rapid communication

An oxonol dye is the most potent known inhibitor of band 3-mediated anion exchange

PHILIP A. KNAUF, FOON-YEE LAW, AND KLAUS HAHN
Department of Biophysics, University of Rochester School of Medicine, Rochester, New York 14642; and Division of Virology, Department of Neuropharmacology, Scripps Research Institute, LaJolla, California 92037

Knauf, Philip A., Foon-Yee Law, and Klaus Hahn. An oxonol dye is the most potent known inhibitor of band 3-mediated anion exchange. *Am. J. Physiol.* 269 (*Cell Physiol.* 38): C1073–C1077, 1995.—When cells are acutely exposed to the oxonol dye, bis(1,3-dibutylbarbituric acid)pentamethine oxonol (diBA), at 0°C, the concentration that gives half inhibition of Cl^- exchange (IC_{50}) is 0.146 ± 0.013 μM (n = 12) initially, but the inhibition increases with time. These characteristics indicate that a rapid initial binding is followed by a slow conformational change that makes the binding tighter. If diBA is allowed to equilibrate with band 3, the IC_{50} is only 1.05 ± 0.13 nM (n = 5), making diBA a more potent inhibitor than 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), for which the IC_{50} under similar conditions is 31 ± 6 nM [T. Janas, P. J. Bjerrum, J. Brahm, and J. O. Wieth. *Am. J. Physiol.* 257 (*Cell Physiol.* 26): C601–C606, 1989]. Inhibition by diBA is very slowly reversible at 0°C ($t_{1/2}$ > 50 h), but the effect is more readily reversible at higher temperatures. DiBA competes with 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) for inhibition, suggesting an external site of action. In contrast to DIDS and DNDS, however, increasing Cl^- concentrations do not decrease the inhibitory effect of diBA, indicating that the inhibition is not competitive. Thus diBA may be useful for investigating conformational changes during anion exchange and for stopping transport without preventing substrate binding. However, when diBA and other oxonols are used to sense membrane potential, they may have undesirable side effects on anion transport processes.

membrane potential; optical methods; mixed inhibition; noncompetitive inhibition; red blood cells; erythrocytes

FLUORESCENT OXONOL DYES have been widely used as optical sensors of membrane potential in a variety of cells (6, 7, 10, 18). Recently, we have reported (19) that one of these dyes, [3-methyl-1-*p*-sulfophenyl-5-pyrazolone-(4)]-[1,3-dibutylbarbituric acid]pentamethine oxonol (WW-781), inhibits red blood cell Cl^- exchange by a two-step mechanism. An initial low-affinity complex is formed with the transport protein, band 3, followed by a slow conformational change (whose rate constant is ~1.1 min^{-1} at 0°C) by which WW-781 becomes more tightly bound to band 3. For cells pretreated with WW-781 and suspended in media with the same WW-781 concentration, the concentration that gives half inhibition of Cl^- exchange (IC_{50}) is only 69 ± 4 nM. Despite this high affinity, the inhibition is completely reversible when cells are washed with media containing albumin to bind the WW-781. Because they interact strongly with functionally essential portions of the transport protein, such high-affinity reversible inhibitors (and reactive derivatives thereof) can be useful tools for studies of transport mechanism and for determining which portions of the protein take part in the transport event.

In this paper, we report the results of investigations with structural analogues of WW-781, which have led to the discovery that one analogue, bis(1,3-dibutylbarbituric acid)pentamethine oxonol (diBA; see Fig. 1, *inset*, for structure), is a far more potent inhibitor of Cl^- exchange than is WW-781. The characteristics of the inhibition indicate that it results from binding to an external site that is different from the external-facing transport site and different from the site of action of the previously most potent inhibitor, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS). Thus diBA and its analogues may provide information about involvement of novel parts of the band 3 protein in the transporting conformational change.

