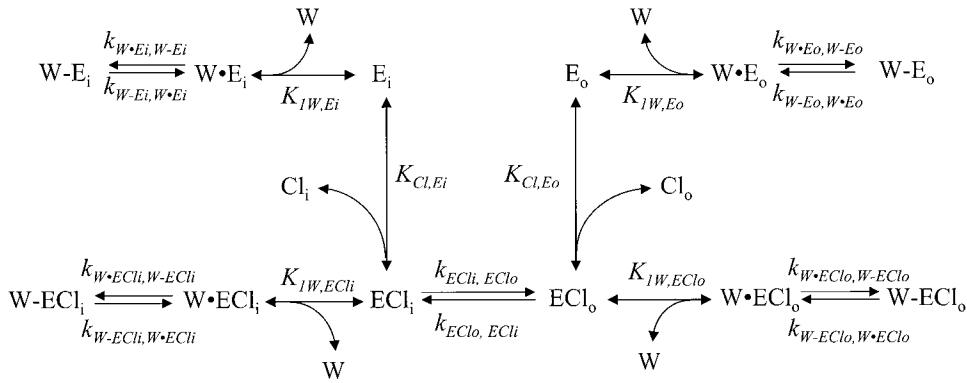
Figure 4. Because WW781 is not a competitive inhibitor, it can in theory bind to all of the various forms of AE1, but the initial binding affinities, indicated by the dissociation constants K_{1W}, may be different. For each form of AE1, the binding may involve two steps, as described for E_o in Scheme II, and the rate constants for conversion from the initial binding form (complex 1) to the other (complex 2) may differ for the different AE1 conformations. Rate constants and dissociation constants are defined as described in materials and methods.

A. At very short times after adding WW781:
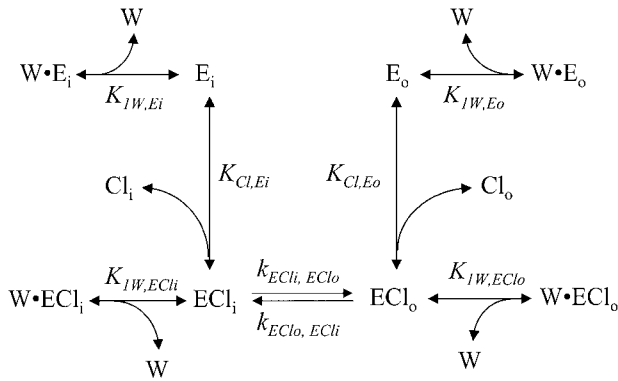
B. At equilibrium:
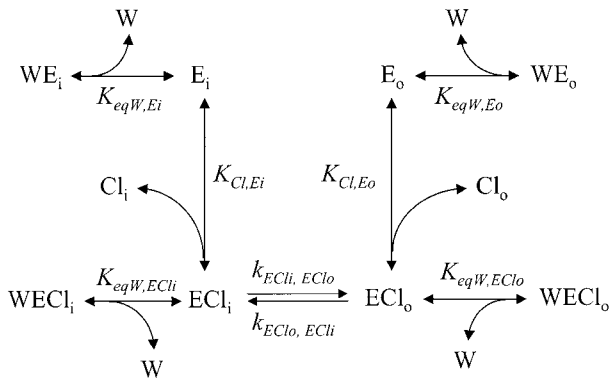
Figure 5. (A) If exposure to WW781 is kept short to minimize formation of complex 2, the dissociation constants will reflect the value of K_{1W} for each form; e.g., $K_{1W,Eo}$ for E_o. (B) If the binding of WW781 is allowed to proceed to equilibrium, the measured dissociation constants will reflect the equilibrium binding to both complexes; e.g., $K_{eqW,Eo}$ for E_o.
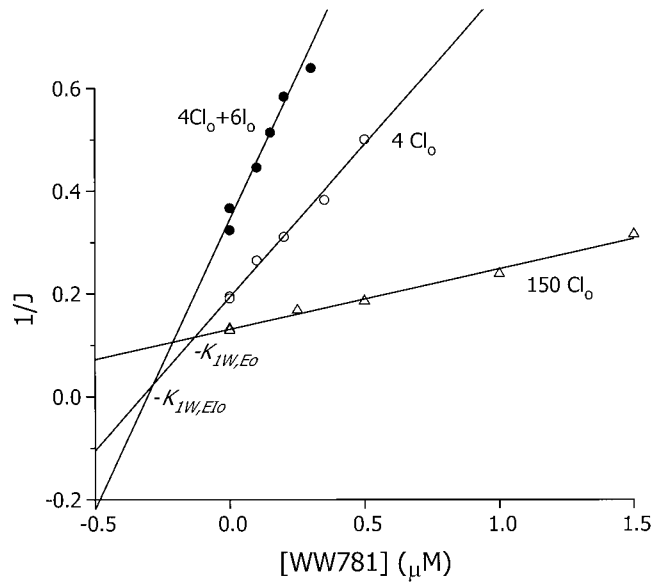

Figure 6. Determination of Dixon plot intersections for cells with the same $[Cl_i]$, $\sim$150 mM, in media with different Cl^- and I^- concentrations. Fluxes were measured in various media after short exposures to WW781 as described in materials and methods and Fig. 2. Except for the solid circle at the highest WW781 concentration, which was not included in fitting the data, the flux samples with WW781 present were all taken at <30 s. The reciprocal of the unidirectional Cl^- exchange flux, J, in mmol/kg-s, is plotted against the WW781 concentration in the medium. △, 150 mM Cl^- medium; ○, 4 mM Cl^- medium with citrate-sucrose replacing Cl^-; ●, 4 mM Cl^- medium with 6 mM I^-. Lines on the reciprocal flux plot are drawn using the parameters obtained from the least-squares best fit of the original flux versus [WW781] data, such as that shown in Fig. 2, to the equation for single-site inhibition (Eq. 1). The negative of the x value of the intersection point for data with 150 mM Cl^- and 4 mM Cl^- media gives $K_{1W,Eo}$, the dissociation constant for binding to E_o; the corresponding value for the intersection of the data with 4 mM Cl^- media containing either 0 or 6 mM I^- gives $K_{1W,EIo}$, the dissociation constant for WW781 binding to EI_o, the form of AE1 with the transport site facing outward and loaded with iodide.

