

Use of Niflumic Acid to Determine the Nature of the Asymmetry of the Human Erythrocyte Anion Exchange System

PHILIP A. KNAUF and NANCY A. MANN

From the Department of Radiation Biology and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642

ABSTRACT Niflumic acid is a noncompetitive inhibitor of chloride exchange, which binds to a site different from the transport or modifier sites. When the internal Cl^- concentration is raised, at constant extracellular Cl^- , the inhibitory potency of niflumic acid increases. This effect cannot be attributed to changes in membrane potential, but rather it suggests that niflumic acid binds to the anion exchange protein band 3 only when the transport site faces outward. When the chloride gradient is reversed, with $\text{Cl}_o > \text{Cl}_i$, the inhibitory potency of niflumic acid decreases greatly, which indicates that the affinity of niflumic acid for band 3 with the transport site facing inward is almost 50 times less than when the transport site faces outward. Experiments in which $\text{Cl}_i = \text{Cl}_o$ show no significant change in the inhibition by niflumic acid when Cl^- is lowered from 150 to 10 mM. These data suggest that the intrinsic dissociation constants for Cl^- at the two sides of the membrane are nearly equal. Thus, the chloride-loaded transport sites have an asymmetric orientation like that of the unloaded transport sites, with ~ 15 times more sites facing the inside than the outside. The asymmetry reflects an ~ 1.5 kcal/mol free energy difference between the inward-facing and outward-facing chloride-loaded forms of band 3. High concentrations of chloride (with $\text{Cl}_i = \text{Cl}_o$), which partially saturate the modifier site, have no effect on niflumic acid inhibition, which indicates that chloride binds equally well to the modifier site regardless of the orientation of the transport site.

INTRODUCTION

According to the ping-pong model of anion exchange, the band 3 transport protein can exist in either of two conformations, one in which the transport site faces the external medium (E_o) and one in which it faces the cytoplasm (E_i). Various lines of evidence (Gunn and Fröhlich, 1979; Jennings, 1980, 1982; Fröhlich, 1982; Knauf et al., 1980, 1984) indicate that the transport system is asymmetric, such that even when $\text{Cl}_i = \text{Cl}_o$, more unloaded transport sites face the inside (cytoplasm) than the outside. As shown previously, this asymmetry can

Address reprint requests to Dr. Philip A. Knauf, Dept. of Radiation Biology and Biophysics, University of Rochester School of Medicine, 601 Elmwood Ave., Rochester, NY 14642.

be measured in terms of an asymmetry factor, A , which is the ratio of E_o to E_i when $Cl_i = Cl_o$. A is equal to $kK_o/k'K_i$, where K_i and K_o are the chloride dissociation constants for the inward- and outward-facing forms of the transport site, respectively, and k is the rate constant for the conformational change from inward-facing to outward-facing, while k' is the rate constant for the reverse conformational change. The asymmetry in the system could thus result either from a difference between K_i and K_o , from a difference between k and k' , or from a combination of these two factors.

The source of the asymmetry can most easily be determined from the distribution of the chloride-loaded forms of the transport system, ECl_o and ECl_i . As shown in the Appendix, Eq. A5, the loaded sites asymmetry ratio, L , defined as ECl_o/ECl_i , is simply equal to k/k' . Thus, if we were to know the value of L , comparison of L with A would reveal whether the asymmetry is due to the rate constants, k and k' , or to the dissociation constants, K_i and K_o . To measure L , we need a probe which binds to the chloride-loaded form of the transport system and which can distinguish ECl_i from ECl_o .

By definition, a noncompetitive inhibitor must bind to the transport system regardless of whether or not the substrate, chloride, is bound to the transport site. That is, it must bind to both chloride-loaded and -unloaded band 3 molecules. Cousin and Motais (1979) reported that niflumic acid is a very potent noncompetitive inhibitor of chloride exchange. They also found that SITS (4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid), a stilbene disulfonate that probably binds to the transport site, interferes with the binding of niflumic acid. Thus, it seemed possible that the niflumic acid site might be near the transport site and that niflumic acid binding might therefore be sensitive to the conformation of the transport site.

In the two preceding papers (Furuya et al., 1984; Knauf et al., 1984), we have shown that if a chloride gradient is imposed across the membrane, this can alter the number of inward- and outward-facing transport sites. In this paper, we have used similar techniques to change the orientation of the transport sites, and thereby to determine whether or not niflumic acid binds preferentially to band 3 when the transport site is in a particular orientation. We have then used this information to determine the loaded sites asymmetry ratio, L , and thus to discover the nature of the asymmetry of the transport system in the chloride-loaded form. Finally, we have obtained evidence from the invariance of the inhibitory effect of niflumic acid at high chloride concentrations that chloride binding to the modifier site is independent of the conformation of the transport site.

Preliminary reports of these data have been published in abstract (Knauf et al., 1981; Knauf and Mann, 1982) and summary (Knauf, 1982) form.

METHODS

Cell Preparation

Blood was obtained by venipuncture from apparently healthy adults with heparin as anticoagulant. The blood was washed twice with ice-cold 150 mM NaCl, 20 mM Na-

phosphate, pH 6, and the white cells were removed by aspiration. The red cells were then washed three times in 150 mM KCl, 20 mM HEPES, 27 mM sucrose, pH 7.2, at 0°C (150K) and were made up to 50% hematocrit. The high (150 mM) chloride cells were then treated with 75 µg/ml nystatin (Sigma Chemical Co., St. Louis, MO) in 150K for 10 min on ice at 10% hematocrit, after which the cells were centrifuged and the treatment was repeated two more times. The cells were then washed five times at room temperature in 150K, made up to 50% hematocrit, and used for flux experiments. The low (10 mM) chloride cells were washed with 10 mM KCl, 20 mM HEPES, 245 mM sucrose, pH 7.2, at 0°C (10K-Hi), and were then treated with nystatin twice in this buffer at 0°C. The final nystatin treatment was done in 10 mM KCl, 20 mM HEPES, 27 mM sucrose, pH 7.2 (10K-Lo), and the cells were then washed five times in 10K-Lo at room temperature before being made up to 50% hematocrit for the flux measurements. For the later experiments in this series, the first nystatin treatment for the low chloride cells was carried out in 30 mM KCl, 130 mM sucrose, 20 mM HEPES, pH 7.2. Also for all solutions, 24 mM sucrose was used instead of 27 mM, and the 10K-Hi buffer contained 285 mM sucrose instead of 245 mM. These changes had no noticeable effect on the outcome of the experiments, but they did serve to reduce cell clumping and to produce cells with a more normal cell volume.

Chloride Exchange Flux

High and low chloride cells were incubated with ³⁶Cl (ICN Chemical and Radioisotope Division, Irvine, CA) and isotope exchange at 0°C was measured as previously described (Knauf et al., 1978), except that Aquasol (New England Nuclear, Boston, MA) was used for liquid scintillation counting. To obtain values for the isotope in the supernatant at infinite time, in some experiments a sample of the flux suspension was stored at room temperature for at least 30 min, after which the cells were centrifuged and a sample of the supernatant was counted for radioactivity. This method gave results that were indistinguishable from those obtained using trichloroacetic acid (TCA) precipitation for infinity values. For experiments without a chloride gradient, low chloride cells were suspended in 10K-Lo or high chloride cells in 150K; for experiments with a gradient, high chloride cells were suspended in 10K-Hi. As shown previously (Furuya et al., 1984), under these conditions at 0°C, the pH gradient across the membrane would change by <0.1 unit during the flux measurements when high chloride cells are plunged into low chloride medium. Fluxes were measured over a range of niflumic acid (kindly provided by Squibb Canada, Montreal, Quebec) concentrations from 0 to 5 µM. Niflumic acid was made up as a 5-mM stock in ethanol, and the ethanol concentration in the flux medium was maintained constant at 0.1% (except where otherwise indicated).