METHODS

Chemicals. Oxonol dyes including diBA were synthesized in Alan Waggoner's laboratory (11) and characterized by K. Hahn and Ratnakar Mujumdar. For diBA, analysis calculated was: C, 64.19; H, 7.8; N, 10.32. Analysis found was: C, 64.42; H, 7.85; N, 10.5 (Atlantic Microlabs). For ^{1}H-nuclear magnetic resonance (300 MHz, deuterated methyl sulfoxide), chemical shifts (in parts per million relative to internal reference tetramethylsilane) and coupling constants (J; in hertz) were: 7.3–7.9 (overlapping multiplets); 7.2 (doublet, J = 9); 3.8 (triplet, J = 7); 1.5 (multiplet), 1.3 (multiplet), 0.9 (triplet, J = 7). 4,4′-Dinitrostilbene-2,2′-disulfonate (DNDS) was obtained from ICN Pharmaceuticals, Plainview, NY. All other chemicals were reagent grade.

Cell preparation. Red blood cells were obtained by venipuncture from apparently healthy volunteers. Cells were washed three times in 150 KH solution [in mM: 150 KCl, 20 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, and 24 sucrose, pH 6.9 at room temperature with KOH]; white cells were removed as previously described (19).

When changes in intracellular Cl^- concentration were required, cells were treated with 30 μg/ml nystatin (Sigma Chemical, St. Louis, MO) as previously described (13).

Flux measurement. Where indicated, cells were pretreated with diBA as previously described (19), except that pretreat-

ment was for 10 min on ice. Cells were loaded with $^{36}Cl^-$, and equilibrium exchange was measured at 0°C as previously described (19), with 25 μl of cells in 30 ml of 150 KH (band 3 concentration ~15 nM), except as otherwise noted. diBA was dissolved in ethanol; equal volumes of ethanol (≤1% vol/vol) were added for control fluxes. Scintillation counts for flux suspensions were similar with and without diBA; there was no indication from external standards that diBA significantly affects counting efficiency. Concentrations of diBA were nominal and did not include corrections for penetration into or binding to the cells.

Data analysis. Data for exit of $^{36}Cl^-$ from the cells were fitted by least squares to an equation for two-compartment equilibrium exchange (19). IC_{50} values were obtained by nonlinear least-squares fits of the flux vs. diBA concentration data to the equation for single-site reversible inhibition. On and off rate constants were determined by nonlinear fits to the two-step inhibition model (19). Nonlinear fits were done with Enzfitter (Elsevier Biosoft), in which SE were estimated by the method of matrix inversion.

RESULTS

Inhibition of Cl^- exchange by diBA. Figure 1 shows $^{36}Cl^-$ efflux data for control cells (open squares) and for cells exposed to 125 nM diBA at *time 0* (solid circles). Even at very short times, the rate constant for Cl^- exchange, given by the slope of the line, is substantially decreased. (This is confirmed by measurements at shorter times, see Fig. 4.) With time, the slope continues to decrease, indicating increasing inhibition.

As discussed previously (19), the simplest model that explains initial inhibition that increases with time involves a two-step binding sequence as follows

$$\text{diBA} + \text{b3} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{diBA}\cdot\text{b3} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{diBA-b3} \qquad (1)$$

where dibA·b3 represents the initial complex of band 3 with diBA (*complex 1*), diBA-b3 represents the second tighter complex (*complex 2*), K_1 is the dissociation constant for formation of *complex 1*, k_2 is the "on" rate constant for formation of *complex 2*, and k_{-2} is the "off" rate constant for the conformational change from *complex 2* back to *complex 1*. Formation of either complex of band 3 with diBA is assumed to inhibit transport completely. The fit to this model is shown by the line through the solid points and corresponds to a k_2 value of 0.8 ± 0.1 min^{-1}.

If cells are pretreated with 125 nM diBA and Cl^- exchange is measured in medium with 125 nM diBA (Fig. 1, solid triangles), the inhibition is far greater. The larger inhibition in this case is explained by the presence of *complex 2* in addition to the rapidly formed *complex 1*. From a series of such experiments, the IC_{50} value (IC_{50eq}) is 1.05 ± 0.13 nM (n = 5). Even this represents an overestimate of the IC_{50}, because it is based on the concentration of diBA added to the cells (nominal concentration) and does not take into account the fact that the actual diBA concentration, as measured by fluorescence (data not shown), rapidly drops to much lower levels, because of binding to band 3 and nonspecific partitioning into the cells (probably into the membrane). If cells are pretreated twice to reduce such losses, the IC_{50} value decreases to 0.38 ± 0.07 nM (n = 2). Moreover, if the diBA concentration measured by fluorescence after the second pretreatment is used instead of the nominal concentration, the IC_{50} becomes 0.12 ± 0.04 nM (n = 2). Further studies are required to understand the partitioning behavior of diBA and to devise methods for maintaining a constant extracellular concentration, but, from the data so far, it is clear that diBA is an exceptionally potent inhibitor of Cl^- exchange.