Dissociation Constant for Binding of WW781 to Various Forms of AE1

Dissociation constant	K_{1W}	K_{eqW}	P*	$K_{2/1W}$‡
$K_{W,Eo}$	0.11 ± 0.01 (5)§	0.0039 ± 0.0008 (4)§	0.0006	28.
$K_{W,EIo}$	0.36 ± 0.07 (2)	0.026 ± 0.011 (3)	0.14	13.
P, relative to $K_{W,Eo}$‖	0.18	0.18		
$K_{W,Ei}$	1.6 ± 0.4 (4)	≥0.046¶		≤34.
P, relative to $K_{W,Eo}$**	0.03			
$K_{W,Ei}/K_{W,Eo}$	14.	≥12.		
$K_{W,EIi}$	1.7 (1)‡‡	0.28 ± 0.07 (2)		
$K_{W,E}$	0.72§§	0.046§§		15.
$K_{W,ECl}$	0.52 − 0.53‖‖	0.17 − 0.19‖‖		1.8 − 2.2
$K_{W,ECl}/K_{W,E}$	0.73 − 0.74	3.6 − 4.0		0.12 − 0.15

*P from unpaired t test for comparison of K_{eqW} and K_{1W} values. ‡Equilibrium ratio of complex 2 to complex 1 (Eq. 3), calculated from Eq. 4. §Values are mean ± SEM, with the number of experiments (n) in parentheses. ‖P from unpaired t test comparison of $K_{W,Eo}$ and $K_{W,EIo}$. ¶Calculated from Eq. A8, using y intercept of the straight line fit in Fig. 10 as $K_{eqW,E}$ and $K_{eqW,Eo}$ value above. **P from unpaired t test of difference between $K_{W,Ei}$ and $K_{W,Eo}$. ‡‡Data points from a second experiment were too scattered for a determination of $K_{1W,EIi}$. §§From y intercepts of straight-line fits in Figs. 3 and 10, respectively. ‖‖Calculated from 600 mM $[Cl_{i=o}]$ data and $K_{W,E}$ by using Eq. A7 with $K_{Cl,E}$ values ranging from 40 to 65 mM.

is significantly larger (P = 0.03) than $K_{1W,Eo}$. This is true despite the fact that the $K_{1W,Ei}$ values show considerably greater scatter than the values of $K_{1W,Eo}$. This is mainly due to the fact that $K_{1W,Ei}$ is larger, so the intersection point occurs at more negative values of WW781, which
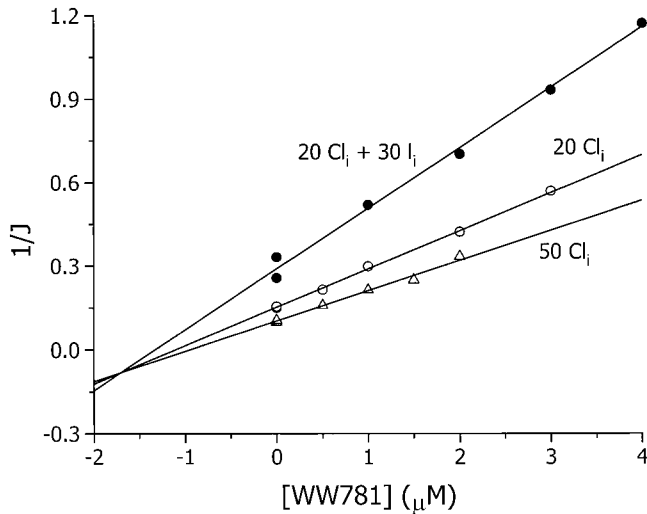

Figure 7. Dixon plot intersections for cells with the same $[Cl_o]$ and different $[Cl_i]$ and $[I_i]$. Cells were loaded with the nystatin technique to contain ~50 or 20 mM Cl^-, or 20 mM Cl^- with 30 mM I^-, and were suspended in 20 mM Cl^- media, with sucrose added to balance the osmolarity. All flux samples with WW781 present were taken in <16 s. Lines were drawn using the parameters obtained from nonlinear least-squares fits to the original flux versus WW781 data. The negative of the x value of the intersection point for cells with 20 mM (○) and 50 mM (△) $[Cl_i]$ is equal to $K_{1W,Ei}$, the dissociation constant for initial binding of WW781 to E_i. The corresponding value for the intersection of the lines for 20 mM $[Cl_i]$ without (○) or with (●) 30 mM $[I_i]$ is $K_{1W,EIi}$, the dissociation constant for initial binding to EI_i (Figs. 4 and 5 A). Note that for this experiment, $K_{1W,EIi}$ is similar to $K_{1W,Ei}$, suggesting that binding of an anion to E_i does not substantially affect the affinity for WW781.

necessarily are farther from the positive values at which actual measurements of fluxes can be done, but the greater difficulty of loading cells with different $[Cl^-]$ and the fact that at low $[Cl_i]$ the ratio of $[Cl_i]/[Cl_o]$ often deviates somewhat from unity also may contribute to the scatter in $K_{1W,Ei}$. The mean $K_{1W,EIo}$ value is somewhat larger than $K_{1W,Eo}$, but the difference is not significant (P = 0.18), and the single value of $K_{1W,EIi}$ is very similar to the $K_{1W,Ei}$ value, suggesting that binding of anions to either E_o or E_i has only a relatively small effect on the affinity of AE1 for the initial binding of WW781.

As shown by Fröhlich (1982) and Fröhlich and Gunn (1986), the average dissociation constant for an inhibitor binding to both of the unloaded forms of AE1, E_i, and E_o, designated $K_{1W,E}$ for the initial binding step, is given by:

$$K_{1W,E} = \frac{1+A}{(1/K_{1W,Ei}) + (A/K_{1W,Eo})}, \qquad (2)$$

where A is the asymmetry factor for the unloaded transporter (without anions bound), defined as the ratio E_o/E_i with $[Cl_i] = [Cl_o]$ (Knauf, 1979; Knauf and Brahm, 1989). If $[Cl_i] = [Cl_o]$, then the K_{1W} value should approach $K_{1W,E}$ as $[Cl^-] \rightarrow 0$, because under these conditions the only forms of the transporter present are E_i and E_o. If we take the y intercept of the best-fit straight line in Fig. 3 as a reasonable estimate for $K_{1W,E}$, we can calculate the $K_{1W,Ei}$ value that would be required to give the observed intercept $K_{1W,E}$ value, assuming the mean value of $K_{1W,Eo}$ shown in Table II and various values of the asymmetry factor, A. A plot of $K_{1W,Ei}$ versus A is shown in Fig. 8. Note that for A values of 0.05–0.1, corresponding to the values obtained by other measurement techniques (Knauf and Brahm, 1989; Gasbjerg and Brahm, 1991), the calculated $K_{1W,Ei}$ values are very similar to the measured values shown in

Table II. For A values over 0.15, however, the calculated value of $K_{1W,Ei}$ rises sharply, and for A values > 0.187, $K_{1W,Ei}$ approaches infinity. Thus the data are consistent with the observed asymmetry for unloaded transport sites as measured by other techniques, and they show further that no value for $K_{1W,Ei}$ can be obtained that is consistent with the $K_{1W,E}$ and $K_{1W,Eo}$ data if $A > 0.187$, providing further evidence that the unloaded transport sites are very asymmetrically distributed, with far more AE1 molecules in the E_i form than in the E_o form.

