Mechanism of Anion Exchange across the Red Cell Membrane by Band 3: Interactions between Stilbenedisulfonate and NAP-taurine Binding Sites†

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ABSTRACT: Anion exchange across the erythrocyte membrane can be inhibited competitively by stilbenedisulfonates, which bind to the external transport site of the band 3 protein, and noncompetitively by external NAP-taurine [2-[*N*-(4-azido-2-nitrophenyl)amino]ethanesulfonate], which it has been suggested binds to a "modifier site" [Knauf, P. A., Ship, S., Breuer, W., McCulloch, L., & Rothstein, A. (1978) *J. Gen. Physiol. 72*, 604–630]. The binding of the two types of inhibitor to erythrocyte membranes is shown in the present study to be competitive, indicating that binding to the same subunit of band 3 is mutually exclusive. Covalent labeling of red cells with a stilbenedisulfonate [4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate (BIDS)] to 80% saturation had no detectable effect upon the K_i for inhibition of [^{32}P]phosphate influx by NAP-taurine, indicating that when bound to adjacent subunits in the band 3 dimer, the two types of inhibitor do not interact. In addition to the external NAP-taurine site, a second high-affinity NAP-taurine site (K_d = 15 μM) was detected on the cytoplasmic side of red cell membranes. This site is less than 51 Å from the disulfonic stilbene binding site, as judged by fluorescence resonance energy transfer from BIDS to NAP-taurine. Binding at this site is not affected by covalent attachment of BIDS, and no clear role for this site in transport could be determined. On the basis of these studies we present a model indicating that disulfonic stilbenes bind to a site which overlaps both the anion transport site and the modifier site on a band 3 monomer and suggests that the modifier site may be part of a transporting gate.

Anion exchange across the red cell plasma membrane is mediated by a well characterized dimeric protein called band 3 (Cabantchik et al., 1978; Knauf, 1979). This transport system has attracted considerable attention, largely because of its abundance [about 25% of total membrane protein; Fairbanks et al. (1971)] and its apparent simplicity. The mechanism by which it exchanges anions, however, remains elusive. Early kinetic studies were interpreted in terms of a mobile carrier model, in which a single site moves in such a way as to face alternately inward and outward, carrying anions across the membrane in opposite directions with each cycle (Wieth, 1972; Gunn et al., 1973). Since net anion flow across the membrane is negligibly small, the constraint was included that the unoccupied site cannot cross the membrane.

Much evidence for the mechanism of anion exchange has come from work with two classes of organic sulfonates that have been used as high-affinity inhibitors of the red cell anion-exchange system. The stilbenedisulfonates are membrane-impermeant agents which competitively inhibit anion exchange only from the external side of the membrane (Cabantchik & Rothstein, 1972, 1974). Several of these compounds have been shown to react specifically with the band 3 protein of erythrocyte membranes at a stoichiometry of one site per band 3 monomer (Lepke et al., 1976; Ship et al., 1977; Jennings & Passow, 1979). Fluorescence resonance energy transfer measurements located the stilbenedisulfonate binding site between 34 and 42 Å from the cytoplasmic domain of the band 3 protein, suggesting some penetration into the bilayer (Rao et al., 1979). In addition, the distance between two disulfonic stilbenes on a band 3 dimer has been measured to be 28–48 Å [transition dipole center-to-center distance; Macara & Cantley (1981)]. The proximity of these rather large inhibitors may explain the apparent negative cooperativity observed when they bind to band 3 (Dix et al., 1979).

The other class of inhibitor is represented by the photoaffinity label 2-[*N*-(4-azido-2-nitrophenyl)amino]ethanesulfonate (NAP-taurine).[1] This probe is transported with low V_{max} and acts as a competitive inhibitor (K_i = 350 μM) of anion exchange when added to the cytoplasmic side of the membrane (Knauf et al., 1978a). When added to the outside, however, it inhibits anion exchange in a noncompetitive manner (K_i = 20 μM). This behavior has been explained by postulating the existence of an external "modifier site", the occupation of which inhibits transport (Dalmark, 1976; Knauf et al., 1978a). Illumination of NAP-taurine in the presence of either intact red cells or inside-out membranes appears to result in the formation of a covalent bond at the external or internal sites, respectively (Knauf et al., 1978b; Grinstein et al., 1978). Grinstein et al. (1978) showed that when 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) reacted with the external site of band 3, NAP-taurine could no longer react from the cytoplasmic side, providing further support for a mobile carrier mechanism.

