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Molecular Mechanisms of Band 3 Inhibitors. 1. Transport Site Inhibitors†

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ABSTRACT: The band 3 protein of red cells is a transmembrane ion transport protein that catalyzes the one-for-one exchange of anions across the cell membrane. ^{35}Cl NMR studies of Cl^- binding to the transport sites of band 3 show that inhibitors of anion transport can be grouped into three classes: (1) transport site inhibitors (examined in this paper), (2) channel-blocking inhibitors (examined in the second of three papers in this issue), and (3) translocation inhibitors (examined in the third of three papers in this issue). Transport site inhibitors fully or partially reduce the affinity of Cl^- for the transport site. The dianion 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) and the arginine-specific reagent phenylglyoxal (PG) each completely eliminate the transport site ^{35}Cl NMR line broadening, and each compete with Cl^- for binding. These results indicate that DNDS and PG share a common inhibitory mechanism involving occupation of the transport site: one of the DNDS negative charges occupies the site, while PG covalently modifies one or more essential positive charges in the site. In contrast, ^{35}Cl NMR line broadening experiments suggest that 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) leaves the transport site partially intact so that the affinity of Cl^- for the site is reduced but not destroyed. This result is consistent with a picture in which DIDS binds near the transport site and partially occupies the site.

Band 3 is a 95-kDa single polypeptide chain that spans the red cell membrane and catalyzes the transmembrane one-for-one exchange of anions [for review see Macara and Cantley (1983) and Knauf (1979)]. The exchange of Cl^- for HCO_3^-—the latter produced by hydration of CO_2—is essential for CO_2 respiration; in fact, band 3 is the most abundant transport protein in the red cell, and the ion flux through band 3 is higher than through any other pathway (Knauf, 1979; Falke et al., 1984a). Recently, substantial progress has been made in elucidating both the structure and mechanism of this ion transport protein. Chemical labeling and proteolysis experiments have demonstrated the existence of at least seven transmembrane segments in the monomer (Jenning & Nicknish, 1984), and the gene sequence suggests as many as 13 transmembrane segments (Kopito & Lodish, 1985). Each monomer acts as an independent catalytic unit in anion transport (Macara & Cantley, 1983), although the monomer is associated in dimers and tetramers in the membrane (Nakashima et al., 1981). ^{35}Cl NMR studies of anion transport sites (Falke et al., 1984b; Falke & Chan, 1985), as well as transport studies [Gunn & Fröhlich, 1979; reviewed by Macara and Cantley (1983)], indicate that the catalytic unit alternates between two conformations, one in which a single transport site faces the internal compartment, and another in which a single transport site faces the external compartment. The Cl^- binding and dissociation reactions at both the inward- and outward-facing transport site conformations are rapid compared to the translocation of the bound Cl^- between these conformations (Falke et al., 1985a), and the translocation of an empty site is negligibly slow. The slowness of empty site translocation explains the observation that net transport of Cl^- is 10^4 times slower than the one-for-one exchange of two Cl^- ions in opposite directions [reviewed by Knauf (1979)]. Thus, the transport cycle catalyzed by band 3 is relatively well understood; however, the molecular nature of the structural changes that occur during the transport cycle is unknown.

Inhibitors of the transport cycle provide a practical approach to structure elucidation. A large number of organic inhibitors of the transport machinery have been described. In the series of three papers presented here, we investigate the molecular mechanisms of some of these inhibitors by using ^{35}Cl NMR

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to monitor (1) the binding of Cl^- to the transport site and (2) the exchange of Cl^- between the transport site and solution. The NMR results indicate that the inhibitors studied can be grouped into three distinct classes.

This paper describes certain sulfonic acid and arginine-specific inhibitors which are transport site inhibitors that occupy the transport site, thereby preventing Cl^- binding. These studies indicate that arginine is an essential residue in the transport site. The second paper (Falke & Chan, 1986a) describes channel blocking inhibitors, which are shown to inhibit the exchange of Cl^- between the transport site and solution by blocking a substrate channel. This third paper (Falke & Chan, 1986b) describes translocation inhibitors that leave the transport site and channel intact but slow the translocation of bound Cl^- across the membrane. These three classes of inhibitors suggest several fundamental features of the structure and mechanism of the anion transport domain of band 3. In the third paper, we present a molecular model for the transport machinery that is consistent with the available evidence.

Materials and Methods

Reagents. Freshly oudated human blood (packed red cells) was a kind gift of the Los Angeles Chapter of the American Red Cross. *p*-Nitrobenzenesulfonate (pNBS)[1] was obtained from Kodak and recrystallized once: 10 g of pNBS was dissolved in 25 mL of 2 N NaOH, H_2O was added, and the resulting suspension was cooled overnight at 4 °C, before isolation of the crystals by filtration and washing with ice-cold saturated NaCl in H_2O. TLC on silica gel plates developed with 1-butanol saturated with H_2O yielded a single spot, and from ϵ_{max} = 9310 at 264 nm the purity was 100 ± 3%. 4,4′-Dinitrostilbene-2,2′-disulfonate sodium salt (DNDS) was recrystallized as previously described (Falke et al., 1984a). NBD-taurine was synthesized (Eidelmann et al., 1981) and purified (Knauf et al., 1978) as previously described, and the purity and structure of the final product were verified by ^1H and ^{13}C NMR. Used without further purification were the following: 4,4′-diisothiocyanostilbene-2,2′-disulfonate sodium salt (DIDS) from Pierce; phenylglyoxal (PG), 1,2-cyclohexanedione (CHD), 1,3-cyclohexanedione (1,3-CHD), and 2,4-dinitrofluorobenzene (DNFB) from Aldrich; *p*-aminobenzenesulfonate sodium salt (pABS) and 2,4,6-trichlorobenzenesulfonate potassium salt (TCBS) from Pfaltz and Bauer; dipyridamole (DP) from Sigma; and niflumic acid (NIF), a kind gift of Squibb & Sons Pharmaceuticals.