Determination of Equilibrium Dissociation Constant, K_{eqW}

To measure the apparent dissociation constant after WW781 comes into equilibrium with both complex 1 and complex 2, designated K_{eqW} (Fig. 5 B), cells were exposed to WW781 for at least 4 min before Cl^- exchange fluxes were measured, as described in materials and methods. On the basis of the rate constants measured for the WW781 complex formation in 150 mM [Cl^-] medium, this should be sufficient time for WW781 inhibition to approach its maximum value. Indeed, it was found that 1 or 2 h of pretreatment produced no significant increase in inhibition (decrease in the apparent K_{eqW} value) as compared with the standard pretreatment protocol, and that the plots of appearance of isotope versus time in media with the same [WW781] as in the pretreatment medium were nearly linear (data not shown), as expected if little further inhibition of Cl^- exchange occurs during the flux measurement.

To see whether or not inhibition under these conditions still fits to a one-site binding model, fluxes were plotted against the WW781 concentration and the data were fitted to the equation for single-site inhibition. Data for cells loaded with 600 mM [Cl^-] are shown in Fig. 9, plotted in the form of a Dixon plot ($1/J$ versus [WW781]). Although there is some scatter at the highest WW781 concentrations, where the fluxes are smallest, in general the fit to a straight line is very good, indicating that equilibrium inhibition can be well described by a single-site model. The straight line through the data in Fig. 9 was drawn using the parameters obtained from a nonlinear fit of the single-site inhibition equation (Eq. 1) to the original flux versus WW781 plot. The negative of the x intercept of this line gives the apparent K_{eqW} under these conditions.

Fig. 10 shows the results of several such experiments at different Cl^- concentrations. Note that in this case the concentrations required for half-inhibition of Cl^- exchange (K_{eqW}) are much lower than those shown in Fig. 3 (K_{1W}), where exposure to WW781 was for very short times. In this case, the slope of a best-fit straight line (solid line) is significantly different from zero ($P = 0.008$), indicating that Cl^- binding does affect the equilibrium of WW781 with complex 2.
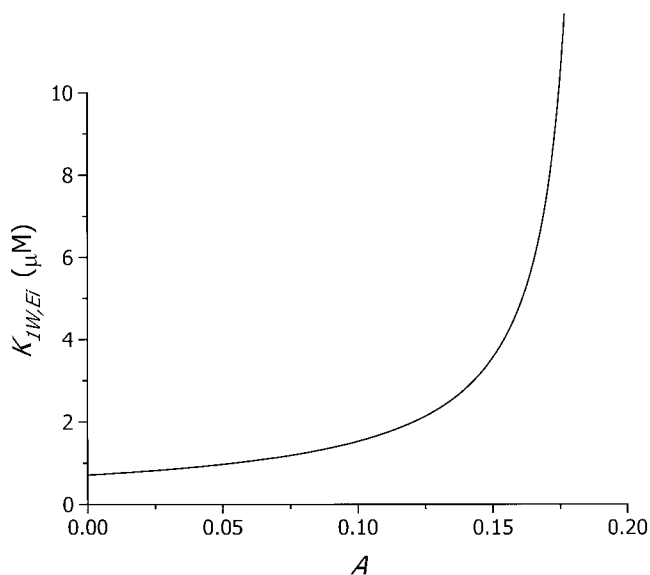

Figure 8. Calculation of $K_{1W,Ei}$ for various values of A. Calculations are based on a value of 0.716 μM for the K_{1W} value extrapolated to zero Cl^- inside and outside ($K_{1W,E}$), obtained from the y intercept of the best-fit straight line in Fig. 3, together with the mean value for $K_{1W,Eo}$ of 0.113 μM (Table II). These were used to calculate values of $K_{1W,Ei}$ consistent with Eq. 2. Note that $K_{1W,Ei}$ approaches infinity at values of A above 0.187, indicating that no solution consistent with the ping-pong model is possible unless A is below this value.

Table I shows the comparison of the mean values of K_{eqW} with those of K_{1W}. For every case except the 600-mM Cl^- data, the difference is significant, and in the latter case it approaches significance ($P = 0.06$). Particularly for the low [Cl^-] condition, the K_{eqW} is substantially lower than K_{1W}, demonstrating that the equilibrium between complex 1 and complex 2 is strongly in favor of complex 2 under these conditions.

Effect of AE1 Conformation on Equilibrium Affinity for WW781

As for the initial binding, the equilibrium binding dissociation constants of the various forms of AE1 for WW781 may differ (Fig. 5 B). For the equilibrium binding, the dissociation constants are designated $K_{eqW,Eo}$, $K_{eqW,EClo}$, etc. To see whether or not the AE1 conformation affects the equilibrium affinity for WW781, we first varied external [Cl^-], which should affect the fraction of E_o relative to the other forms of AE1. As shown in Table I, the K_{eqW} with 2 mM [Cl^-] outside was significantly lower than that with 150 mM [Cl^-] outside, indicating that the E_o form has a higher affinity for WW781 than the mixture of the other forms of AE1 that prevails in 150 mM [Cl^-] medium.

To determine $K_{eqW,Eo}$, the dissociation constant for equilibrium binding to E_o, the effects of WW781 were
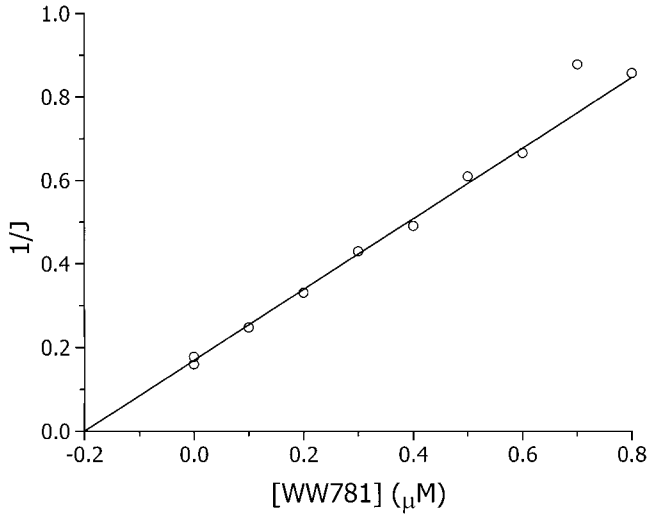
Figure 9. Dixon plot of inhibition after pretreatment with WW781 for cells loaded with 600 mM Cl$^-$. Cells were loaded with 600 mM Cl$^-$ by the nystatin technique, as described in materials and methods ([Cl$_i$]/[Cl$_o$] = 0.98), and were pretreated with the indicated concentrations of WW781 for $\geq$2 min, and then loaded with ^{36}Cl in the same medium. Fluxes were measured in 600 mM Cl$^-$ medium with the same concentration of WW781 as that during the pretreatment and ^{36}Cl loading. All flux data fit well to straight lines (r^2 > 0.99), indicating that the inhibitory effect was near maximal at the beginning of the flux measurement, and did not increase significantly thereafter. The reciprocal of the flux, J, in mmol/kg dry solids·s is plotted against the WW781 concentration. The straight line was drawn with the best-fit parameters obtained from a nonlinear fit of the equation for single-site inhibition (Eq. 1) to the flux versus [WW781] data, obtained with the uninhibited flux, J_u, fixed at the mean value of the control fluxes, 5.92 mmol/kg dry solids·s, and with K_{eqW} = 0.200 $\pm$ 0.009 μM, corresponding to the negative of the x intercept of the Dixon plot.