For some experiments with high chloride cells, nystatin treatment was omitted and the cells were simply washed with 150K and loaded with ³⁶Cl. For experiments with valinomycin, the valinomycin (Calbiochem-Behring Corp., San Diego, CA) concentration was 10 µM and 1.33% ethanol was present in the flux medium.

Chloride ratios were measured as previously described (Furuya et al., 1984). ID₅₀ values for niflumic acid were determined from either the x-intercept or 1/slope of modified Dixon plots, such as that shown in Fig. 1, as discussed in the text. Membrane potential (*E_m*) values were calculated as described in Furuya et al. (1984), taking into account the variations in the Cl⁻ ratio and assuming the values for intracellular K⁺ indicated in the tables.

Experiments with Reversed Chloride Gradients

Blood was obtained as described above and the red cells were washed three times in 165

mM NaCl. According to the procedure of Jennings (1982), the cells were then resuspended in 100 mM Na₂SO₄, 20 mM Na₃ citrate, pH 6 (S-6), at room temperature and were titrated to pH 6 at room temperature with 1 N HCl. The cells were then washed four times in S-6 buffer. Before each wash, they were incubated 10 min at 37°C to promote exchange of intracellular chloride for sulfate. The cells were then made up to 50% hematocrit in S-6 buffer, and incubated for ~20 min at 37°C with 2 μCi/ml ³⁵SO₄ (Amersham Corp., Arlington Heights, IL). They were then washed four times in ice-cold S-6 buffer and brought back to 50% hematocrit. For flux measurements, cells were added at a final hematocrit of 2% to either S-6 buffer or C-6 buffer (150 mM NaCl, 20 mM Na₃ citrate, pH 6) at 27°C, containing various concentrations of niflumic acid (0–50 μM) and 1% ethanol.

Aliquots of the flux suspensions were centrifuged in an Eppendorf model 3200 Microcentrifuge (Eppendorf Division, Brinkmann Instruments, Westbury, NY) at various times, and the supernatants were counted for radioactivity. For infinity samples, 1 ml of the flux suspension was added to 0.2 ml of 30% TCA, and the supernatant was counted. Corrections for the quenching effect of TCA were made, and fluxes were calculated as previously described (Knauf et al., 1978). For the experiments in C-6 medium, samples were taken within <20% of the *t*_{1/2} for isotope exchange, so that the chloride gradient would not be altered very much during the course of the flux determination. Chloride ratios were measured at various times after addition of cells to C-6 medium as previously described (Furuya et al., 1984).

RESULTS

Effects of Chloride Gradients on Niflumic Acid Inhibition

If niflumic acid binds preferentially to band 3 when it is in the *E*_o or *E*Cl_o form, then changes in internal chloride concentration at constant external chloride should have exactly the same effects on the inhibitory potency of niflumic acid (Appendix, Eq. A12) as on that of H₂DIDS (4,4'-diisothiocyano-1,2-diphenyl-ethane-2,2'-disulfonic acid) or NAP-taurine [*N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate]. That is, when Cl_i > Cl_o, the concentration of niflumic acid required to inhibit chloride exchange by 50%, the ID₅₀, should decrease relative to the value (ID₅₀*) when Cl_i = Cl_o. If, on the other hand, niflumic acid binds preferentially to the *E*_i and *E*Cl_i forms, then the ID₅₀ should increase when Cl_i > Cl_o. Of course, if niflumic acid exhibits no preference and binds equally well to *E*_o, *E*_i, *E*Cl_o, and *E*Cl_i, then there will be no effect of a chloride gradient on the ID₅₀.

Two groups of cells were prepared. High chloride cells were treated with nystatin in a medium with 150 mM chloride, while low chloride cells were treated with nystatin in 10 mM KCl medium, as described in Methods. For some experiments, fresh cells, untreated with nystatin, were used for high chloride cells. High chloride cells were suspended in media with either 150 mM chloride (no gradient) or 10 mM chloride (Cl_i > Cl_o), with sucrose added for osmotic balance. Low chloride cells were suspended in 10 mM chloride medium (no gradient).

The results of such an experiment are shown in Fig. 1 on a modified Dixon plot, on which the slope is equal to 1/ID₅₀ and the *x*-intercept is -ID₅₀. For the high chloride (solid squares) or low chloride (open triangles) cells with Cl_i ≅ Cl_o,

the slopes and intercepts are very similar, as expected if niflumic acid is a noncompetitive inhibitor whose ID_{50} is unaffected by the substrate, chloride. In contrast, the high chloride cells in low chloride medium, with a Cl_i/Cl_o of 15.6 (solid circles), showed a much higher slope, which corresponds to a more than twofold decrease in ID_{50} , and an x -intercept closer to the origin. Thus, the inhibitory potency of niflumic acid was greater in cells with a 15-fold chloride gradient than for cells with nearly equal chloride inside and outside.

Data for several such experiments are summarized in Table I. ID_{50} values were calculated from the x -intercepts and from the reciprocal of the slopes of

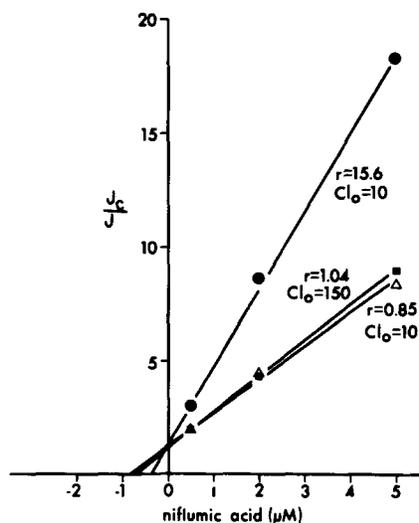


FIGURE 1. Modified Dixon plot of the inhibition of chloride exchange by niflumic acid with different values of r (Cl_i/Cl_o). The control flux, J_c , divided by the flux in the presence of inhibitor, J , is plotted against the niflumic acid concentration in micromolar. Cells were prepared and fluxes were measured as described in Methods. Values for the ID_{50} determined from the x -intercept and $1/\text{slope}$, respectively, are 0.72 and 0.64 for the cells with 156 mM Cl^- in 150 mM Cl^- medium ($r = 1.04$); 0.84 and 0.69 for the cells with 8.5 mM Cl^- in 10 mM Cl^- medium ($r = 0.85$); and 0.38 and 0.29 for the cells with 156 mM Cl^- in 10 mM Cl^- medium ($r = 15.6$).

modified Dixon plots. In almost all cases, the standard deviation for the slope data was smaller than that for the corresponding intercept data, which suggests that the slope method may provide a slightly more accurate value for the ID_{50} . When fresh or nystatin-treated high chloride cells were compared, in no case did both the intercept and slope show a significant difference, so for further analysis the two groups of cells were combined. As can be seen from comparison of condition B with condition A in Table I, when external chloride was 10 mM, both the intercept and slope indicated a highly significant effect of changes in internal chloride on the ID_{50} for niflumic acid. From the slope data, the mean value of the ratio of the ID_{50} with a chloride gradient to that without a gradient (or with a very small gradient), ID_{50}^* , was 0.386. The results are similar to those

obtained with H₂DIDS and NAP-taurine (Knauf et al., 1984), and are compatible with a model in which niflumic acid only binds to band 3 when it is in the E_o or ECl_o form.