A stock solution of 40 mM DIDS in 1:1 EtOH/H_2O was used immediately; 2 mM DP in 1:1 EtOH/H_2O was kept in the dark; PG ($^5/_2$ times the final concentration) or DNFB (2 times the final concentration) was dissolved in reaction buffer stock solution by bath sonication for 15 min without cooling, just before use; and NIF was dissolved in buffer containing an equivalent amount of NaOH just before use.

Membrane Preparation. Intact red cells (Falke et al., 1984b), leaky isolated red cell membranes or ghosts (Falke et al., 1984a), inside-out and right-side-out vesicles (Falke et al., 1984b), stripped ghost membranes (Falke et al., 1985b), and sonicated ghost membranes (Falke et al., 1984b) were all prepared exactly as previously described.

Covalent Inhibitors. DIDS. Intact red cells modified and unmodified with DIDS were prepared by washing red cells 3 times in phosphate-buffered saline (PBS), with simultaneous removal of the buffy coat (Falke et al., 1984a). Then the cells were incubated at 21 °C for 30 min in PBS ± 70 μM DIDS, pelleted at 3000 rpm for 30 min in a Sorvall GSA rotor, resuspended in 150 mM NaCl and 10 mM Tris, pH 8.4, with HCl at 21 °C, and then pelleted and washed once in PBS before making ghost membranes.

PG, CHD, and DNFB. Leaky ghost or stripped ghost membranes were modified with PG as previously described (Falke & Chan, 1984). Samples (3 parts) were diluted with buffer (2 parts) to yield membranes in 80 mM boric acid, pH to 8.0 with NaOH, ±300 mM NaCl, ±200 μM DNDS, and 0–20 mM PG. For the CHD reaction, the same protocol was used to produce a reaction suspension containing membranes and 0–100 mM CHD (Falke & Chan, 1984). For the DNFB reaction, a reaction suspension was prepared as follows (Rudloff et al., 1983). Membranes were first washed with 10 mM NaH_2PO_4, pH to 7.4 with NaOH, and 50 mM NaCl. The washes (48000 *g* for 15 min) were continued until the membranes were diluted by a factor of ≥100. The washed membranes were resuspended to their original volume with the same buffer and then diluted with an equal volume of buffer containing DNFB to give membranes in the indicated buffer with 0–4 mM DNFB. Exceptions were DNFB reactions carried out in the absence of Cl^-, for which NaCl was omitted from the washing buffer. Reactions with PG, CHD, and DNFB were carried out at 37 °C for 60 min (PG, CHD) or 30 min (DNFB) unless otherwise indicated. In one experiment the reversibility of CHD modification was examined by washing CHD membranes with 200 mM NH_2OH·HCl, pH to 8.0 with NaOH (same washing protocol as above). The membranes were incubated in this buffer 12 h at 4 °C, followed by 2 h at 37 °C. Where indicated the modified membranes were washed free of excess reagent by pelleting at 48000 *g* for 15 min, followed by aspiration of the supernatant and resuspension of the pellet in 80 mM boric acid, pH to 8.0 with NaOH (PG, CHD), or 100 mM NH_4Cl and 5 mM NaH_2PO_4, pH to 8 with NH_4OH and NaOH (DNFB). The washing was continued until a dilution factor of ≥100 was obtained. The membranes were resuspended to their original volume in the same buffer containing 20% D_2O and then sonicated as previously described (Falke et al., 1984b), or crushed by ultracentrifugation at 70000*g* in a Beckman SW-27 rotor for 1 h at 4 °C. Finally, the suspension was diluted with an equal volume of 2 × NMR buffer to yield the final buffer composition indicated in the figure legends.

Noncovalent Inhibitors. DNDS, pNBS, DP, NIF, NBDT, and TCBS were each added to modified or unmodified membranes as previously described for DNDS (Falke et al., 1984a).

Samples containing DP were analyzed for free DP by pelleting membranes suspended in 150–250 mM NH_4Cl, 5 mM NaH_2PO_4, and 20% D_2O, pH to 8.0 with NH_4OH and NaOH (2 mL), at 35000 *g* for 30 min, 0 °C. An aliquot of supernatant (1.2 mL) was brought to 0.5% sodium dodecyl sulfate (SDS) by addition of 20% SDS. The resulting solution yielded the A_{416} of free DP when read against a blank made from a membrane sample lacking DP. The concentration of bound DP was calculated as the difference of the total and free DP concentrations.

The quantitation of the stoichiometry of DP binding to band 3 required determination of the band 3 concentration. Lowry

[1] Abbreviations: pNBS, *p*-nitrobenzenesulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; PG, phenylglyoxal; pABS, *p*-aminobenzenesulfonate; TLC, thin-layer chromatography; NBDT or NBD-taurine, 2-[(7-nitrobenzofurazan-4-yl)amino]ethanesulfonate; CHD, 1,2-cyclohexanedione; 1,3-CHD, 1,3-cyclohexanedione; DNFB, 2,4-dinitrofluorobenzene; TCBS, 2,4,6-trichlorobenzenesulfonate; DP, dipyridamole; NIF, niflumic acid; Tris, tris(hydroxymethyl)aminomethane.

interferences prevented direct determination of total protein in membranes labeled with PG, CHD, or DNFB; however, the protein concentration can be calculated from the cholesterol concentration. Independent determinations of the ratio (mg of total protein)/(mg of cholesterol) for four different preparations of leaky ghosts yielded the ratio 3.6 ± 0.1 [determination of protein and cholesterol as described in Falke et al. (1984a, 1985b)]. Assuming that 30% of the total protein is band 3 (Falke et al., 1984b), a membrane concentration of 1.0 mg of cholesterol/mL corresponds to 1.1 mg of band 3/mL, or 11 μM band 3. Using this approach, the band 3 concentration can be compared to the bound DP concentration to ascertain the stoichiometry of binding.