measured in cells with ~150 mM [Cl$^-$] inside and different external [Cl$^-$] concentrations. Combined data for four such experiments are shown in Fig. 11. In this case, particularly at the low external Cl$^-$ concentration (○), inhibition is very pronounced even at the lowest WW781 concentrations (Fig. 11 A). Although it was not possible to obtain data between 0 and ~0.1 μM WW781, because the binding to AE1 causes depletion of the WW781 in the medium under these conditions (Raha et al., 1993), the data at higher concentrations fit quite well to the equation for single-site inhibition, as shown by the linearity of Dixon plots of the data (Fig. 11 B). Thus, it was possible to determine $K_{eqW,Eo}$ values from the negative of the x value of the intersection point of the Dixon plot lines for different [Cl$_o$], by using the parameters obtained from fits to the flux versus [WW781] data to calculate the intersection point. As expected from the K_{eqW} data, $K_{eqW,Eo}$ is significantly lower than $K_{1W,Eo}$ (Table II). Similar data were obtained with iodide added to the low [Cl$^-$] external medium to determine $K_{eqW,EIo}$ (Table II).
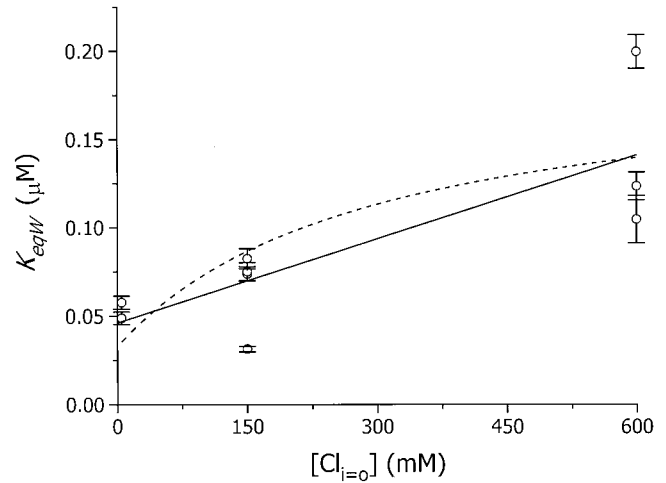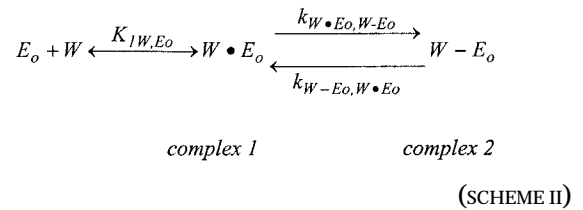

Figure 10. K_{eqW} as a function of [Cl$^-$], with [Cl$_i$] = [Cl$_o$]. K_{eqW} values were obtained from experiments such as that shown in Fig. 9, with cells loaded with various [Cl$^-$] by the nystatin technique. Bars indicate SEM. The solid line shows the best-fit straight line to the data, with y intercept of 0.046 $\pm$ 0.016 μM and slope of 0.00016 $\pm$ 0.00004 μM/mM (P = 0.008). The dashed line shows the best fit to Eq. A6, with $K_{Cl,E}$ fixed at 50 mM (Gasbjerg and Brahm, 1991), $K_{eqW,ECl}$ = 0.19 $\pm$ 0.06 μM, and $K_{eqW,E}$ = 0.033 $\pm$ 0.012 μM, with the aberrant lowest point for 150 mM [Cl$^-$] excluded from the fit.

As shown in Fig. 4, for each form of AE1, an equation for the two-step binding can be written, analogous to the equation for the total AE1 present. For E$_o$ (Scheme II),

$$E_o + W \underset{K_{1W,Eo}}{\longleftrightarrow} W \bullet E_o \underset{k_{W-Eo,W \bullet Eo}}{\overset{k_{W \bullet Eo,W-Eo}}{\rightleftarrows}} W - E_o$$

complex 1   complex 2

(SCHEME II)

where $k_{W \bullet Eo,W-Eo}$ and $k_{W-Eo,W \bullet Eo}$ are the rate constants for formation and breakdown of complex 2 (Scheme II). The constant describing the equilibrium ratio between complex 2 and complex 1 for E$_o$ is defined as:

$$K_{2/1W,\,Eo} = \frac{[W-E_o]}{[W \bullet E_o]}\bigg|_{eq} = \frac{k_{W \bullet Eo,\,W-Eo}}{k_{W-Eo,\,W \bullet Eo}}. \quad (3)$$

$K_{2/1W,Eo}$ can be determined from $K_{1W,Eo}$ and $K_{eqW,Eo}$ in a manner analogous to equation 6 of Raha et al. (1993), as follows:

$$K_{2/1W,\,Eo} = (K_{1W,\,Eo}/K_{eqW,\,Eo}) - 1. \quad (4)$$

From the data in Table II, $K_{2/1W,Eo}$ is 28, corresponding to a free energy difference, ΔG^0 [= exp($-K_{2/1W,Eo}/RT$)], between complex 2 and complex 1 (for the E$_o$ form of AE1) of -7.6 kJ/mol. As in the case of the K_{1W}