Possible Effects of Membrane Potential and pH

Although Cl⁻/OH⁻ and Cl⁻/HCO₃⁻ exchange will cause the interior of high chloride cells to become alkaline when they are suspended in a well-buffered low chloride medium, this pH shift occurs so slowly at 0°C (Furuya et al., 1984) that a <0.1 pH unit shift would occur during the course of these flux measurements.

TABLE I
Effects of Cl_o and Cl_i on the Inhibitory Potency of Niflumic Acid

Condition	Cl _i	Cl _o	Cl _o /Cl _i	x-intercept ID ₅₀	Slope ID ₅₀	E _m
(A) n = 9	142.96* ±5.17	10	0.071 ±0.003	0.288 ±0.027	0.286 ±0.011	+62.5 ±0.9
(B) n = 5	9.40 ±0.80	10	1.094 ±0.088	0.822 ±0.063	0.740 ±0.085	-1.8 ±2.0
(C) n = 15	147.15 ±3.48	150	1.029 ±0.026	0.649 ±0.033	0.603 ±0.020	-0.5 ±0.6
Statistical analysis			A vs. B	A vs. C	B vs. C	
x-intercept						
P			0.0005	<0.0001	0.35	
ID ₅₀ /ID ₅₀			0.350	0.444	1.267	
95%			0.246	0.341	0.966	
Confidence interval			0.487	0.559	1.620	
1/slope						
P			0.006	<0.0001	0.19	
ID ₅₀ /ID ₅₀			0.386	0.474	1.227	
95%			0.286	0.419	0.831	
Confidence interval			0.572	0.534	1.646	

* All values are given as mean ± SEM.

Thus, it is unlikely that changes in intracellular pH play any major role in the increase of niflumic acid inhibition when Cl_i > Cl_o.

Membrane potential changes, on the other hand, caused by net efflux of chloride, should take place rapidly upon exposing the high chloride cells to 10 mM chloride medium. In the preceding papers (Furuya et al., 1984; Knauf et al., 1984), possible effects of membrane potential were tested by reversing the sign of the membrane potential with valinomycin. In the case of niflumic acid, however, this method cannot be used, since even when there is little or no change in membrane potential, addition of valinomycin causes an apparent increase in the ID₅₀ for niflumic acid (compare Table II, part C with Table I, part C). We therefore elected to see whether or not the effect of chloride gradient on the ID₅₀ could be observed in the presence of valinomycin, when the intracellular

and extracellular K^+ concentrations were so manipulated that the membrane would become hyperpolarized when $Cl_i > Cl_o$, rather than depolarized. Since niflumic acid is a noncompetitive inhibitor, and since the half-time for chloride exchange is longer in cells with 150 mM Cl^- than in cells with 10 mM Cl^- , which permits more accurate flux measurements, we compared cells with high chloride inside and outside to cells with a chloride gradient.

The results are shown in Table II. For both the cells with and without a chloride gradient, the ID_{50} values in the presence of valinomycin were slightly higher than the corresponding values in the absence of valinomycin (Table I). In the experiments without valinomycin (Table I), the membrane potential changed from about -1.8 mV without a gradient to 62.5 mV with $Cl_i > Cl_o$. In the presence of valinomycin, the potential changed from either 55.7 (B) or 0 (C) mV

TABLE II
Effects of Chloride Gradient on the ID_{50} for Niflumic Acid with $10 \mu M$ Valinomycin Present

Condition	K_i	Cl_i	Cl_o^*	Cl_o/Cl_i	x-intercept ID_{50}	Slope ID_{50}	E_m
(A) $n = 6$	150	135.66 [†] ± 5.77	10	0.074 ± 0.003	0.440 ± 0.027	0.445 ± 0.018	-56.9 ± 0.6
(B) $n = 3$	10	155.20 ± 2.41	150	0.967 ± 0.013	0.810 ± 0.055	0.797 ± 0.026	$+55.7$ ± 0.0
(C) $n = 2$	150	152.71 ± 4.36	150	0.985 ± 0.025	0.935 ± 0.055	0.915 ± 0.085	0.0 ± 0.0
(B + C) $n = 5$		154.20 ± 2.00	150	0.974 ± 0.012	0.860 ± 0.047	0.844 ± 0.042	
Statistical analysis				A vs. B + C x-intercept	A vs. B + C 1/slope		
P				0.0002	0.0003		
ID_{50}/ID_{50}				0.512	0.527		
95%				0.406	0.441		
Confidence interval				0.641	0.634		

* $K_o = Cl_o$.

† Values are given as mean \pm SEM.

without a gradient to -56.9 mV with a chloride gradient (A). Thus, the direction of the potential change associated with a chloride gradient was reversed in the presence of valinomycin. Yet even with valinomycin, the chloride gradient caused almost a twofold decrease in the ID_{50} for niflumic acid, which was significant at the $P < 0.0005$ level. These results strongly argue that changes in membrane potential are not the primary cause for the increased inhibitory effect of niflumic acid when $Cl_i > Cl_o$, and thus that the change in internal chloride concentration itself is probably the direct cause of the altered inhibitory potency.

Experiments with Reversed Chloride Gradients

The experiments above show that niflumic acid binds preferentially to E_o and E_{Cl_o} , but they do not address the question of whether or not niflumic acid can

bind to the E_i and E_{Cl} forms and, if so, with what affinity. To measure this, we made use of Jennings' (1980, 1982) technique for recruiting nearly all of the band 3 molecules into either the inward- or outward-facing form.

Since sulfate is transported much more slowly than chloride, if sulfate is present at the inside of the membrane and chloride at the outside, inward transport of chloride will occur rapidly. Once the transport site is in the inward-facing conformation, however, it will become loaded with sulfate and will therefore return very slowly to the outward-facing form. Thus, nearly all of the transport sites will be inward-facing under these conditions, and the inhibitory potency of niflumic acid should reflect its affinity for the inward-facing form of band 3. This can be compared with a situation in which sulfate is present at both sides of the membrane, when the ID_{50} will reflect the affinity of niflumic acid for both inward- and outward-facing forms, weighted by the proportion of those forms present (see Appendix, Eq. A23). We therefore measured the inhibitory effect of niflumic acid on sulfate efflux under both of these circumstances, at pH 6.0 and 27°C, so that the sulfate flux would be large enough to measure.

TABLE III
Effect of a Reversed Chloride Gradient ($Cl_o > Cl_i$) on the ID_{50} for Niflumic Acid in Sulfate-loaded Cells at 27°C

Experiment	ID_{50} (μM) for niflumic acid			
	Sulfate medium (S-6)		Chloride Medium (C-6)	
	x-intercept	1/slope	x-intercept	1/slope
N-108	10.55	8.34	73.6	68.9
N-110	8.94	7.04	44.1	39.2
N-111	7.74	5.71	48.3	38.4
Mean	9.08	7.03	55.3	48.8
SEM	0.81	0.76	9.2	10.0

The results of three such experiments are shown in Table III. In each case, the ID_{50} for niflumic acid with chloride outside was much higher than with sulfate inside and outside, which indicates a strong preference of niflumic acid for the form of band 3 with the transport site facing outward. The mean ID_{50} with an inwardly directed chloride gradient is nearly seven times larger than without a gradient. While this fact alone would indicate that the affinity for niflumic acid of the inward-facing form is much lower than that of the outward-facing form, the true affinity difference is much greater than that suggested by the ratio of ID_{50} values. Even with sulfate inside and outside, most of the transport sites face the cytoplasm, so the true affinity of the outward-facing form is greatly underestimated by the ID_{50} under these circumstances. From comparisons of sulfate influx into chloride or sulfate-loaded cells, Jennings (1982) calculates that at pH 6 and 27°C only one-eighth of the transport sites face outward, so there are seven times as many inward-facing as outward-facing sites under these circumstances. Our measurements of sulfate efflux into chloride or sulfate media are compatible with this estimate. When this intrinsic asymmetry is taken into

account, one can calculate from Eq. A25 that the affinity of niflumic acid for the E_o and ECl_o forms is 48.5 times as great as its affinity for the inward-facing forms. Even this very large difference may still be an underestimate, since chloride ratio measurements at different times during the flux measurements indicate that the chloride gradient decays rapidly, so even the weak effects of niflumic acid with an inwardly directed chloride gradient may be due to some interaction with the E_o and ECl_o forms, which gradually increase as the gradient dissipates.