Determination of Transport Site Line Broadening. ^{35}Cl NMR samples were prepared, spectra were obtained by using standard parameters, and line broadenings were measured and normalized exactly as previously described (Falke et al., 1984a,b, 1985b). The transport site line broadening is that part of the total line broadening which is inhibited by 1 mM DNDS or 300 mM HCO_3^- [the latter anion is used to determine the transport site line broadening of DNFB-modified membranes, since DNFB and DNDS compete for binding to band 3 (Rudloff et al., 1983)].

Statistics. All averages are given as the mean ± 1 standard deviation for $n \geq 3$.

Results

Determination of Inhibitor Mechanism by ^{35}Cl NMR. The ^{35}Cl NMR technique provides a sensitive assay for Cl^- binding at band 3 transport sites on both sides of the red cell membrane (Falke et al., 1984a,b). The physical basis of this technique is the large ^{35}Cl NMR spectral width of Cl^- in a macromolecular binding site; the bound Cl^- spectral width is typically $>10^4$ times larger than the line width of Cl^- in solution. As a result, when solution Cl^- samples a binding site sufficiently rapidly, the site can cause measurable broadening of the solution Cl^- line width. This increase in line width, or line broadening, contains information on the structure and motions of the site and on the rate of Cl^- migration between the site and solution (Lindman & Forsen, 1976; Falke et al., 1984a). The line broadening due to a given site is directly proportional to the fraction of total Cl^- bound to the site, and the contributions to the line broadening due to different sites are additive. Thus the line broadening due to a heterogeneous population of sites is given by (Falke et al., 1984a)

$$\delta = \sum_j \frac{\alpha_j [E_j]_T}{K_{Dj}} \frac{[Cl^-]^{-1}}{[Cl^-]^{-1} + K_{Dj}^{-1}} \quad (1)$$

where the sum is over the different types of sites E_j. α_j is a constant characteristic of the jth type of site: in the limit of slow Cl^- exchange between the site and solution $\alpha_j = k_{OFF}/\pi$, where k_{OFF} is the rate constant for Cl^- dissociation from the site. K_{Dj} is the Cl^- dissociation constant for the jth type of site. Equation 1 assumes that the bound Cl^- returns to solution before binding to another site and that the bound Cl^- concentration is negligible relative to the total Cl^- concentration.

In the red cell membrane system, multiple types of Cl^- binding sites are observed (Falke et al., 1984a). The contribution of the transport site to the total red cell membrane line broadening can be determined by using DNDS, an anionic reversible inhibitor of the transport site, to identify the transport site line broadening. For a homogeneous class of sites the line broadening becomes (Falke et al., 1984a)

$$\delta_j = \frac{\alpha_j [E_j]_T}{K_{Dj}} \frac{[Cl^-]^{-1}}{[Cl^-]^{-1} + K_{Dj}^{-1}} \quad (2)$$

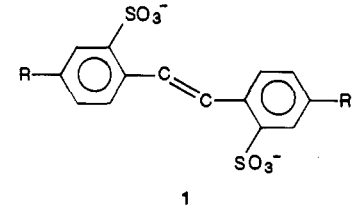
Equation 2 indicates that a high-affinity site ($K_{Dj} \leq [Cl^-]$) gives rise to a square hyperbola on a plot of line broadening vs. $[Cl^-]^{-1}$, while a low-affinity site ($K_{Dj} \gg [Cl^-]$) gives rise to a straight line of zero slope.

Equation 2 also suggests that in principle there are two types of line broadening inhibitors, which can be resolved by appropriate experiments. Inhibitors of the site itself cause $[E_j]_T$ to decrease and/or K_{Dj} to increase; in either case the binding of inhibitor and that of Cl^- are mutually disfavored. As a result, such inhibitors show a characteristic decrease in inhibitory potency when $[Cl^-]$ is increased. In contrast, inhibitors of Cl^- exchange between the site and solution cause α_j to decrease but are unaffected by occupation of the site with Cl^-; thus these inhibitors are identified by the absence of a relationship between inhibitory potency and $[Cl^-]$. A similar decrease in α_j would be observed for an inhibitor that alters the structure of the site so that the quadrupolar coupling constant of bound Cl^- decreases while the affinity of Cl^- for the site is unaffected (no change in $[E_j]_T$ or K_{Dj}). However, such a structural change is highly unlikely and is not considered further. Thus (a) inhibitors of Cl^- binding to a site and (b) inhibitors of Cl^- exchange between the site and solution both inhibit the $^{35}Cl^-$ line broadening but can be resolved by their differing dependence on $[Cl^-]$. The present paper shows that this approach can identify transport site inhibitors.