167   Knauf et al.
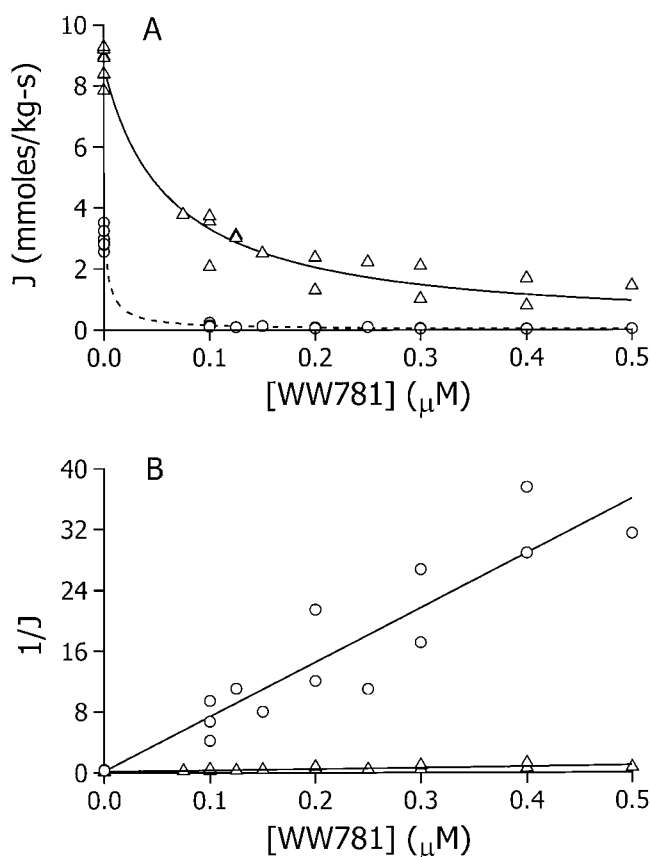
Figure 11. (A) Flux as a function of [WW781] for cells with ~150 mM [Cl_i] equilibrated with WW781 in either 150 ($\triangle$) or 2 ($\bigcirc$) mM Cl^- medium, with citrate-sucrose replacing Cl^-. For the 150 mM Cl^- medium, cells were exposed to WW781 for ≥2 min, and then loaded with ^{36}Cl in the same medium, as described in Fig. 9. The flux medium had the same concentration of WW781 as that during the pretreatment. For the 2 mM Cl^- medium, where fluxes are much slower, cells were not pretreated with WW781, but fluxes were measured by waiting to take timed samples until at least 4 min had elapsed after addition of the cells to WW781-containing medium, by which time there was little or no further increase in inhibition. Combined data are from four separate experiments. Lines show the best fits to the equation for single-site inhibition (Eq. 1), with fixed J_u of 8.601 mmol/kg dry solids-s and K_{eqW} of 0.062 ± 0.005 μM for the 150 mM Cl^- medium, and J_u of 2.967 mmol/kg-s and K_{eqW} of 0.0055 ± 0.0029 μM for the 2 mM Cl^- medium. (B) Dixon plot of data in A. $\bigcirc$, 2 mM Cl^- medium; $\triangle$, 150 mM Cl^- medium. Lines drawn using the parameters from the best fits to the data in A. The mean and SEM of the intersection points for the lines from the individual experiments, whose x value is equal to $-K_{eqW,Eo}$, are shown in Table II.

values, binding of iodide to E_o causes an apparent increase in K_{eqW} that is not statistically significant, but which corresponds to a $K_{2/1W,EIo}$ value of 12.5, suggesting that binding of iodide to E_o destabilizes complex 2 and thus decreases the equilibrium ratio of complex 2 to complex 1.

From the y intercept of the straight line fit to the K_{eqW} values in Fig. 10, it is possible to obtain a value for

$K_{eqW,E}$, the equilibrium dissociation constant for binding to both forms of AE1 without Cl^- bound (E_o and E_i), with [Cl_o] = [Cl_i], which is 0.046 μM. As for the K_{1W} values, this can be used together with the value of $K_{eqW,Eo}$ to calculate values of $K_{eqW,Ei}$ that are consistent with the ping-pong model (see Eq. A8). As shown in Fig. 12, the minimum value of $K_{eqW,Ei}$ consistent with the model is equal to $K_{eqW,E}$, 0.046 μM, and the maximum value of A is 0.0926. Thus, the equilibrium binding data provide further evidence for at least 10-fold asymmetry in favor of E_i versus E_o, in agreement with other determinations of this parameter (Knauf and Brahm, 1989; Gasbjerg and Brahm, 1991).

Although it is not possible to measure the dissociation constants for WW781 binding to the individual Cl^--loaded forms of AE1, ECl_i, and ECl_o, it is possible to estimate a value for the dissociation constant, $K_{eqW,ECl}$ (see Eq. A5), for the combination of ECl_o and ECl_i, whose ratio is a constant determined by the value of the asymmetry factor for Cl^--loaded forms of AE1, A_{Cl} (= $k_{ECli,EClo}/k_{EClo,ECli}$ = ECl_o/ECl_i) (Knauf, 1989). Calculations using Eq. A7, together with the mean value of the K_{eqW} at 600 mM [Cl^-] and values of $K_{Cl,E}$, the apparent Cl^- dissociation constant with [Cl_i] = [Cl_o], ranging from 40 to 65 mM, give values for $K_{eqW,ECl}$ of 0.166–0.185 μM. Similar calculations for $K_{1W,ECl}$ range from 0.524 to 0.529 μM. The ratio of complex 2 to complex 1 for the Cl^--loaded forms ($K_{2/1W,ECl}$) is 1.8–2.2, as compared with 14.6 for the unloaded forms ($K_{2/1W,E}$). Cl^- binding thus appears to reduce the tendency of WW781 to form the second complex with AE1.

An alternate method for determining $K_{eqW,E}$ and $K_{eqW,ECl}$ involves fitting all of the K_{1W} or K_{eqW} data to Eq. A6, which gives the half-inhibitory concentration as a function of $K_{W,E}$, $K_{W,ECl}$, and [Cl^-], under conditions where [Cl_i] = [Cl_o]. If this is done for the K_{1W} data in Fig. 3, assuming that $K_{Cl,E}$ = 50 mM (Gasbjerg and Brahm, 1991), the dashed line is obtained, with $K_{1W,E}$ = 0.99 μM and $K_{1W,ECl}$ = 0.51 μM. When the calculations of Fig. 8 are repeated with the higher value of $K_{1W,E}$, the minimum value of $K_{1W,Ei}$ rises to 0.99 μM and the maximum value of $A ≤ 0.129$.

Similar calculations for the K_{eqW} data in Fig. 10 (without the lowest point at 150 mM [Cl^-]) (dashed line) give $K_{eqW,ECl}$ = 0.19 μM and $K_{eqW,E}$ = 0.033 μM, which corresponds to the minimum possible value of $K_{eqW,Ei}$. Consistent results are obtained with $A ≤ 0.134$.

From the estimates of $K_{eqW,ECl}$, it is further possible to calculate the values of $K_{eqW,ECli}$ that are compatible with the other measured parameters. This requires some knowledge of $K_{eqW,EClo}$, which cannot be directly measured. However, if $K_{eqW,EClo}$ lies somewhere between the value of $K_{eqW,Eo}$ and $K_{eqW,EIo}$, the value with I^- bound (which seems reasonable because the smaller Cl^- ion would be expected to have less effect on WW781 bind-
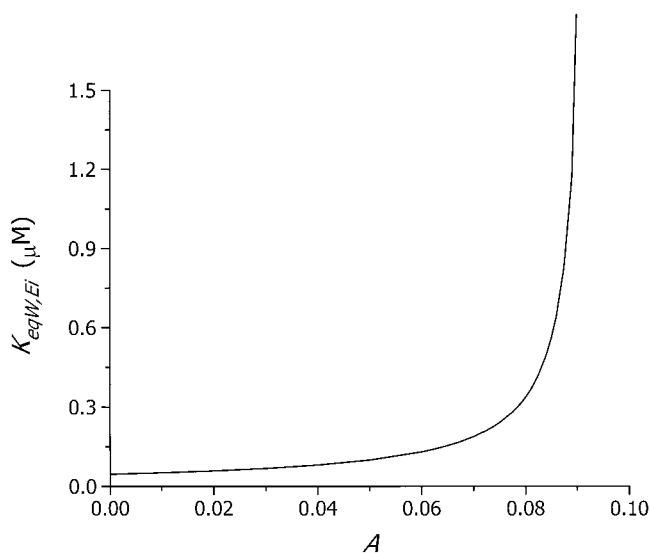
Figure 12. Calculated values of $K_{eqW,Ei}$, the dissociation constant for equilibrium binding of WW781 to E_i, as a function of the asymmetry ratio, A. Calculations are based on Eq. A8, using the $K_{eqW,E}$ value of 0.046 μM, obtained from the y intercept of the straight line in Fig. 10, and the mean $K_{eqW,Eo}$ value of 0.0039 μM from Table II.