Model for Niflumic Acid Inhibition of Chloride Exchange

A model for the action of niflumic acid on the anion exchange system is shown in Fig. 2. Because chloride has no apparent effect on niflumic acid binding, niflumic acid must bind to a site different from the transport site and different from the modifier site (at which chloride inhibits). Niflumic acid binds with high affinity only to the E_o or ECl_o forms of the band 3 protein, and not at all or with about 50-fold lower affinity to the forms of band 3 with the transport site facing inward. Thus, when the transport site is in the inward-facing conformation, part or all of the niflumic acid binding site must be blocked, as shown on the right side of Fig. 2. Since niflumic acid is a noncompetitive inhibitor, it must block the conformational change from ECl_o to ECl_i . As it binds preferentially to E_o and ECl_o , it will therefore trap the band 3 protein in these forms, at the expense of the E_i and ECl_i forms.

Asymmetry of the Chloride-loaded Transport Sites

Because it binds preferentially to E_o and ECl_o , niflumic acid can be used to determine whether or not the loaded sites asymmetry ratio, L , is the same or different from the unloaded sites asymmetry ratio, A . Fig. 3 shows schematically how this can be done, for a system with a five-to-one asymmetry in favor of the unloaded sites ($A = 0.2$), by measuring the inhibitory potency of niflumic acid as a function of chloride concentration, keeping $Cl_i = Cl_o$. At very low chloride concentrations, when most of the sites are unloaded (left-hand panel), the inhibitory potency of niflumic acid will depend on the ratio E_o/E_i , that is, on A . At higher chloride concentrations, when most of the sites are loaded, the inhibition will depend on ECl_o/ECl_i , that is, on L . If $L = 1$, as in the middle panel, there will be more outward-facing sites when the chloride concentration is increased, and hence the inhibition by niflumic acid will increase. If, on the other hand, $L = A$, as in the right-hand panel, there will be no change in inhibitory potency as the chloride concentration is increased.

The calculated effect of changes in chloride concentration on the ID_{50} for niflumic acid for various L values, assuming that $A = 0.0638$ and $K_c = 65$ mM, is shown in Fig. 4. If $L = 1$, the ID_{50} at 10 mM Cl^- should be 3.09 times the value at 150 mM Cl^- . Note that the most dramatic effects are seen at very low Cl^- concentrations. As L decreases toward A , the effect of chloride concentration decreases until when $L = A$, the ID_{50} does not change with Cl^- . If L is less than A , that is, if the loaded sites are more asymmetrically distributed than the

unloaded sites, then the ID_{50} will actually decrease as the chloride concentration is lowered from 150 to 10 mM.

The data in Table I (C vs. B) show no significant effect of changing the chloride concentration from 150 to 10 mM on the ID_{50} for niflumic acid. These results are incompatible with the idea that $L = 1$, since no threefold increase in ID_{50} was observed. Thus, the data are inconsistent with the hypothesis that the chloride dissociation constants K_i and K_o entirely account for the asymmetry of

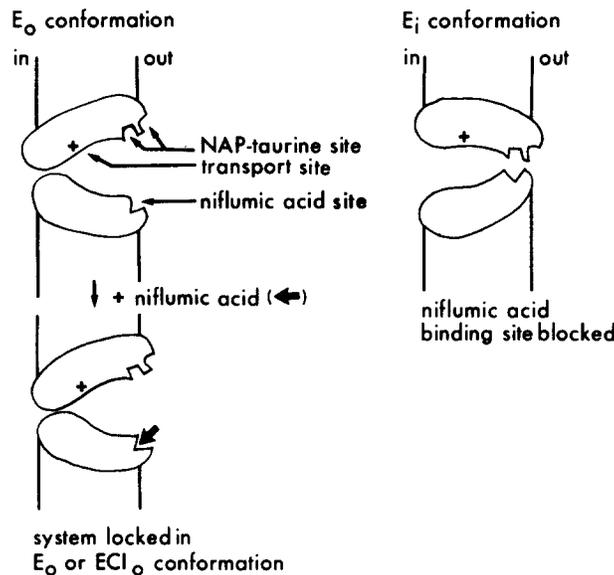


FIGURE 2. Model for niflumic acid interaction with anion exchange system. The transport-related conformational change from outward-facing (E_o) to inward-facing (E_i) is shown schematically as involving the reorientation of two domains of a single band 3 monomer. Niflumic acid binds to a site separate from the transport site. Thus, niflumic acid can bind (left-hand side) to both the E_o form (shown) and the chloride-loaded ECl_o form (not shown) of band 3. When band 3 is in the E_i or ECl_i form (right-hand side), the niflumic acid site is blocked (or the affinity for niflumic acid is greatly reduced). Thus, niflumic acid binding can be used to sense the conformation of the loaded and unloaded transport sites. Since niflumic acid binds preferentially to E_o and ECl_o , and since it presumably inhibits transport by blocking the conformational change from ECl_o to ECl_i , niflumic acid probably locks the band 3 protein in the E_o or ECl_o form.

unloaded sites. The small but insignificant increase in ID_{50} observed from the slope data would suggest an L value of 0.1 or less, quite near to the value of A (0.0638). This would correspond to a less than twofold ratio of K_i to K_o . Thus, the asymmetry is clearly dominated by the ratio of k to k' . The simplest hypothesis compatible with the data would be that the asymmetry is entirely due to differences in the rate constants, with k' being more than 15 times greater than k , and with the chloride dissociation constants being identical at the two sides of the membrane.

niflumic acid, with $Cl_i = Cl_o$, should decrease with increasing chloride concentration. This same result is obtained if, as we have recently shown (Knauf and Mann, 1984; Knauf et al., 1984), intracellular rather than extracellular Cl^- binds to the modifier site, provided that Cl^- binds only when the transport site faces outward.

Fig. 6 shows the results of such experiments by Cousin and Motais (1979), replotted as ID_{50} values. The correlation coefficient squared is 0.011, and the slope of a best-fit line is 0.00007 ± 0.00017 SD, not significantly different from zero. This contrasts with the significant decrease in ID_{50} to be expected if binding of chloride to the modifier site were to occur only when the transport site is in the E_o or ECl_o form (dashed and dotted lines in Fig. 6).