We examine here the inhibitors (1) pABS, DNDS, and DIDS, which all possess negative sulfonate charges capable of occupying the anion transport site, and (2) phenylglyoxal, an arginine-specific reagent capable of modifying essential positive charge(s) in the transport site.

pABS (p-Aminobenzenesulfonate). pABS is a useful control sulfonic acid since its concentration of half-maximal inhibition of anion transport is high [apparent K_D = 61 ± 26 mM at 100 mM sulfate (Barzilay & Cabantchik, 1979b)]. Similarly, pABS has essentially no effect on the transport site line broadening. The total line broadening due to low-affinity and transport sites on both sides of leaky ghost membranes is 6.4 ± 0.2 Hz/(mg of ghost protein/mL) and is decreased to 6.2 ± 0.1 Hz/(mg/mL) by 1 mM pABS, while 1 mM DNDS causes a 12-fold greater decrease in line broadening to 4.0 ± 0.0 Hz/(mg/mL) (spectral parameters, 8.8 MHz, 3 °C; buffer composition, 250 mM NH_4Cl, 5 mM NaH_2PO_4, and 20% D_2O, pH to 8.0 with NH_4OH and NaOH). The linebroadening inhibition observed for DNDS is not a general property of sulfonic acids.

DNDS (4,4'-Dinitrostilbene-2,2'-disulfonate). DNDS is a well-studied inhibitor that is used in this study as the prototype transport site inhibitor since the available data suggest that this molecule fully occupies the transport site with one of its negative charges. DNDS belongs to the stilbenedisulfonate family, which has the general structure



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where R = $-NO_2$ for DNDS, and only the trans isomer binds to band 3 (Fröhlich & Gunn, 1981). The stilbenedisulfonates bind with a stoichiometry of 1 molecule per band 3 monomer, and the binding sites on adjacent monomers in a multimer are independent except when R is sufficiently large (Macara & Cantley, 1983). The stilbenedisulfonate binding site on band

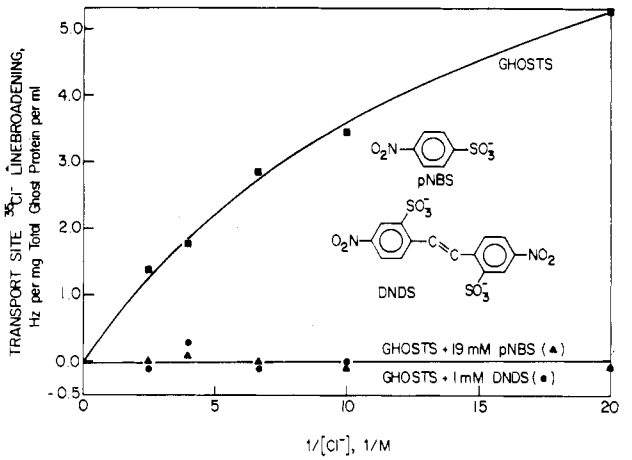

FIGURE 1: DNDS and pNBS inhibit transport site $^{35}Cl^-$ line broadening. Shown is the line broadening due to leaky ghosts. The solid curves are nonlinear least-squares best-fit curves for a set of low-affinity (lower curve, $y = A$, $K_D \gg 0.4$ M) chloride binding sites plus a homogeneous set of high-affinity [upper curve, $y = A + Bx/(K_D x + 1)$, $K_D = 60 \pm 5$ mM] transport sites. Both inward- and outward-facing transport sites contribute to the transport site line broadening, which is the difference between the two curves (Falke et al., 1984a). Each sample contained the indicated [NH_4Cl] as well as 2.5 mM NaH_2PO_4 and 20% D_2O, pH to 8.0 with NH_4OH and NaOH. Sufficient citric acid (pH to 8.0 with NaOH) was added to bring the ionic strength up to that of the sample containing the highest [NH_4Cl] = 500 mM. Spectral parameters: 8.8 MHz, 3 °C.

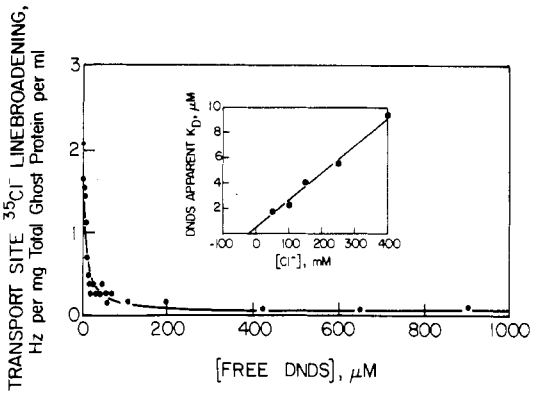

FIGURE 2: Competition between DNDS and Cl^- for binding to the transport site. Displacement of Cl^- from the transport site by DNDS. Shown is the line broadening due to both inward- and outward-facing transport sites on leaky ghosts. The solid curve is the nonlinear least-squares best-fit curve [$y = A + Bx/(K_D x + 1)$] for a homogeneous set of sites with an apparent K_D of 6.4 ± 0.5 μM for DNDS binding. The buffer was 250 mM NH_4Cl, 5 mM NaH_2PO_4, and 20% D_2O, pH to 8.0 with NH_4OH. Spectral parameters: 8.8 MHz, 3 °C. Inset: Displacement of DNDS from the transport site by Cl^-. The apparent K_D for DNDS binding to transport sites, determined as described above, is plotted for different [Cl^-]. The data are best fit by a linear least-squares straight line that yields $K_D = 0.5$ μM for DNDS binding at zero chloride concentration and $K_D = 20 \pm 10$ mM for chloride binding. Samples and determination of the transport site line broadening are as in Figure 1. Spectral parameters: 8.8 MHz, 3 °C.

3 is buried within the bilayer in a hydrophobic region that restricts free rotation of the inhibitor (Macara & Cantley, 1983). The reversible binding of stilbenedisulfonates is decreased by the presence of anion transport substrates, suggesting that these inhibitors occupy the transport site with a negative charge. DNDS has been shown to compete with Cl^- for binding to the transport site (Fröhlich, 1982) and can be used to verify the effectiveness of the NMR approach.