When pretreated cells are suspended in flux medium with no diBA (Fig. 1, open triangles), the inhibition (based on nominal concentrations) is only slightly smaller, with an IC_{50} value of 4.8 ± 0.3 nM (n = 5). According to the model, in this case the inhibition is due solely to *complex 2*, because *complex 1* rapidly dissociates. The similarity of inhibition to that seen with diBA in the flux medium (Fig. 1, solid triangles) fits with the concept that most of the inhibition under those conditions is due to *complex 2*. The fact that the inhibition does not decrease during the time of the flux, even with no diBA in the medium (Fig. 1, open triangles), indicates that k_{-2} is very small, so no appreciable decomposition of *complex 2* occurs during the time of the flux.

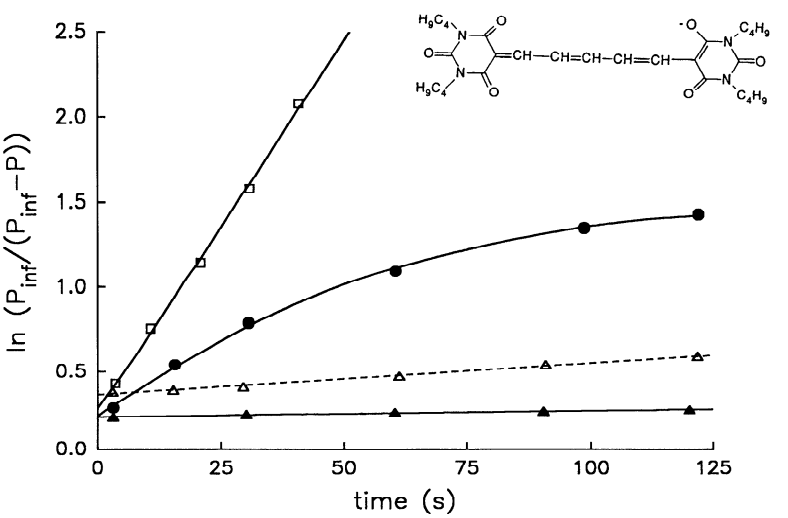

Fig. 1. Effects of bis(1,3-dibutylbarbituric acid)pentamethine oxonol (diBA) on $^{36}Cl^-$ equilibrium exchange efflux. Red blood cells were prepared, loaded with $^{36}Cl^-$, and fluxes were measured in 150 KH medium at 0°C as described in METHODS. On y-axis $\ln|P_{inf}/(P_{inf} - P)|$ is plotted, where P_{inf} is counts per minute of $^{36}Cl^-$ in the medium at infinite time (obtained by counting 2 samples of cell suspension) and P represents counts in supernatant at particular time indicated on x-axis. When plotted in this fashion, slope of the line equals rate constant for Cl^- equilibrium exchange, which, when multiplied by Cl^- content of cells, gives unidirectional Cl^- efflux. □, Control with 1% vol/vol ethanol (rate constant = 0.044 ± 0.001 s^{-1}); △, cells pretreated for 10 min with 0.125 μM diBA; ▲, cells pretreated with 0.125 μM diBA and with 0.125 μM diBA in flux medium; ●, cells exposed to 0.125 μM diBA at *time 0*. Lines represent least-squares best fits to a straight line, except for filled circles, where the line is a least-squares best fit to the equation for 2-step binding (*Eq. 5* of Ref. 19), assuming that off rate constant (k_{-2}) is negligible and with on rate constant (k_2) = 0.83 ± 0.10 min^{-1} and dissociation constant (K_1) = 0.130 ± 0.015 μM. *Inset*, structure of diBA.