Figure 13. Calculated values of $K_{eqW,ECli}$, the dissociation constant for equilibrium binding of WW781 to ECl_i, as a function of the asymmetry ratio for Cl^--loaded sites, A_{Cl}. Calculations are based on Eq. A10, with $K_{eqW,ECl} = 0.17$ μM, determined from the 600 mM [Cl^-] data, and with different values of $K_{eqW,EClo}$ for Cl^-, ranging from $K_{eqW,Eo}$ (0.0039 μM, Table II, dotted line), $K_{eqW,EIo}$ − 1 SEM (0.015 μM, dashed line), $K_{eqW,EIo}$ (0.026 μM, Table II, solid line), $K_{eqW,EIo}$ + 1 SEM (0.037 μM, dash-dot line), and $K_{eqW,EIo}$ + 2 SEM (0.048 μM, short-dashed line). Calculations with $K_{eqW,ECl} = 0.19$ μM, from the nonlinear fit to the data in Fig. 10, give similar results, except that the minimum value of $K_{eqW,ECli}$ is 0.19 μM and the curves rise at slightly lower values of A_{Cl}.

ing than does the larger iodide), then calculations of $K_{eqW,ECli}$ as a function of A_{Cl} can be done using Eq. A10, as shown in Fig. 13. The minimum value of $K_{eqW,ECli}$ consistent with the data is 0.17–0.19 μM (depending on which $K_{eqW,ECl}$ value is used), considerably higher than the minimum value of $K_{eqW,Ei}$ (0.033–0.046 μM). Regardless of which value of $K_{eqW,EClo}$ is chosen, there is some value of A_{Cl} at which $K_{eqW,ECli}$ becomes infinite; that is, no consistent solution can be found. Thus, (based on the assumption that $K_{eqW,EClo}$ is within 1 SEM $K_{eqW,EIo}$ value) the data provide evidence that $A_{Cl} <$ 0.28, consistent with previous nuclear magnetic resonance (NMR) experiments indicating that A_{Cl} is similar to A (Liu et al., 1996). Furthermore, the fact that calculations assuming that $K_{eqW,EClo} = K_{eqW,Eo}$ (Fig. 13, leftmost dotted line) only give consistent solutions with values of A_{Cl} much lower than that estimated by NMR suggests that external Cl^- binding probably does increase the equilibrium dissociation constant for WW781 binding to E_o; i.e., $K_{eqW,EClo} > K_{eqW,Eo}$.

Measurements of the $K_{eqW,Ei}$ and $K_{eqW,EIi}$ parameters by techniques similar to those used for the K_{1W} parameters in Fig. 7 are difficult because the effects of WW781 on the fluxes are so much larger than those of either [Cl_i] or [I_i] that the Dixon plot lines tend to be nearly parallel and the intersection points are difficult to determine with any precision. The two experiments that were performed to measure $K_{eqW,EIi}$, however, gave values of 0.21 and 0.35 μM (data not shown), which, while not statistically different from the corresponding $K_{eqW,Eo}$ and $K_{eqW,EIo}$ values (Table II), are reasonable in
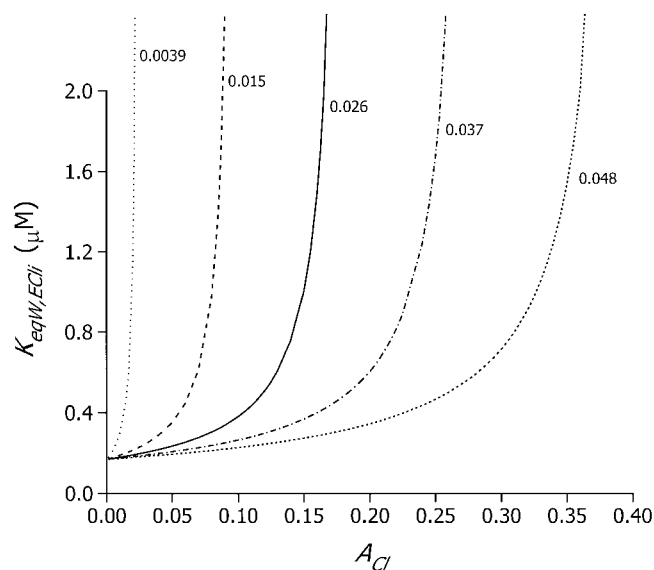
light of the constraints imposed on $K_{eqW,ECli}$ (≥ 0.17 μM) according to the analyses in Fig. 13.

DISCUSSION

Accuracy of Initial and Equilibrium Estimates of WW781 Affinities

The methods used to distinguish the effects of initial binding of WW781 from the effects after equilibrium is reached have the disadvantage that, since some complex 2 is formed even at early times, apparent values of K_{1W} obtained from early time samples are always at least slightly contaminated by additional inhibition caused by the second binding step. The measurements of equilibrium parameters have the opposite problem in that the extent of the second binding step will be underestimated if the time of exposure to WW781 is not sufficient to ensure that equilibrium has been attained. Fortunately, the effects of these errors are in opposite directions, resulting in underestimates of K_{1W} and overestimates of K_{eqW}. Since $K_{eqW} < K_{1W}$, such errors will tend to bring the measured K_{1W} and K_{eqW} values closer together than they actually are. The fact that K_{1W} is over nine times larger than K_{eqW} for [Cl^-] ≤ 150 mM (Table I) argues that the method, while not completely accu-

rate, is at least able to detect a considerable difference in the initial and equilibrium affinities for WW781. The significant effect of increasing [Cl⁻] on K_{eqW} (Fig. 10), but not on K_{1W} (Fig. 3) provides further evidence that even this first-order method is sufficient to distinguish different effects on the two parameters.