Line broadening experiments yield information on both the sidedness and the inhibitory mechanism of DNDS. In the intact red cell, intracellular sites are NMR-invisible so that only outward-facing low-affinity sites and transport sites are observed (Falke et al., 1984b). In this system externally added 100 μM DNDS reduces the total line broadening due to outward-facing sites from $(1.4 \pm 0.2) \times 10^{-9}$ Hz/(cell/mL) to $(0.5 \pm 0.1) \times 10^{-9}$ Hz/(cell/mL) (spectral parameters, 8.8 MHz, 3 °C; buffer composition, 250 mM NH_4Cl, 5 mM NaH_2PO_4, and 20% D_2O, pH to 8.0 with NH_4OH and NaOH). Thus the inhibitory binding site for the membrane-impermeant DNDS is exposed to the extracellular solution. In the leaky ghost system both inward- and outward-facing transport sites are observed; here 1 mM DNDS completely and specifically inhibits the line broadening due to all transport sites on both sides of the membrane [Figure 1; see also Falke et al. (1984b)]. The observed line broadening inhibition is due to DNDS binding to a homogeneous population of sites on band 3, since the binding can be described by an apparent dissociation constant $K_{Dapp} = 6.4 \pm 0.5$ μM ([Cl^-] = 250 mM) calculated for identical sites (Figure 2): this value is quantitatively consistent with the value $K_{D,app} = 4.4$ μM interpolated from direct studies of the DNDS–band 3 association (Fröhlich, 1982). Thus DNDS binds to homogeneous sites exposed to the external solution and thereby inhibits the line broadening of both inward- and outward-facing transport sites.

The mechanism of line broadening inhibition by DNDS involves inhibition of Cl^- binding to the transport site, since the apparent DNDS dissociation constant is a linearly increasing function of [Cl^-] (Figure 2, inset). The apparent dissociation constants for DNDS and Cl^- at zero anion concentration that are calculated from these data (0.5 ± 0.2 μM at [Cl^-] = 0 mM and 20 ± 10 mM at [DNDS] = 0 mM, respectively) agree closely with values obtained from direct binding studies (0.6 μM and 39 ± 4 mM, respectively) (Fröhlich, 1982). Thus line broadening studies support previous studies which indicate that DNDS binds to an externally accessible site and thereby competitively inhibits Cl^- binding to the transport site.

In principle DNDS could inhibit the line broadening due to two anion binding sites by simultaneously occupying both sites with its two negative charges. Thus it is important to ascertain whether one negative charge alone can produce the same effect on the line broadening as caused by DNDS. It has previously been shown that the monovalent substrate anions I^-, HCO_3^-, and Br^- each inhibit the transport site line broadening to the same extent as DNDS by binding to a homogeneous class of sites (Falke et al., 1984a; Falke & Chan, 1985). Similarly, the monovalent *p*-nitrobenzenesulfonate (pNBS), which is structurally related to DNDS and is known to compete with extracellular substrate for binding to transport sites, inhibits the transport site line broadening to the same extent as DNDS (Figure 3). Line broadening inhibition by pNBS stems from pNBS binding to a class of sites that can be accurately described as identical sites (Figure 3). Together the monovalent anion data indicate that the occupation of the transport site with a single negative charge is sufficient to specifically and completely eliminate the transport site line broadening. These results are completely consistent with a picture in which one of the negative charges on DNDS occupies the outward-facing transport site so that DNDS and Cl^- binding are mutually exclusive. When the site is saturated by sufficiently high [DNDS], all the transport sites on both sides of the membrane are recruited to the outward-facing conformation and inhibited by bound DNDS (Falke et al., 1984b).

DIDS (4,4'-Diisothiocyanostilbene-2,2'-disulfonate). Like

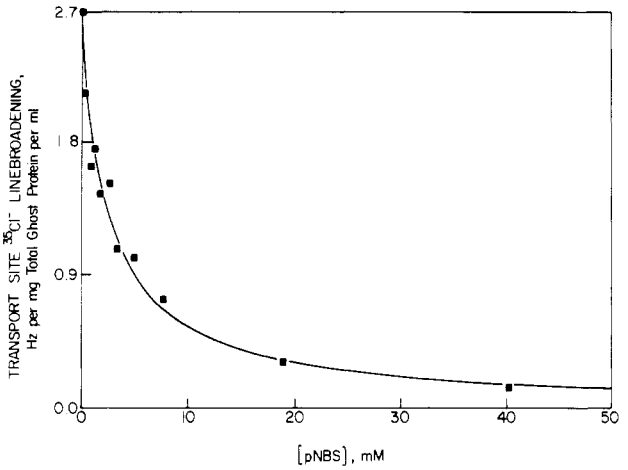

FIGURE 3: Titration of transport site $^{35}Cl^-$ line broadening with pNBS. Shown is the line broadening due to both inward- and outward-facing transport sites on leaky ghosts. The solid curve is a nonlinear least-squares best-fit curve for a set of homogeneous pNBS binding sites [$y = A[1 - x/(x + K_D)]$, $K_D = 3 \pm 1$ mM]. Samples contained 200 mM NH_4Cl, 17% D_2O, and 5 mM NaH_2PO_4, pH to 8.0 with NH_4OH and NaOH. Spectral parameters: 8.8 MHz, 3 °C.