Reversibility of diBA inhibition. To obtain better information about the rate of reversal of diBA inhibition, cells were pretreated with diBA, and then fluxes were measured in medium with no diBA for longer time periods. Figure 2 shows $^{36}Cl^-$ efflux data for cells pretreated with 250 nM diBA. The exchange rate constant (slope) increases with time, but, even at 40 min, the rate constant is far lower than the control (Fig. 1, squares; note difference in time scale). A fit of the two-step model to Fig. 2 gives k_{-2} of $1.4 \pm 0.1 \times 10^{-4}$ min^{-1}, corresponding to a half time of > 80 h; the mean k_{-2} for three experiments of this kind is $2.2 \pm 0.7 \times 10^{-4}$ min^{-1}, giving a half time of > 50 h. The very high affinity of diBA for band 3 is thus due to the very low off rate constant, which causes band 3 to be trapped in the inhibited form as *complex 2*.

To see whether the diBA inhibition is fully reversible, and to investigate the effects of temperature on k_{-2}, we washed diBA-pretreated cells at either room temperature or 37°C (Table 1). Washing at higher temperature was far more effective in removing diBA inhibition. The slow rate of release of diBA from *complex 2* observed at 0°C (low k_{-2}) makes reversal of inhibition very slow at 0°C (Fig. 2); the more rapid reversal of inhibition at higher temperatures thus indicates that k_{-2} increases dramatically with increasing temperature.

Site of action of diBA. As might be expected because of its hydrophobic nature and single delocalized negative charge (see Fig. 1, *inset*), diBA seems to penetrate rapidly into cells, as judged by its disappearance from the medium (data not shown). To see whether diBA inhibits by binding to external or internal sites, we measured the effects of diBA on Cl^- exchange in the presence or absence of DNDS, an inhibitor that binds to an external site (8). The data are plotted in Fig. 3 as a Dixon plot of $1/J_{Cl}$, where J_{Cl} is the Cl^- exchange flux, against the diBA concentration. The data with or without DNDS fall on parallel lines, indicating that DNDS and diBA are mutually exclusive inhibitors (9). The simplest interpretation is that diBA inhibits by binding to an external site that partially overlaps the DNDS site, although an allosteric link between DNDS and diBA binding (to an external or internal site) is also possible. The idea that the diBA site is external is reinforced by the fact that the structurally similar dye, WW-781, inhibits by binding to an external site (19).

Table 1. *Reversal of diBA inhibition*

No. of Washes	^{36}Cl Flux, %control
21°C	
0	3.8 ± 0.1
3	53.1 ± 1.3
6	70.9 ± 0.2
37°C	
0	3.2 ± 0.3
3	70.9 ± 1.1
6	87.5 ± 0.1

Values are means ± range of 2 measurements with blood from same donor. Cells were pretreated with 0.125 μM bis-(1,3-dibutylbarbituric acid) pentamethine oxonol (diBA) and were then washed indicated number of times with 150 KH solution containing 0.5% wt/vol bovine serum albumin at indicated temperature (cells were resuspended for 10 min before each centrifugation). $^{36}Cl^-$ exchange flux is expressed with respect to control cells that were pretreated with same amount of ethanol as that added with diBA and were then washed 3 times (21°C experiment) or 6 times (37°C experiment).

Mechanism of diBA inhibition. To see whether diBA overlaps the external Cl^- binding site, we measured the effects of diBA on Cl^- exchange at different Cl^- concentrations, keeping intracellular Cl^- concentration equal to extracellular Cl^- concentration (Fig. 4). Because Cl^- should compete with diBA for the first binding step (formation of *complex 1*), we measured inhibition by diBA at very short times (<21 s). Under these conditions, very little *complex 2* is formed, so the IC_{50} should