In view of the fact that intracellular Cl^- binds to the modifier site, it could be possible that such binding takes place only when the transport site faces inward. In this case, assuming a dissociation constant for Cl^- at the modifier site of 335

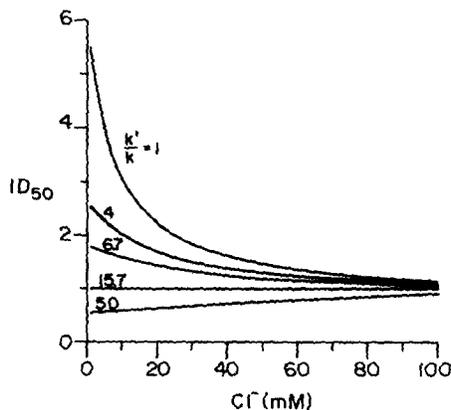


FIGURE 4. Predicted effect of chloride concentration (with $Cl_i = Cl_o$) on the ID_{50} for niflumic acid. For these calculations, it is assumed that $1/A = 15.7$ (Gunn and Fröhlich, 1979) and that $K_c = 65$ mM (Brazy and Gunn, 1976). The ID_{50} for niflumic acid, divided by the value of ID_{50} at 150 mM chloride, is plotted against the chloride concentration in millimolar. The lines show the predicted results for various values for the loaded site asymmetry, $ECl_i/ECl_o = k'/k = 1/L$.

mM (Dalmark, 1976), the ID_{50} value at 600 mM Cl^- would be 2.7 times its value at 0 mM Cl^- (Eq. A19), in sharp contrast to the experimental data.

One further case needs to be considered: if internal Cl^- binds to the modifier site only when the transport site faces inward, but external Cl^- binds to the NAP-taurine site only when the transport site faces outward (Knauf et al., 1984), and if the chloride dissociation constants for these two different sites are equal, increases in Cl^- concentration with $Cl_i = Cl_o$ will have equal but opposite effects on the transport site conformation, predicting no change in the ID_{50} for niflumic acid (Eq. A20), as was observed. This possibility, however, seems rather unlikely. If one supposes that the modifier site has a Cl^- dissociation constant of 335 mM (Dalmark, 1976), while the NAP-taurine site has a Cl^- dissociation constant of 165 mM (Knauf et al., 1978), then at 600 mM Cl^- the ID_{50} for niflumic acid would be 63% of the value at 0 mM Cl^- , a prediction that is still in disagreement

with the experimental results in Fig. 6. Thus, it seems highly probable that the binding of Cl^- to the modifier site is not affected by the conformation of the transport site.

DISCUSSION

Preferential Binding of Niflumic Acid to E_o and ECl_o

The data presented above show that changes in internal chloride concentration strongly affect the inhibitory potency of niflumic acid (Table I and Fig. 1). Although niflumic acid fluxes have not been measured, from the rates of penetration of similar carboxylic acids it would seem likely that there is little

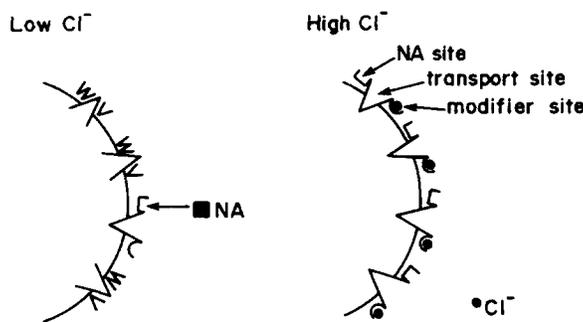


FIGURE 5. Predicted effects of chloride binding to the modifier site on the orientation of the transport site and on the inhibitory potency of niflumic acid, if chloride binds to the modifier site only when the transport site faces outward. The diagram is shown with external Cl^- binding to the modifier site, as originally proposed by Schnell et al. (1978). If, as more recent evidence indicates (Knauf and Mann, 1984), internal Cl^- binds to the modifier site, the predicted effects on transport site orientation are exactly the same, provided that Cl^- binds to the modifier site only when the transport site faces outward. At low chloride concentrations, most of the transport sites face inward, and thus few band 3 molecules are in the proper conformation for binding of niflumic acid to its site or for binding of chloride to the modifier site. At very high chloride concentrations, sufficient to cause binding of chloride to most of the modifier sites, by the law of mass action more transport sites will be recruited toward the outward-facing conformation. Since this is the form of band 3 which can bind niflumic acid, the inhibitory potency of niflumic acid will increase (see Eq. A18).

influx of niflumic acid during the short (<3 min) flux measurements at 0°C . Thus, there is probably a transmembrane effect of internal chloride on the binding of external niflumic acid. Membrane potential shifts do not seem to cause the change in inhibitory potency, since the same effects are seen when the direction of the membrane potential changes is reversed (Table II). The observations fit well to the predictions of the ping-pong model for anion exchange, if it is assumed that niflumic acid only binds to band 3 when the transport site faces outward (Fig. 2). Since niflumic acid inhibits the conformational change, it must trap the band 3 protein in the form with the transport site facing outward.

Niflumic acid thus joins a list of inhibitors, including H₂DIDS (Furuya et al., 1984), DIDS (Grinstein et al., 1979), NAP-taurine (Knauf et al., 1984), phlorizin, and positively charged furosemide derivatives (Passow et al., 1980*a, b*), all of which bind preferentially to band 3 when the transport site faces outward and/or which recruit the system toward the outward-facing form.

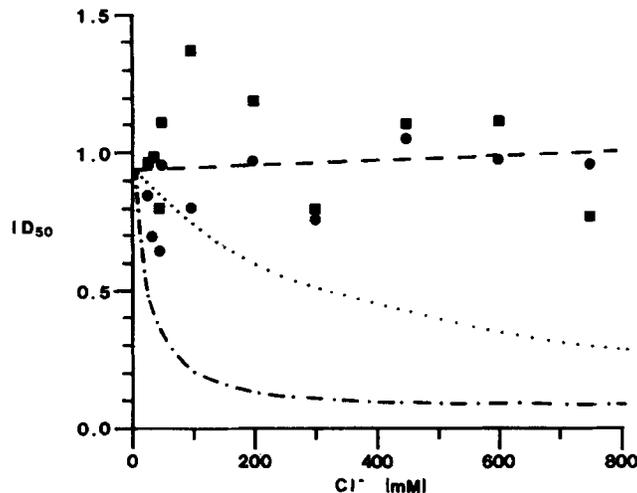


FIGURE 6. Comparison of predicted and actual effects of high chloride concentrations on the ID₅₀ for niflumic acid. Data are taken from Fig. 9 of Cousin and Motais (1979). The original values for inhibition of chloride exchange at 0.5 μ M (■) and 2 μ M (●) niflumic acid were recalculated as ID₅₀ values, using the formula $ID_{50} = I(1 - i)/i$, where I is the niflumic acid concentration and i is the fractional inhibition of chloride flux. These ID₅₀ values were then divided by the average of the values above 400 mM Cl and are plotted against the chloride concentration in millimolar. The dashed line shows the least-squares best fit line for the data points, which had a slope of 0.00007 ± 0.00017 SD, not significantly different from zero. The value of the correlation coefficient squared (r^2) was 0.011 and the y-intercept was 0.927 ± 0.060 SD. The dashed-dotted line was calculated assuming that chloride only binds to the modifier site when the transport site faces outward (Eq. A18) and that the apparent dissociation constant for chloride at the modifier site is 335 mM (Dalmark, 1976). Because most of the modifier sites are not accessible to chloride according to this model, this corresponds to a value for K_i of 20.2 mM. If one assumes that K_i is actually 335 mM, the dotted line is obtained. In both cases, the predictions assuming that chloride binds to the modifier site only when the transport site faces outward are inconsistent with the experimental data.