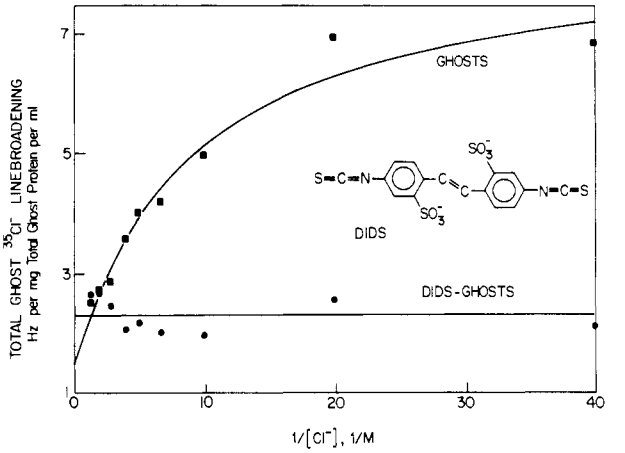

FIGURE 4: DIDS inhibits transport site $^{35}Cl^-$ line broadening. Shown is the line broadening due to leaky ghosts made from red cells labeled or unlabeled with DIDS. The solid curves are nonlinear least-squares best-fit curves for a set of low-affinity (lower curve, $y = A$, $K_D \gg 0.4$ M) chloride binding sites plus a homogeneous set of high-affinity [upper curve, $y = B + Cx/(K_D x + 1)$, $K_D = 100 \pm 20$ mM] transport sites. The samples each contained the indicated [NaCl] as well as 20% D_2O and 25 mM Tris, pH to 7.5 with NaOH. Sufficient citric acid (pH to 8.0 with NaOH) was added to bring the ionic strength up to that of the [NaCl] = 200 mM sample. Spectral parameters: 8.8 MHz, 21 °C.

DNDS, DIDS has been thought to occupy the transport site so that Cl^- binding is prevented. Here it is shown that DIDS does reduce the affinity of the transport site for Cl^-, suggesting that it binds near the site; however, DIDS does not fully block Cl^- binding to the site. Structurally, DIDS is similar to DNDS (R = —N=C=S in **1**), but the mechanistic differences between DIDS and DNDS stem from the covalent reaction of DIDS with a lysine residue on the 60-kDa chymotryptic fragment of band 3 (Ramjeesingh et al., 1980; Kopito & Lodish, 1985). The rapidity of this reaction has prevented studies of the effect of substrate anions on the reaction rate; however, Cl^- is known to competitively inhibit the noncovalent binding of the closely related H_2DIDS (Shami et al., 1978) (the latter has the same structure as DIDS except that the stilbene double bond is reduced to a single bond—see **1**). The location of the stilbenedisulfonate binding site is external since DIDS and H_2DIDS inhibit each other when added to the external compartment. Thus it has previously been assumed that DIDS is a competitive inhibitor of Cl^- binding to the outward-facing transport site [reviewed by Macara and Cantley (1983) and Knauf (1979)].

Line broadening studies verify that DIDS and Cl^- compete for binding. DIDS reduces the line broadening due to transport sites (Figure 4); however, this inhibition of the transport site line broadening is not complete. The line broadening expected when transport sites are completely inhibited can be estimated by following the square hyperbola due to the sites to the y intercept at $1/[Cl^-] = 0$, where the transport site line broadening = α[bound Cl^-]/[Cl^-] = 0. The transport site line broadening due to ghosts saturated with DNDS or pNBS is the same as that due to transport sites at $1/[Cl^-] = 0$, indicating complete inhibition by these inhibitors (Figure 1). In contrast, the line broadening due to DIDS-modified membranes appears to be larger than this estimated zero point, *suggesting* incomplete inhibition (Figure 4). However, due to uncertainty in the data and in the y intercepts of both the upper and lower curves in Figure 4, ambiguity exists in the extent of transport site line broadening inhibition by DIDS.

In order to examine more closely the extent of transport site line broadening inhibition by DIDS, it is important to directly compare the line broadening inhibition due to DIDS and DNDS. To do this, a quantity of red cells was divided into two parts and treated equally, except one part was labeled by incubation with DIDS while the other part was incubated without DIDS. Membranes were isolated from both parts, and then the $^{35}Cl^-$ line broadening was measured in the presence and absence of DNDS (Table I). The line broadening due to the DIDS-labeled ghost is significantly larger than the line broadening due to unlabeled ghosts to which DNDS is added. The difference in line broadening is not simply due to incomplete DIDS labeling, since the addition of DNDS to DIDS-labeled membranes has no further inhibitory effect (Table I). Thus, complete reaction with DIDS produces less line broadening inhibition than observed for saturation of the transport sites with DNDS. The simplest interpretation of this result is that the total line broadening which remains after modification by DIDS is composed of both (1) low-affinity Cl^- binding sites that are insensitive to DIDS and DNDS and that are normally subtracted out on plots of transport site line broadening and (2) incompletely inhibited transport sites that have been converted to low-affinity sites ($K_{D'} \gg 0.5$ M), since the square hyperbola due to high-affinity sites is transformed by DIDS to a straight line of zero slope (Figure 4, eq 2). This interpretation suggests that covalently attached DIDS does not completely inhibit binding to Cl^- to the transport site, in contrast to DNDS and the other competitive inhibitors which yield the same complete inhibition of the transport site line broadening as DNDS [I^-, HCO_3^-, Br^- (Falke et al., 1984a), and pNBS (Figure 3)].