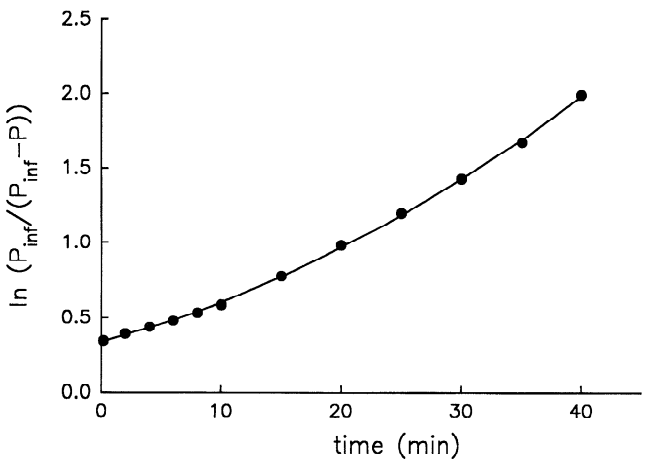

Fig. 2. $^{36}Cl^-$ efflux from diBA-pretreated red blood cells into medium without diBA. Cells were prepared and $^{36}Cl^-$ equilibrium exchange was measured as described in METHODS and in legend to Fig. 1. Cells were pretreated with 0.25 μM diBA for ≥ 10 min at 0°C before flux measurement. Solid line represents a least-squares best fit to the equation for reversal of inhibition in a 2-step binding model (*Eq. 4* of Ref. 19), with $k_{-2} = 1.39 \pm 0.08 \times 10^{-4}$ min^{-1}. Note that k_{-2} can be determined in this way without any information about K_1 or k_2.

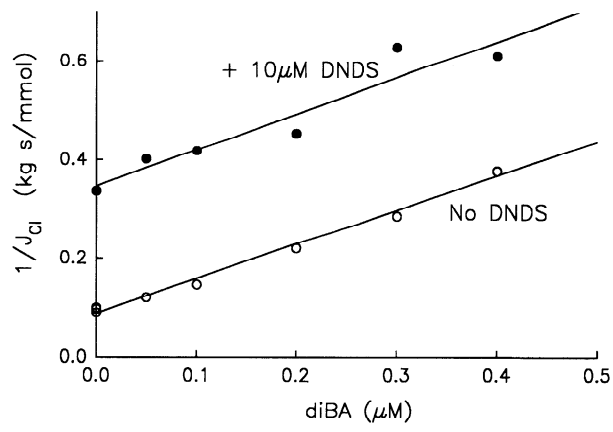

Fig. 3. Dixon plot for inhibition of Cl^- exchange by diBA in presence or absence of 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS). Reciprocal of Cl^- exchange flux in mmol·kg dry solids^{-1}·s^{-1}, measured as described in METHODS and Fig. 1, is plotted against diBA concentration ([diBA]). Lines were calculated from nonlinear least-squares fits of flux vs. diBA concentration data to equation $J_{Cl} = J_u/\{1 + ([diBA]/IC_{50})\}$, where J_u is flux (J_{Cl}) without diBA present and IC_{50} is concentration that gives half inhibition of Cl^- exchange. Dixon plot slopes calculated from these fits are 0.61 ± 0.05 kg·s·mmol^{-1}·μM^{-1} without DNDS and 0.74 ± 0.11 with 10 μM DNDS. Nearly parallel lines are consistent with hypothesis that DNDS and diBA each prevent binding of other, i.e., that the 2 inhibitors are mutually exclusive. Two other experiments gave similar results.

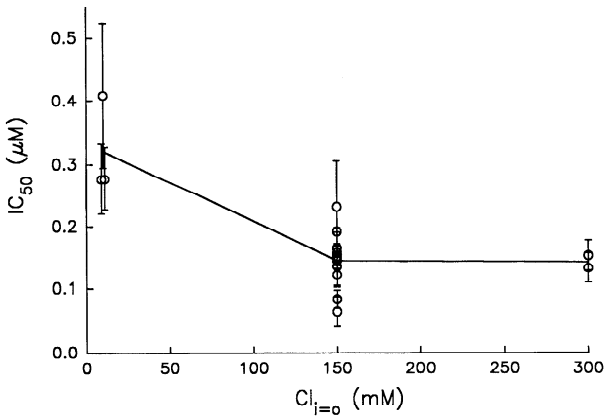

Fig. 4. IC_{50} for diBA measured at short times as function of Cl^- concentration, with intracellular Cl^- concentration equal to extracellular Cl^- concentration ($Cl_{i=o}$). IC_{50} values were obtained from nonlinear least-squares fits of equation for single-site inhibition (see Fig. 3) to data for flux vs. [diBA]. For 10 mM Cl^-, 2 of the points are displaced slightly to make them visible. Fluxes were measured at short times (<21 s) to avoid formation of *complex 2*, so IC_{50} values should be good approximations of K_1, apparent dissociation constant for initial binding step. Bars represent SE. IC_{50} value at 10 mM Cl^- (0.32 ± 0.04 μM, $n = 3$) is not significantly higher ($P = 0.063$) than that at 150 mM Cl^- (0.15 ± 0.01 μM, $n = 12$); value at 300 mM Cl^- (0.14 ± 0.01 μM, $n = 2$) is nearly identical to that at 150 mM Cl .