Source of Intrinsic Asymmetry in the Transport System

Both our data (Table I) and that of Cousin and Motais (Fig. 6), when compared with theoretical predictions for different values of the loaded sites asymmetry ratio, L (Fig. 4), indicate that $L \cong A$. Since $A = kK_o/k'K_i$ and $L = k/k'$, this implies that $K_i \cong K_o$. Thus, A becomes approximately equal to k/k' , and the asymmetry in the transport system is dominated by differences in the rate constants for the

conformational change from inward-facing to outward-facing and vice versa, with k being 15 times less than k' . In terms of equilibrium thermodynamics, a 15-fold ratio of k' to k would suggest that the ECl_i form of band 3 has a Gibbs free energy that is 1.5 kcal/mol lower than that of the ECl_o form. The large apparent asymmetry in the transport system can thus be attributed to a comparatively small free energy difference between the two conformations of band 3, a difference that is not unexpected for two conformations of a fairly complex protein. The asymmetry may play no functional role in transport, but may simply be a consequence or side effect of the fact that band 3 can exist in two conformations, which simply happen to differ in their free energy.

These conclusions are based on the assumptions that the binding of Cl^- to the transport site at the two sides of the membrane is at or very near equilibrium, and that the rate-limiting step in transport is the conformational change from ECl_i to ECl_o or vice versa. This is very likely to be the case, but has not yet been proven to be so for Cl^- . O. Fröhlich (personal communication) has made similar calculations for the "hexagon" ping-pong model of chloride exchange (Fröhlich, 1982; Fröhlich et al., 1983), in which the rates of anion association and dissociation from the transport sites are taken into account. From these calculations, Eq. A16 can be derived by making the assumption that the rates for Cl^- dissociation from the transport site at both inside and outside are much faster than the rate constants for the transport-related conformational change (k and k'). It is thus only necessary that the dissociation rates be rapid in order for the invariance of niflumic acid ID_{50} with Cl^- concentration to provide evidence that $A \approx L$.

Passow et al. (1980b) have observed effects of chloride concentration (with $Cl_i = Cl_o$) on the reaction of FDNB (1-fluoro-2,4-dinitro-benzene) with a lysine residue (Lys-a) near the transport site, which they have interpreted to mean that $A \neq L$. At very low chloride concentrations, reaction of FDNB is slow, which is attributed to the fact that most of the transport sites face toward the cytoplasm (*trans*), so $A \ll 1$. At higher chloride concentrations, the reaction rate increases, which Passow et al. suggest correlates with an increase in the outward-facing (*cis*) form. Since this effect is unlikely to represent a direct electrostatic interaction between chloride and the site of FDNB reaction (Passow et al., 1980a), it probably indicates some change in conformation of the band 3 protein. If indeed the conformational change that FDNB senses involves a reorientation of transport sites, such that more transport sites face the outside when chloride is increased, then these data would suggest that $K_i > K_o$ (or that $K_{101} > K_{111}$, in the terminology of Passow et al., 1980b). This would contradict our finding that $K_i \approx K_o$.

Interestingly, Passow et al. (1980a) report that a half-maximal effect of chloride is seen at a concentration close to K_c , the chloride concentration that half-saturates the chloride transport system (with $Cl_i = Cl_o$). This may suggest that the change in FDNB reaction with Lys-a is related to a conformational change which occurs upon binding of chloride, especially since work with electron spin resonance probes has provided evidence for such an effect (Ginsburg et al., 1982). If the change in FDNB reactivity were related to a change in transport site orientation, the effect should be seen at much lower chloride concentrations, as shown in Fig. 4. A problem with the FDNB method is that it can potentially

sense conformational changes related to either chloride binding or transport site reorientation. Our data can be reconciled with that of Passow et al. (1980*a, b*) if FDNB is sensing the former when chloride is increased.¹ The niflumic acid method has the advantage that since niflumic acid is a noncompetitive inhibitor, its binding is by definition insensitive to the binding of chloride, so it can provide information about transport site orientation uncontaminated by effects caused by the binding of chloride. Thus, we believe that the results with niflumic acid are more likely to provide correct information about the nature of the asymmetry of the transport system than are the measurements of FDNB reaction with Lys-a. A final resolution of this question, however, will require further studies of possible artifacts in the two methods.

One problem with all methods for determining the relative values of A and L is that they all depend to some extent on the value of A . We have used the A value of 0.0638 determined from the data of Gunn and Fröhlich (1979), as this seems to be the most direct and accurate means of determining A . It should be noted, however, that many other ways of measuring A (Knauf et al., 1984), including the niflumic acid data presented here (Table I), would suggest less asymmetry, with A possibly being as great as 0.2. This would, of course, affect the predicted dependence of ID_{50} on chloride concentration for different L values, and would suggest an L value nearer to 1 than that calculated with $A = 0.0638$. Thus, the conclusions about the nature of the transport system asymmetry are dependent on the value of A , and may have to be revised if new information should lead to a different estimate of A . Even if this should be the case, the niflumic acid data would still be useful in determining a new value for the A/L ratio.

It should also be noted that since L depends on the rate constants for a conformational change which occurs with anions bound to the transport site, these rates may differ for different anions. Thus, the conclusion that $A \cong L$ is valid only for chloride. Jennings' (1980, 1982) data would indicate that when the transport system is largely saturated with sulfate, the L ratio for sulfate is <1 . Thus, for sulfate as well as chloride, most of the loaded transport sites seem to face the inside. On the other hand, Gunn and Fröhlich (1979) have concluded from bromide-chloride exchange experiments that the maximum velocities for inward and outward translocation of bromide are very similar. This would suggest that L may be near 1 for bromide, and since A does not depend on the

¹ Another possible explanation for the differences between our data and that of Passow et al. (1980*a*) could stem from the different temperatures at which the experiments were done, 0°C in our case and 37°C in theirs. To explain the FDNB data on the basis that the unloaded sites are more asymmetrically distributed than the loaded sites, the K_i/K_o ratio would have to be larger at 37°C than at 0°C (where $K_i \cong K_o$). This could hardly be due to an increase in K_i , since at 0°C K_c is nearly equal to K_i (see Eq. A8 for the case where $k' \gg k$ and $K_i = K_o$), and K_c shows little temperature dependence (Brahm, 1977). A decrease in K_o with increasing temperature is also unlikely, since a low K_o value would predict effects on FDNB reactivity at much lower Cl^- concentrations than K_c , in contrast to the observations. Thus, while further work needs to be done on the temperature dependence of anion transport asymmetry, it seems unlikely that temperature effects are responsible for the differences between our conclusions and those of Passow et al. (1980*a*).

nature of the monovalent anion in the system, A would still be 0.0638 and would therefore be unequal to L . We have attempted to confirm this by performing experiments in bromide media similar to those reported in Table I, but difficulties in obtaining linear Dixon plots for niflumic acid in bromide media precluded analysis of the data.

Involvement of the Modifier Site in Transport

The data of Cousin and Motais (1979) in Fig. 6, when considered in light of theoretical predictions (Appendix, Eqs. A11 and A18), demonstrate that chloride binding to the modifier site is unaffected by the conformation of the transport site. In our analysis, we have assumed that when chloride is bound to the modifier site, transport is completely inhibited. If we assume, as do Passow et al. (1980b), that there is some chloride transport even with the modifier site fully occupied, then at very high chloride concentrations the ratio of outward-facing to inward-facing transport sites will depend on the rates at which the conformational change from inward-facing to outward-facing or vice versa occurs with the modifier site occupied. If transport at very high chloride does reflect such partial inhibition, then the niflumic acid data would provide evidence that the relative rates for translocation inward and outward with the modifier site occupied are not very different from the relative rates with the modifier site empty.