Time Course of Binding

In general, the differential equations describing the time course of the binding and transport inhibition are exceedingly complex, since each form of AE1 has its own individual parameters, as shown in Fig. 4. If, however, the forms of AE1 without WW781 equilibrate rapidly with each other, as well as with their corresponding complex 1 forms, then the time course of complex 2 formation should be described by a single rate constant. This seems to be the case, at least at 150 mM [Cl⁻], where our previous analysis was consistent with a simple exponential approach to equilibrium for both the "on" and "off" binding reactions (Raha et al., 1993). This apparent rate constant, however, will depend on the relative abundance of the different forms of AE1, together with the magnitude of their individual rate constants, so the time course of binding is expected to be affected by changes in AE1 distribution caused, for example, by changes in [Cl$_i$] and [Cl$_o$]. Further experiments, using techniques that permit direct measurements of binding, are needed to explore this possibility.

Preference for Forms of AE1

Both for the initial binding and equilibrium association, WW781 exhibits a strong preference (by >10-fold) for the E$_o$ form of AE1 as compared with the E$_i$ form. Previous brief reports of this work (Knauf et al., 1990, 1992; Mendoza et al., 1990) gave a value of >100 for the ratio of the equilibrium dissociation constants for E$_i$ and E$_o$, but this was based on a particular estimate of the loaded-site asymmetry ratio, A_{Cl}, which gave a higher value of $K_{eqW,Ei}$ (see Fig. 12). The present data, which only give a lower limit for $K_{eqW,Ei}$, do not preclude the possibility that the $K_{eqW,Ei}/K_{eqW,Eo}$ ratio could be as large as 100, but they provide no evidence for a ratio >12 (Table II). Even this is a larger selectivity factor than that exhibited by any other inhibitor (Knauf et al., 1992), except the disulfonic stilbenes such as DNDS (Fröhlich, 1982; Knauf et al., 1993). In contrast to the disulfonic stilbenes, where binding of Cl⁻ greatly diminishes the apparent affinity for the inhibitor, in a manner that resembles competitive inhibition (Shami et al., 1978; Fröhlich, 1982), for the initial binding step of WW781 there was no significant effect of [Cl⁻] (Fig. 3), and for the equilibrium binding (Table II) we observed only about a fourfold increase in the dissociation constant for binding to the Cl⁻-loaded forms ($K_{eqW,ECl}$) as compared with the unloaded forms ($K_{eqW,E}$).

Conclusions about the WW781 affinity of the Cl⁻-loaded forms, based as they are on the [Cl⁻] dependence of the K_{eqW}, must be tempered by the possibility that binding of Cl⁻ to the inhibitory modifier site may also affect the affinity for WW781. Although the dissociation constant for binding of Cl⁻ to the modifier site (over 300 mM; Dalmark, 1976; Gasbjerg and Brahm, 1991) is much higher than that (around 50 mM; Gasbjerg and Brahm, 1991) for binding to the transport site, the inevitable scatter in measurements of dissociation constants (because the parameter measured, Cl⁻ exchange inhibition, is a less-than-linear function of the dissociation constant) makes it impossible from our experiments to precisely determine the Cl⁻ affinity of the site that affects WW781 binding. Models in which the change in WW781 affinity is assumed to be due to binding to the transport site, however, indicated by the dashed lines in Figs. 3 and 10, fit the observations reasonably well. Nevertheless, the possibility that modifier site binding of Cl⁻ causes some or all of the change in WW781 affinity cannot be excluded.

Although the binding of an oxonol analogue of WW781, diBA(5)C$_4$, is mutually exclusive with the disulfonic stilbene, DNDS (Knauf et al., 1995), the different effects of [Cl⁻] on the affinity of DNDS (Fröhlich, 1982) as compared with diBA(5)C$_4$ (Knauf et al., 1995) and WW781 (Figs. 3 and 10) indicate that, if the binding sites overlap, they do so only partially. Thus, the fact that WW781 binding is affected both by the orientation of the transport site (E$_i$ versus E$_o$) and by the binding of Cl⁻ suggests that an additional part of the AE1 protein, probably adjacent to but distinct from the disulfonic stilbene binding site, is affected by the conformational changes that occur during substrate binding and transport-site reorientation. This in turn implies that these conformational changes are not simply local reorientations of a few amino acids, but likely involve more global changes in protein structure such as have been observed with other proteins such as hemoglobin and alcohol dehydrogenase.

Effects of WW781 Binding on AE1 Conformation

Because of the preference for AE1 forms with the transport site facing outward (E$_o$ and ECl$_o$), the addition of even as little as 1 μM of WW781 should cause a pronounced reorientation of AE1 toward the outward-facing conformations. Calculations for various cases based on values for A and A_{Cl} consistent with the present and previous data are shown in Fig. 14. With 5 mM [Cl⁻] inside and outside, AE1 is highly biased toward the E$_i$ form, and >93% of AE1 is in some inward-facing form. Addition of WW781 causes this to shift so that ~60% is in the outward-facing form. A Cl⁻ gradient across the membrane, with 150 mM inside and 2 mM outside, normally results in ~56% of the sites facing outward,

but with WW781 this is increased to >98%. Even with a much smaller gradient, such as 150 mM [Cl_i] and 30 mM [Cl_o], the preference for outward-facing forms is >80% in the presence of WW781 (data not shown). With symmetric 150 mM Cl^- concentrations, where >90% of the sites face inward, WW781 causes rearrangement toward a more uniform distribution among the various forms, with ~55% of the transport sites facing outward.

Because of this property, WW781 may be a useful tool for changing the orientation of sites, for studies aimed at detecting differences between the inward and outward-facing conformations, one of the central questions that must be answered if AE1 anion exchange is to be understood at the molecular level. The reorientation by WW781 toward outward-facing sites may also help to explain the early observation by Freedman and Novak (1983) that WW781 appears to cause an increase in net Cl^- permeability, P_{Cl}. Considered together with the observation of Fröhlich et al. (1983) that P_{Cl} increases when the fraction of AE1 in the E_o conformation is increased by lowering [Cl_o], the skewing of the AE1 distribution to outward-facing forms by WW781 may explain the increase in P_{Cl}.

Information About the Asymmetry of the AE1 Mechanism

The data presented here add to a growing body of evidence that the AE1 transport sites are very asymmetrically distributed. The conclusion that $A \leq 0.1$ is in good agreement with most of the data from flux measurements and other techniques (Knauf and Brahm, 1989; Gasbjerg and Brahm, 1991), although some of the data that give higher estimates of A (Hautmann and Schnell, 1985; Knauf and Brahm, 1989) are incompatible with this result. The idea that $A_{Cl} \leq 0.28$ agrees with ^{35}Cl NMR binding measurements, which give an average A_{Cl} value of ~0.1, based on a measured external Cl^- dissociation constant (K_o) of ~40 mM in eosin-5-maleimide–treated cells (Liu et al., 1996). The values of A and A_{Cl} used to calculate Fig. 14 predict a slightly lower K_o of ~33 mM, nearer to the value of 23 ± 9 mM from more recent improved NMR measurements (Kennedy, S.D., C. Wu, and P.A. Knauf, unpublished data). For both the Cl^--loaded and unloaded forms of AE1, therefore, the Gibbs free energy for the form with the transport site facing inward seems to be ~5 kJ/mol lower than that for the outward-facing form.