be a good approximation of K_1, the dissociation constant for rapid initial binding (*Eq. 1*). For a competitive inhibitor, the IC_{50} should rise steeply with increasing Cl^- concentration ($[Cl^-]$), along a straight line whose x-intercept is about −65 mM (22). In fact, the IC_{50} shows an apparent decrease (which is not statistically significant, $P = 0.063$) as $[Cl^-]$ is increased from 10 to 150 mM, and then remains constant as $[Cl^-]$ is raised to 300 mM. This demonstrates that diBA is not a competitive inhibitor, i.e., it can bind to band 3 regardless of whether Cl^- is bound or not. The apparent decrease in inhibitory potency at low $[Cl^-]$ suggests that the forms of band 3 without Cl^- bound may have a lower affinity for diBA. Dipyridamole, another inhibitor that interferes with disulfonic stilbene binding, requires Cl^- to be present for inhibition of anion exchange at neutral pH (15, 20). Although the effects of Cl^- seen with diBA resemble those seen with dipyridamole under similar conditions (20), further experiments are required to reveal whether or not dipyridamole and diBA share a common site and mechanism of binding. Interestingly, for WW-781, a structural analogue of diBA, increasing the $[Cl^-]$ from 5 to 150 mM had no effect on the initial exposure (K_1) IC_{50} value (Figs. 3 and 6 of Ref. 19).

DISCUSSION

As shown in Fig. 1, when the binding of diBA to band 3 is allowed to come into equilibrium (Fig. 1, solid triangles), diBA is the most potent known inhibitor of red blood cell anion exchange, with an IC_{50eq} ~30 times lower than that of the next best inhibitor, DIDS, even though the IC_{50eq} for diBA is overestimated because it is based on the nominal concentration added, and much diBA is removed from the medium by entry into cells or binding to cell membranes (data not shown). The high potency of diBA (but not the selectivity, see below) rivals that of ouabain for the Na^+-K^+-ATPase and is due to a very high affinity for formation of *complex 1* (see *Eq. 1* and Fig. 4), coupled with a conformational change to *complex 2* that results in a very slow release of the bound diBA (Fig. 2).

For a two-step binding process, it can easily be shown that (*Eq. 6* of Ref. 19)

$$IC_{50eq} = K_1/(1 + k_2/k_{-2}) \quad (2)$$

Thus, if k_2 is large compared with k_{-2}, i.e., if formation of *complex 2* is highly favored, then IC_{50eq} is very small compared with the IC_{50} at short times (which is equal to K_1). If we insert the mean values for K_1 (0.146 ± 0.013 μM; $n = 12$), k_2 (0.79 ± 0.11 min^{-1}; $n = 6$), and k_{-2} (2.2 ± 0.7 × 10^{-4} min^{-1}; $n = 3$) in 150 mM Cl^- medium, the calculated IC_{50eq} value is 0.04 nM, slightly smaller than the value of 0.12 ± 0.04 nM, obtained by using measured rather than nominal diBA concentrations. Because of the limited accuracy of the determinations of the on and off rate constants[1] and because of complexities introduced by the decrease in diBA concentration during the experiments, a discrepancy between the predicted and actual IC_{50eq} values of this magnitude is not unexpected.

In terms of structural requirements for binding, it is somewhat surprising that both K_1 and IC_{50eq} for diBA are much lower than the corresponding values for WW-781 [580 ± 60 and 69 ± 4 nM, respectively (19)], despite the fact that diBA has only a single delocalized negative charge, whereas WW-781 has, in addition, a sulfonic acid moiety. Contrary to what one might expect, in view of the high inhibitory potency of sulfonates such as DIDS and DNDS, it seems that the sulfonic acid in WW-781 does not play an important role in the binding to band 3, or else it seems that other aspects of the diBA structure more than compensate for the absence of the sulfonate.