Table I: DIDS Does Not Completely Inhibit DNDS-Sensitive $^{35}Cl^-$ Line Broadening

	total $^{35}Cl^-$ line broadening[a] [Hz/(mg of total ghost protein/mL)]	
[DNDS] (total) (mM)	ghosts	DIDS-modified ghosts
0	5.1 ± 0.1	3.9 ± 0.0
1	2.4 ± 0.1	3.8 ± 0.2

[a] Spectral parameters: 8.8 MHz, 21 °C. Buffer composition: 250 mM NH_4Cl, 5 mM NaH_2PO_4, and 20% D_2O, pH to 8.0 with NH_4OH and NaOH.

The sidedness of the residual transport site line broadening is difficult to ascertain, since DIDS treatment changes the

Table II: Effect of DIDS on $^{35}Cl^-$ Line Broadening due to Sealed Inside-Out Vesicles (IOV)

	total $^{35}Cl^-$ line broadening[a] [Hz/(mg of total ghost protein/mL)]	
	predicted for intracellular surface	obsd for IOV[b]
inhibited by DIDS (transport sites)	0.7 ± 0.2	0.6 ± 0.2
uninhibited by DIDS	2.7 ± 0.7	2.2 ± 0.2

[a] Spectral parameters: 8.8 MHz, 3 °C. Buffer composition: 250 mM NaCl, 5 mM NaH_2PO_4, and 20% D_2O, pH to 8.0 with NaOH. [b] Assays of sealing and sidedness by acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase [conducted as in Falke et al. (1984b)] indicate 73 ± 1% sealed IOV and 11 ± 1% sealed ROV.

physical properties of the red cell membrane. In particular, DIDS interferes with the membrane systems used to specifically observe Cl^- binding sites on the extracellular membrane surface: DIDS increases the spontaneous lysis of red cells during NMR experiments, and leaky ghosts isolated from DIDS-labeled red cells are a poor starting material for preparation of sealed right-side-out vesicles and crushed ghosts (data not shown). Thus it has not been possible to specifically examine the line broadening due to extracellular sites alone. However, sealed inside-out vesicles (IOV) can be prepared from DIDS-labeled membranes (Table II) so that sites on the intracellular membrane surface can be specifically examined (Falke et al., 1984b). IOV with and without DIDS labeling yield line broadenings that are indistinguishable from the line broadenings predicted for the intracellular surface of leaky ghosts in the presence and absence of 1 mM DNDS, respectively (Table II). Such results suggest that the effect of DIDS on the intracellular sites is the same as that of DNDS; thus DIDS eliminates the line broadening due to inward-facing transport sites, and the residual transport site line broadening observed after DIDS labeling stems solely from outward-facing transport sites.

The simplest picture consistent with the data is that covalently bound DIDS binds in the vicinity of the outward-facing transport site and inhibits the inward-facing sites by recruitment to the outward-facing conformation. The bound DIDS does not fully occupy the outward-facing site, so that Cl^- binding can still occur but K_{Dj} (eq 2) is increased. The magnitude of the residual transport site line broadening indicates that Cl^- exchange between the partially inhibited site and solution remains intact so the α_j (eq 2) is not decreased. In fact, the magnitude of the residual line broadening is sufficiently large to suggest that an increase in α_j occurs that counteracts the decrease in line broadening expected for an increase in K_{Dj} (eq 2). Such a situation is feasible since the outward-facing transport site is in the slow-exchange limit, where $\alpha_j = k_{OFF}/\pi$. This analysis suggests that the increase in K_{Dj} stems from an increase in k_{OFF}, which causes a corresponding increase in α_j so that significant line broadening can be observed for the inhibited site. Together, these data suggest a mechanism for DIDS line broadening inhibition that involves recruitment of transport sites to the outward-facing conformation, to which DIDS covalently binds and competitively inhibits Cl^- binding by partial occupation of the transport site.

Thus the lysine with which DIDS reacts may not provide an essential positive charge in the transport site, since modification by DIDS does not completely destroy Cl^- binding and its associated transport site line broadening. pH studies have, in contrast, implicated arginine as a residue providing one or more essential positive charges (Wieth & Bjerrum, 1982; Falke & Chan, 1985). The presence of such arginines can be tested with arginine-specific reagents.

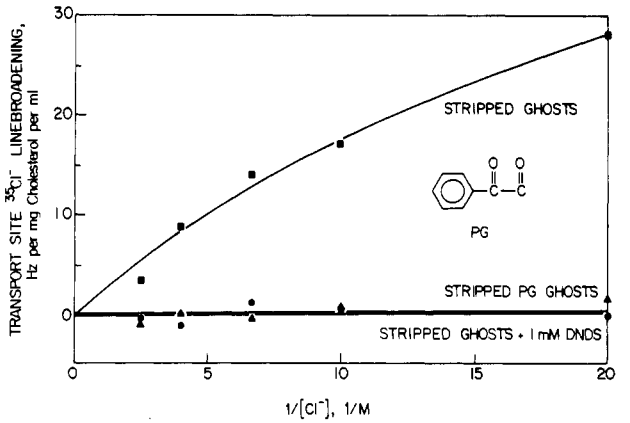

FIGURE 5: Phenylglyoxal inhibits transport site $^{35}Cl^-$ line broadening. Shown is the line broadening due to both inward- and outward-facing transport sites on leaky ghosts that were labeled or unlabeled with PG and then stripped and sonicated. The solid curves are nonlinear least-squares best-fit curves for a set of inhibited (lower curve, $y = 0$) and intact [upper curve, $y = Bx/(K_D x + 1)$, $K_D = 100 \pm 20$ mM] transport sites. Sample composition is as in Figure 1. Spectral parameters: 8.8 MHz, 3 °C.