Passow et al. (1980b) have suggested that there are really two conformations of the modifier site, designated R and S, corresponding to states in which the transport site faces inward (*trans*) or outward (*cis*), respectively. Passow and Fasold (1981) note, however, that in both of these states the modifier site may face the same side of the membrane. Our evidence indicates that the two states have very similar chloride affinities. These results do not rule out the possibility that the modifier site may play some role in the transport-related conformational change. For example, it might be involved in forming the transition state between the inward-facing and outward-facing states. It is clear, however, that the final conformations of the modifier site in the two forms of band 3 (inward- and outward-facing transport site) must not differ substantially, since chloride can bind equally well in both states. This would rule out models in which the state of the modifier site changes depending on the conformation of the transport site (Macara and Cantley, 1981, 1983).

Our data also indicate that the binding of extracellular Cl^- to the external NAP-aurine site (Knauf et al., 1978; Knauf and Mann, 1984) is unaffected by the conformation of the transport site. This may seem paradoxical, since binding of external NAP-aurine to this site is strongly affected by the transport site orientation (Knauf et al., 1984). These findings can be rationalized, however, when one considers the fact that external NAP-aurine has a much higher affinity for this site than does external Cl^- . The additional noncovalent interactions which are involved in the tight binding of NAP-aurine may thus be affected by transport site orientation, while the portion of the binding site that interacts with Cl^- may be unaffected. This is indicated in Fig. 2 and also in Fig. 7 of the preceding paper, where the NAP-aurine binding site is shown as being composed of two parts, only one of which is affected by the transport site conformation.

Implications for Future Work

As Passow et al. (1980*b*) have pointed out, in general the fraction of outward-facing transport sites will depend on the chloride concentration. At low chloride concentrations, it will depend on A , at higher chloride concentrations on L , and at very high chloride concentrations on the properties of the form of band 3 with chloride bound to the modifier site. Our evidence suggests that in the case of chloride, the distribution of transport sites is very similar under all these circumstances, with far more sites facing inside than outside.

This is very useful from an experimental standpoint, since there are many ways of artificially forcing the system into the outward-facing form, but comparatively few ways of achieving the opposite orientation. Thus, both chloride gradients and chemical probes can be used to perturb the orientation of the transport sites, so that more sites face outward than in the native situation. These changes in transport site orientation can then be probed by using proteolytic enzymes or other probes of protein conformation, to gain further information regarding the transport-related conformational change and, ultimately, the molecular mechanism of the transport process. Conversely, the methods developed here for measuring the asymmetry in the transport site orientation can be used to determine the effects of various partial inhibitors or agents that perturb protein structure on the asymmetry of the transport system and on the individual rate constants and dissociation constants.

APPENDIX

Definitions

As in the preceding papers, (Furuya et al., 1984; Knauf et al., 1984), the band 3 protein (E) is assumed to exist in two conformations: E_i , in which the transport site faces inward (toward the cytoplasm), and E_o , in which the transport site faces the outside medium. Cl to the right of E designates binding of Cl^- to the transport site. The dissociation constants for Cl^- at the inside and outside are defined as:

$$K_i = \frac{(E_i)(Cl_i)}{ECl_i} \quad \text{and} \quad K_o = \frac{(E_o)(Cl_o)}{ECl_o}. \quad (A1)$$

Binding of Cl^- to the modifier site is designated by placing Cl to the left of E . Assuming that internal Cl^- binds to the modifier site (Knauf and Mann, 1984; see Knauf et al., 1984), regardless of the conformation of the transport site, the dissociation constant, K_z , is defined as:

$$K_z = \frac{(E_o)(Cl_i)}{ClE_o} = \frac{(E_i)(Cl_i)}{ClE_i} = \frac{(ECl_o)(Cl_i)}{ClECl_o} = \frac{(ECl_i)(Cl_i)}{ClECl_i}. \quad (A2)$$

Niflumic acid in the medium (N) is assumed to bind to a site different from both the transport and modifier sites:

$$K_n = \frac{(E_o)(N)}{E_oN} = \frac{(ECl_o)(N)}{ECl_oN} = \frac{(ClE_o)(N)}{ClE_oN} = \frac{(ClECl_o)(N)}{ClECl_oN}. \quad (A3)$$

Since the net chloride flux is very small relative to the chloride exchange under all

conditions studied here, the unidirectional efflux, J_o , is assumed to be equal to the unidirectional influx, J_i . Thus,

$$J_o = kECl_i = J_i = k'ECl_o, \quad (A4)$$

where k is the rate constant for the conformational change from ECl_i to ECl_o and k' is the rate constant for the change from ECl_o to ECl_i . The asymmetry ratio for the loaded transport sites, L , can therefore be defined as:

$$L = \frac{ECl_o}{ECl_i} = \frac{k}{k'}. \quad (A5)$$

As in the preceding paper (Knauf et al., 1984), the asymmetry ratio for the unloaded sites, A , is defined as:

$$A = \left(\frac{E_o}{E_i} \right)_{Cl_i=Cl_o} = \frac{kK_o}{k'K_i}. \quad (A6)$$

The maximum flux, J_m , assuming no modifier site inhibition, is:

$$J_m = kE_t/(1 + k/k'), \quad (A7)$$

where E_t is the total amount of the anion exchange protein, band 3. K_c , the concentration of Cl^- that half-saturates the transport system when $Cl_o = Cl_i$, is given by (Knauf et al., 1984):

$$K_c = \frac{K_i k' + K_o k}{k' + k}. \quad (A8)$$

Effects of Cl_i on the ID_{50} for Niflumic Acid

CASE 1 Suppose that niflumic acid binding only takes place when the transport site faces outward. Then the equation for total band 3 is:

$$E_t = E_i + E_o + ECl_i + ECl_o + E_oN + ECl_oN + ClE_i + ClE_o + ClECl_i + ClECl_o + ClE_oN + ClECl_oN. \quad (A9)$$

Terms for the binding of Cl^- to the external NAP-aurine site (Knauf et al., 1984) have not been included, as these make no difference in the final form of the equations obtained so long as the binding of external Cl^- to this site does not depend on the orientation of the transport site (as seems to be the case). Even if the binding of external Cl^- does depend on the orientation of the transport site, no effects will be seen at external Cl^- concentrations that are low relative to the dissociation constant of the external Cl^- site (~ 165 mM; Knauf et al., 1978). This is true for most of the experiments presented; the case of high chloride concentrations is discussed at greater length below. Substituting Eqs. A1-A4 and A7 into A9, and solving for $1/J_o$, we obtain:

$$\frac{1}{J_o} = \frac{1}{J_m(1 + k/k')} \left[\frac{K_i}{Cl_i} + \frac{K_o k}{Cl_o k'} + 1 + \frac{k}{k'} + \frac{NK_o k}{K_n Cl_o k'} + \frac{N}{K_n} \frac{k}{k'} \right] \left[1 + \frac{Cl_i}{K_s} \right]. \quad (A10)$$

When the niflumic acid concentration is equal to the ID_{50} , the concentration that inhibits Cl^- exchange by 50%, the terms containing niflumic acid must be equal to the other terms in the same brackets. Thus:

$$ID_{50} = K_n \left[\frac{Cl_o k'}{K_o k + Cl_o k} \left(\frac{K_i}{Cl_i} + 1 \right) + 1 \right]. \quad (A11)$$

Forming the ratio of ID_{50} to ID_{50}^* , the value of ID_{50} when $Cl_o = Cl_i$, and substituting Eqs. A6 and A8, if Cl_o is constant and Cl_i is varied, we obtain:

$$\frac{ID_{50}}{ID_{50}^*} = \frac{(Cl_o/Cl_i) + A + (Cl_o/K_c)(1 + A)}{1 + A + (Cl_o/K_c)(1 + A)}. \quad (A12)$$

This is exactly the same equation that was obtained for H_2DIDS (Furuya et al., 1984) and for NAP-taurine (Knauf et al., 1984). Thus, regardless of whether a probe competes with Cl^- for the transport site (H_2DIDS) or a different site (NAP-taurine), or behaves as a completely noncompetitive inhibitor (niflumic acid), if it binds only when the transport site faces outward, variations of Cl_i at constant Cl_o have exactly the same effect on the ID_{50} . This is also true for an uncompetitive inhibitor (as pointed out by one of the reviewers of this paper) and probably for many other more complex types of inhibition as well.