The high potency of diBA implies that it must have very strong interactions with portions of band 3. The fact that DNDS interferes with diBA inhibition (Fig. 3) suggests that the binding site is external and overlaps the DNDS site. This overlap might be expected on the basis that diBA, like the *trans* form of DNDS (the form that inhibits transport), is probably a very planar molecule (5), so it might fit into some part of the DNDS binding site. However, Cl^- has a very strong, apparently competitive, effect on DNDS binding,[2] very different from its effects on diBA binding. Cl^- may have some effect on the affinity for diBA (Fig. 4), but Cl^- and diBA

[1] There is considerable scatter in the determinations of k_2 and k_{-2}. In 6 experiments, the mean k_2 is 0.79 ± 0.11 (SE) min^{-1} (based on fits to *Eq. 5* of Ref. 19, assuming that k_{-2} is negligible); the value of k_{-2} for experiments with times up to 40 min was 2.2 ± 0.7 × 10^{-4} min^{-1} ($n = 3$); the value of k_{-2} including other experiments with shorter times (likely to be less accurate) was much higher (1.9 ± 0.8 × 10^{-3} min^{-1}; $n = 8$). If the latter data for k_{-2} are inserted into *Eq. 2*, the calculated IC_{50eq} is 1.9 nM, higher than the measured value even using nominal concentrations (1.05 ± 0.13 nM). Regardless of which values are used for the constants, the qualitative predictions of *Eq. 2*, that $k_2 \gg k_{-2}$, are fulfilled.

[2] Although increases in Cl^- concentration very greatly reduce the inhibitory potency of disulfonic stilbenes such as DNDS, as expected for competitive inhibitors, recent evidence (1, 14, 21) strongly suggests that these probes may bind to a site different from the substrate binding site.

do not compete for binding to the external-facing transport site. Thus diBA must bind to a different site, at which it strongly interferes with the transporting conformational change. Because diBA is not a competitive inhibitor, it can bind to various conformations of band 3, with the transport site either inward or outward facing and empty or loaded with substrate. The initial binding and the conformational shift to *complex 2* may, however, be strongly affected by the band 3 conformation, in which case diBA may be a useful probe for the conformational state of band 3. In addition, because of its high affinity, diBA may serve as a fluorescent marker for its binding site and may also be useful for stopping anion translocation by band 3 without blocking the transport site, for nuclear magnetic resonance studies of Cl^- binding to the transport site (16).

Although diBA is an extremely potent inhibitor, it is not entirely selective for band 3-mediated anion exchange. At higher concentrations ($IC_{50} \approx 1.2–1.8$ μM at 21–22°C), diBA inhibits volume-activated Cl^- fluxes and currents in HL-60 promyelocytic leukemic cells (2, 3), which probably take place through Cl^- channels (3). Cl^- exchange in the same cells, which is probably mediated by the band 3 analogue AE2, is also inhibited, but with an IC_{50} of $\sim 1.4 \pm 0.2$ μM (K. R. Hallows, unpublished data), over 1,000 times higher than for band 3 (AE1).

Finally, this work, like our previous studies with WW-781, sounds a note of caution concerning the use of oxonols as fluorescent probes of membrane potential. The possibility of side effects of oxonols on Cl^- transport processes has been recognized by some researchers, such as Freedman et al. (6, 18), but not in other studies, including ones using diBA (17, 23). Bis(1,3-dibutylbarbituric acid)trimethine oxonol [diBA-(3)-C_4], another oxonol that has been used to measure potential (4), also inhibits Cl^- exchange, with an IC_{50eq} of ~8 nM (Law and Knauf, unpublished data). The high-affinity inhibition is strongly dependent on structure and is not seen with all oxonols. Nevertheless, it seems important to consider the possibility that such probes may inhibit anion exchange (or even conductive anion flow, as in HL-60 cells; Ref. 3), thereby affecting the membrane potential.

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Address for reprint requests: P. A. Knauf, Dept. of Biophysics, Univ. of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642.

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