PG (Phenylglyoxal). PG is an arginine-specific covalent modification reagent that holds great potential as a label for transport site arginines: here the ability of PG to inhibit Cl^- binding to the transport site is tested. PG reacts covalently with deprotonated arginine in a reaction that is highly specific for an arginine exhibiting relatively low pK_a's, such as those in anion binding sites, and the stoichiometry of labeling is generally 2 molecules of PG per arginine (Riordan, 1979). When the extracellular surface of the red cell membrane is labeled, 2 molecules of PG per band 3 monomer are incorporated into the 35-kDa chymotryptic fragment. These two PG molecules are sufficient to inhibit anion transport, suggesting that modification of one arginine can produce complete inhibition (Wieth et al., 1982; Bjerrum et al., 1983). The PG reaction is slowed by the substrate Cl^- or the transport site inhibitor DNDS, indicating that the essential arginine could be within the transport site itself (Wieth et al., 1982; Zaki, 1983; Falke & Chan, 1984).

PG completely and specifically eliminates the line broadening due to band 3 transport sites on both sides of the red cell membrane (Figure 5). The specificity of line broadening inhibition is better for stripped ghosts (Figure 5) than for unstripped ghosts, indicating that stripping removes virtually all of the PG-sensitive low-affinity Cl^- binding sites (Falke & Chan, 1984). The rate of the inhibitory PG reaction is slowed by a factor of 2–3 by 300 mM Cl^- or 200 μM DNDS (Falke & Chan, 1984): these results are consistent with the idea that Cl^-, DNDS, and PG are mutually exclusive species that each occupy the transport site. The sidedness of the PG labeling cannot be determined from the data of Figure 5 because both sides of the membrane are labeled under the conditions used here. Thus the simplest interpretation of these results is that PG covalently modifies one or more essential arginines in either the inward- or outward-facing conformations of the transport site, or in both conformations.

Discussion

The transport site inhibitors examined here can be grouped into two classes: (1) those that fully occupy the site and prevent Cl^- binding (DNDS and PG) and (2) those that do not fully occupy the site so that the affinity of Cl^- for the site is reduced but not destroyed (DIDS). Both classes inhibit the

line broadening due to both inward- and outward-facing transport sites.

All of the results presented are consistent with the ping-pong model for the Cl^- transport cycle, in which a single transport site is alternately exposed to opposite sides of the membrane. DNDS, pNBS, and DIDS each bind to the outward-facing transport site [Barzilay and Cabantchik (1979a,b) and Cabantchik and Rothstein (1972), respectively], and the ping-pong model predicts that such inhibitors will recruit the sites to the inhibited outward-facing conformation. Thus such inhibitors should inhibit the transport site line broadening on both sides of the membrane, as is observed (Figure 1 and 4; Falke et al., 1984b). In addition, the titration curves for DNDS and pNBS inhibition can be described by a single apparent K_D for inhibitor binding (Figures 2 and 3) as predicted by the ping-pong model. Similarly, the dependence of this apparent K_D on $[Cl^-]$ (Figure 2, inset) is predicted by the ping-pong model (Falke & Chan, 1985). These examples add to the large number of ^{35}Cl NMR results that are completely consistent with a ping-pong transport cycle (Gunn & Fröhlich, 1979; Falke et al., 1984b, 1985a; Falke & Chan, 1985).

A striking characteristic of transport site inhibitors is their central planar hydrophobic region. This structural feature suggests that the inhibitor binding site is in a hydrophobic cleft. Consistent with this picture is a model in which the transport site lies at a hydrophobic interface between two adjacent transmembrane α-helices: such a model is presented in more detail in the third paper of three in this issue (Falke & Chan, 1986b).

A fundamental conclusion of the present study is that PG covalently modifies at least one essential arginine in the transport site. Since it reacts with the deprotonated guanidino group, PG typically exhibits specificity for arginine residues in anion binding sites where the guanidino charge is destabilized by a hydrophobic environment and/or nearby positive charge(s). The observation that the rate of the inhibitory PG reaction is slowed by Cl^- and DNDS (Wieth et al., 1982; Zaki, 1983; Falke & Chan, 1984) strongly supports the idea that PG inhibition stems from modification of one or more essential arginine residues within the transport site itself. Thus Cl^- and DNDS, which occupy the transport site, can protect the site against modification. Labeling of the extracellular membrane surface modifies 1–2 essential arginines in the outward-facing transport site (Bjerrum et al., 1983), and additional arginines are modified when both sides of the membrane are labeled (Bjerrum et al., 1983; Zaki, 1984), as in the present study. The pH dependence of anion transport (Wieth & Bjeerum, 1982) and of the transport site line broadening (Falke & Chan, 1985) requires arginine and rules out lysine in either or both of the inward- and outward-facing transport site conformations of a ping-pong transporter. Modifying both sides of the membrane with PG may therefore produce two populations of band 3, in which essential arginines are modified in the inward- and outward-facing transport sites, respectively. However, unlike the arginine(s) strongly implicated in the outward-facing transport site, the arginine(s) that may exist in the inward-facing transport site has (have) yet to be demonstrated.

In summary, DNDS, DIDS, and PG inhibit anion transport and the transport site line broadening by fully or partially occupying the band 3 transport site. DNDS and PG are mutually exclusive inhibitors of Cl^- binding to the site, while DIDS may reduce the affinity of Cl^- for the site without destroying binding. The second paper of three in this issue (Falke & Chan, 1986a) summarizes a second class of inhibitors, which block a channel leading from the transport site to solution.

Registry No. pNBS, 138-42-1; DNDS, 128-42-7; DIDS, 53005-05-3; PG, 1074-12-0; Cl^-, 16887-00-6; L-arginine, 74-79-3.

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