CASE II Niflumic acid binds to band 3 regardless of the transport site conformation. In this case, the equation for $1/J_o$ becomes:

$$\frac{1}{J_o} = \frac{1}{J_m(1 + k/k')} \left[\frac{K_i}{Cl_i} + \frac{K_o k}{Cl_o k'} + 1 + \frac{k}{k'} \right] \left[1 + \frac{Cl_i}{K_z} \right] \left[1 + \frac{N}{K_n} \right]. \quad (A13)$$

When $N = ID_{50}$, the term in niflumic acid in the right-hand bracket (N/K_n) must equal 1, so:

$$ID_{50} = K_n. \quad (A14)$$

Since K_n is constant, a variation in neither Cl_i nor Cl_o will have any effect on the ID_{50} .

Effects of Chloride Concentration on ID_{50} with $Cl_o = Cl_i$

If niflumic acid binds to all forms of band 3, ID_{50} will not vary with Cl^- concentration (Eq. A14). If, however, niflumic acid binds only when the transport site faces outward, in general changes in Cl^- concentration (with $Cl_i = Cl_o$) will affect the ID_{50} , particularly at low chloride concentrations. From Eq. A11, if $Cl_o = Cl_i$:

$$ID_{50} = K_n + (K_n k' / k)(1 + K_i / Cl_o) / (1 + K_o / Cl_o). \quad (A15)$$

In the special case where the unloaded sites asymmetry ratio, A , is equal to the loaded sites asymmetry, L , however, $K_i = K_o$ (compare Eqs. A5 and A6). Thus, the terms containing Cl_o cancel out and:

$$ID_{50} = K_n(1 + k'/k). \quad (A16)$$

Since K_n , k , and k' are constants, the ID_{50} is constant as the chloride concentration is varied.

Sensitivity of the Modifier Site to the Conformation of the Transport Site

All of the above derivations have been based on the assumption that Cl_i binds equally well to the modifier site, regardless of the transport site conformation. If, however, internal Cl^- only binds to the modifier site when the transport site faces outward, then the equation for $1/J_o$ becomes:

$$\frac{1}{J_o} = \frac{1}{J_m(1 + k/k')} \left[\frac{K_i}{Cl_i} + 1 + \left(\frac{K_o k}{Cl_o k'} + \frac{k}{k'} \right) \left(1 + \frac{Cl_i}{K_z} \right) \left(1 + \frac{N}{K_n} \right) \right]. \quad (A17)$$

Setting the terms in niflumic acid equal to the terms without niflumic acid, and solving for the ID_{50} , with $Cl_i = Cl_o$:

$$ID_{50} = K_n \left[1 + \frac{k'}{k} \left(1 + \frac{K_i}{Cl_o} \right) / \left(1 + \frac{K_o}{Cl_o} \right) \left(1 + \frac{Cl_o}{K_z} \right) \right]. \quad (A18)$$

Thus, even if $K_i = K_o$, the ID_{50} will decrease at high values of Cl_o , because of the Cl_o/K_z term in the denominator. This effect will be most pronounced if $k'/k > 1$, as it seems to be for this system. Since $Cl_i = Cl_o$, the same expression is obtained regardless of whether internal or external Cl^- is assumed to bind to the modifier site.

If, on the other hand, internal (or external) Cl^- binds to the modifier site only when the transport site faces inward, the corresponding expression for the ID_{50} is:

$$ID_{50} = K_n \left[1 + \frac{k'}{k} \left(1 + \frac{K_i}{Cl_o} \right) \left(1 + \frac{Cl_o}{K_z} \right) / \left(1 + \frac{K_o}{Cl_o} \right) \right]. \quad (A19)$$

In this case, even with $K_i = K_o$, the term in Cl_o in the numerator will cause ID_{50} to increase with increasing Cl^- concentration, as more band 3 molecules are oriented into the inward-facing (E_i , ECl_i , $ClECl_i$, . . .) forms, which have a low affinity for niflumic acid.

A special case occurs when it is assumed that internal Cl^- binds to the modifier site only when the transport site faces inward, but external Cl^- binds to the NAP-aurine site (with dissociation constant K_y) only when the transport site faces outward. For this case, the ID_{50} (with $Cl_i = Cl_o$) is given by:

$$ID_{50} = K_n \left[1 + \frac{k'}{k} \left(1 + \frac{K_i}{Cl_o} \right) \left(1 + \frac{Cl_o}{K_z} \right) / \left(1 + \frac{K_o}{Cl_o} \right) \left(1 + \frac{Cl_o}{K_y} \right) \right] \quad (A20)$$

Obviously, if $K_i = K_o$ and $K_z = K_y$, the ID_{50} will be invariant with Cl^- concentration. If $K_z < K_y$, the ID_{50} will increase with Cl^- ; if $K_z > K_y$, the ID_{50} will decrease.

Experiments with Reversed Chloride Gradient

With chloride outside and sulfate inside, as Jennings (1980) has pointed out, virtually all of the transport sites will face the inside (cytoplasm), since inward Cl^- transport is rapid but outward SO_4^{2-} transport is slow. Under these conditions, niflumic acid will bind to the inward-facing form of band 3 with a dissociation constant K_n' :

$$K_n' = \frac{(E_i + ES_i)(N)}{(E_iN + ES_iN)} \quad (A21)$$

where ES_i denotes the inward-facing transport site loaded with sulfate. (Modifier site effects are ignored in this calculation, but should have no effect if Cl^- binds to the modifier site regardless of the transport site orientation.)

With sulfate at both sides of the membrane, the ID_{50} for niflumic acid, K_{50} , will reflect the intrinsic asymmetry of the transport sites as well as the affinities of niflumic acid for band 3 molecules with inward (K_n') and outward-facing (K_n) transport sites:

$$K_{50} = \frac{(E_o + ES_o + E_i + ES_i)N}{(E_oN + ES_oN + E_iN + ES_iN)} \quad (A22)$$

Substituting Eqs. A3 and A21 into A22:

$$K_{50} = \frac{(E_o + ES_o + E_i + ES_i)N}{(E_o + ES_o)(N/K_n) + (E_i + ES_i)(N/K_n')} \quad (A23)$$

The asymmetry ratio for both loaded and unloaded sites, with $S_i = S_o$, may be defined as B :

$$B = \frac{E_o + ES_o}{E_i + ES_i} \quad (\text{A24})$$

Substituting Eq. A24 into A23 and solving for K_n :

$$K_n = \frac{BK_{ss}K_n'}{K_n'(1 + B) - K_{ss}} \quad (\text{A25})$$

B can be determined from the rates of sulfate-sulfate and sulfate-chloride exchange as described by Jennings (1980). At pH 6, B is $\sim 1/7$ (Jennings, 1982). Since K_{ss} and K_n' are determined experimentally, K_n can be calculated, as well as the ratio K_n'/K_n .

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