The data presented here demonstrate the usefulness of an oxonol inhibitor to distinguish the various conformations of AE1 and to both measure and alter the distribution among the various forms. Further work is needed to define the structural requirements that confer conformational selectivity on WW781, and to determine whether or not other oxonols with different structures exhibit the same or different conformational preferences.
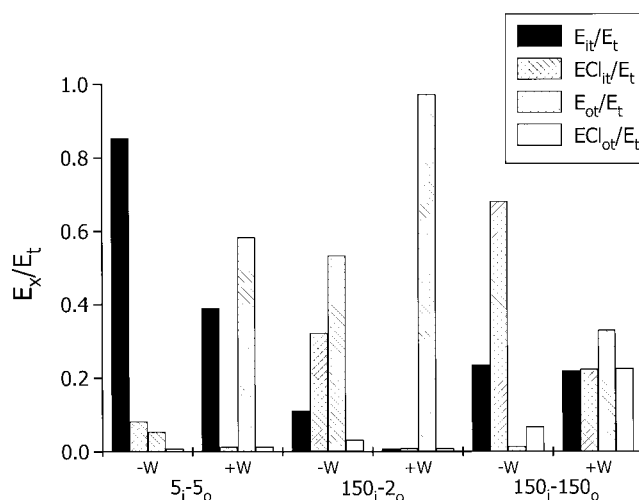

Figure 14. Calculated fractions of AE1 in various conformations for different conditions without (−W) and with (+W) 1 μM WW781. The fraction of AE1 in each form (see Fig. 4) is shown as a fraction of the total AE1 (E_t); for the cases with WW781, the total of the free and WW781-complexed forms is shown; for example, $E_{ot} = E_o + WE_o$, assuming equilibrium binding of WW781. Values on the x axis indicate the Cl^- concentrations inside and outside the cells (mM). Calculations are based on following values for parameters: $A = 0.064$, $A_{Cl} = 0.1$, $K_{Cl,E} = 50$ mM, $K_{eqW,Eo} = 0.0039$ μM (Table II), $K_{eqW,EClo} = 0.0264$ μM (= $K_{eqW,EIo}$, Table II), $K_{eqW,Ei} = 0.1$ μM (chosen to be consistent with A, Fig. 12), $K_{eqW,ECli} = 0.35$ μM (chosen to be consistent with A_{Cl}, Fig. 13).

APPENDIX

Dependence of K_W on [Cl^-] with [Cl_i] = [Cl_o]

K_W is defined as the concentration of WW781 that causes 50% inhibition of transport. Depending on whether experiments are done under initial binding conditions or equilibrium conditions, K_W can be thought of as either K_{1W} or K_{eqW}.

$K_{Cl,E}$, the concentration of Cl^- that gives half-maximal Cl^- exchange, with [Cl_i] = [Cl_o], is given in equation A8b in Gasbjerg and Brahm (1991) and equation 1 in Liu et al. (1996) (Eq. A1):

$$K_{Cl,E} = K_{Cl,Ei}(1 + A)/(1 + A_{Cl}), \quad (A1)$$

where A is the asymmetry ratio (E_o/E_i) for the forms of AE1 with the transport site unloaded and with [Cl_i] = [Cl_o], and A_{Cl} is the asymmetry ratio (ECl_o/ECl_i) for the Cl^--loaded forms of AE1. $A = (k_{ECli,EClo} K_{Cl,Eo})/(k_{EClo,ECli} K_{Cl,Ei})$ and $A_{Cl} = k_{ECli,EClo}/k_{EClo,ECli}$, where the various constants are defined as described in **materials and methods** and Figs. 4 and 5. If we define ECl as the sum of ECl_i plus ECl_o and E as the sum of E_i + E_o, then their ratio is given by:

$$[ECl]/[E] = (1 + A_{Cl})[Cl^-]/\{K_{Cl,Ei}(1 + A)\} = [Cl^-]/K_{Cl,E}. \quad (A2)$$

171   Knauf et al.

K_W can be defined as an apparent dissociation constant:

$$K_W = [W]([E] + [ECl])/([WE] + [WECl]), \quad (A3)$$

where W is WW781, WE represents the sum of the E forms associated with WW781; that is, the sum of WE_i plus WE_o, and WECl represents the sum of $WECl_i + WECl_o$ (Figs. 4 and 5).

We can also define the apparent dissociation constant for binding of WW781 to the unloaded forms of AE1 as:

$$K_{W,E} = [E][W]/[WE], \quad (A4)$$

and that for the Cl^--loaded forms as:

$$K_{W,ECl} = [ECl][W]/[WECl]. \quad (A5)$$

Substituting Eqs. A2, A4, and A5 into A3, we obtain:

$$K_W = K_{W,E}(1 + [Cl^-]/K_{Cl,E})/ \\ (1 + [Cl^-]K_{W,E}/K_{Cl,E}K_{W,ECl}). \quad (A6)$$

This equation was used to determine the dashed lines in Figs. 3 and 10. It can be solved for $K_{W,ECl}$ if $K_{W,E}$ is known as well as the K_W for any given $[Cl^-]$:

$$K_{W,ECl} = K_W K_{W,E}[Cl^-]/ \\ \{K_{W,E}[Cl^-] + K_{Cl,E}(K_{W,E} - K_W)\}. \quad (A7)$$

This equation was used to calculate $K_{W,ECl}$ values from the K_W values with 600 mM $[Cl^-]$.

As shown by Fröhlich (1982) and Fröhlich and Gunn (1986), and as is obvious from Eq. A6, the K_W extrapolated to zero $[Cl^-]$ is equal to $K_{W,E}$, which is given by (see equations 39, 35a, and 35b of Fröhlich and Gunn, 1986; note that their a/b = A):

$$K_{W,E} = (1 + A)/(1/K_{W,Ei} + A/K_{W,Eo}), \quad (A8)$$

which corresponds to Eq. 2 for $K_{1W,E}$. Similarly, $K_{W,ECl}$ is given by (see equation 42b of Fröhlich and Gunn, 1986; note that their $K_2 = A_{Cl}$) (Eq. A9):

$$K_{W,ECl} = (1 + A_{Cl})/(1/K_{W,ECli} + A_{Cl}/K_{W,EClo}). \quad (A9)$$

If $K_{W,ECl}$ is known, $K_{W,ECli}$ can be calculated as a function of A_{Cl} and $K_{W,EClo}$, as in Fig. 13, as follows:

$$K_{W,ECli} = K_{W,ECl}/(1 + A_{Cl} - A_{Cl}K_{W,ECl}/K_{W,EClo}). \quad (A10)$$

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