Although the experiments above suggest that the modifier site is separate from the transport site, other alternatives exist. One possibility is that transport on one subunit of the dimer is inhibited by NAP-taurine binding to the transport site on the adjacent subunit. Negative cooperativity has already been observed for stilbenedisulfonate binding to band 3 (Dix et al., 1979; Macara & Cantley, 1981) although recent experiments indicate that transport occurs independently on the two subunits (Macara & Cantley, 1981). A second possibility is that the transport site and modifier site are both part of a transporting gate and that the stilbenedisulfonates overlap both sites. Circumstantial evidence in favor of this latter possibility has been provided by Cabantchik et al. (1976), who showed that photoreaction of NAP-taurine with red cells substantially

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[1] Abbreviations used: NAP-taurine, 2-[*N*-(4-azido-2-nitrophenyl)-amino]ethanesulfonate; NBD-taurine, 2-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]ethanesulfonate; DADS, 4,4′-diaminostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; BIDS, 4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate; BADS, 4-benzamido-4′-aminostilene-2,2′-disulfonate; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulfonate; H_2DIDS, 2,2′-(1,2-ethanediyl)bis[5-isothiocyanobenzenesulfonate]; NPM, *N*-pyrenemaleimide; NaDodSO_4, sodium dodecyl sulfate; IO, inside out; RO, right side out.

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reduced the binding of DIDS to band 3. NAP-taurine and DIDS react with the same 17 000-dalton transmembrane fragment at band 3 (Knauf et al., 1978a). Moreover, NAP-taurine appears able to react externally with band 3 only when the system is in an outward-facing conformation (Knauf et al., 1980).

Experimental Procedures

Materials. NAP-taurine and DIDS were purchased from Pierce Chemical Co., *N*-pyrenemaleimide was from Regis Chemical Co., and DADS was from Eastman. Triton X-100 was from Rohm and Haas. Trypsin and trypsin inhibitor were from Sigma. BADS and BIDS were prepared from DADS by the method of Rao et al. (1979). [U-^{14}C]Sucrose was from Amersham Radiochemicals; [^{3}H]water and [^{32}P]phosphoric acid were from New England Nuclear.

Preparation of Labeled Erythrocyte Ghosts and Vesicles. Ghosts were prepared from newly outdated red cells as previously described (Macara & Cantley, 1981). DIDS-labeled red cells, BIDS ghosts, and NPM ghosts were prepared by the methods of Rao et al. (1979) and were either stored at 4 °C or, for storage periods of more than 1 week, quick-frozen in dry ice–acetone and kept at −70 °C. Thawed ghosts were not refrozen. The extent of saturation of red cells by BIDS was varied by changing the amount of BIDS present in the reaction mixture. The BIDS and band 3 stoichiometries were determined as described by Rao et al. (1979). Inside-out (IO) and right-side-out (RO) vesicles were prepared from appropriately labeled red cells by the method of Steck & Kant (1976) with the modifications described by Grinstein et al. (1978). The sidedness of the vesicles was determined from the acetylcholinesterase activity in the presence and absence of Triton X-100 (Steck & Kant, 1974). Protein concentration was determined either by the method of Lowry et al. (1951) or from the absorption at 280 nm of membranes solubilized in 1% $NaDodSO_4$ [assuming an absorptivity of 1.09 mL/(mg·cm)]. Peripheral proteins were stripped from ghosts when necessary by incubation in 10 mM NaOH and 0.1 mM EDTA for 5 min at 0 °C (Grinstein et al., 1978). The ghosts were then spun down at 40000*g* for 15 min, washed 3 times with 5 mM sodium phosphate, pH 7.5, and then made up to their original volume in 28.5 mM sodium citrate, pH 7.4. Trypsinization was carried out at 100 μg/mL trypsin for 1 h at 0 °C in 5 mM sodium phosphate and 0.1 mM $MgSO_4$, pH 7.5, at a protein concentration of 1 mg/mL. Digestion was stopped by addition of 200 μg/mL soybean trypsin inhibitor, and the ghosts were washed as described above.

Inhibition of [^{32}P]Phosphate Influx. [^{32}P]Phosphate (specific activity 1.0–1.25 Ci/mol) was incubated at a concentration of 4 mM with red cells at 40% hematocrit in 28.5 mM sodium citrate and 205.3 mM sucrose, pH 7.4. After 40 min at 4 °C, the cells were centrifuged in a microfuge and washed once with 1.0 mL of cold 100 mM sodium phosphate, pH 7.5. The pellet was treated with 1.0 mL of 10% Cl_3CCOOH and centrifuged, and 0.1 mL of the supernatant was counted for ^{32}P in Aquasol. It was established that influx was linear over the 40-min incubation period. Inhibition of influx by external NAP-taurine was performed at 4 °C to hinder influx of the inhibitors (Cabantchik et al., 1976). Inhibition from the inside of the cells was measured by equilibrating with the inhibitor for 30 min at 37 °C and then washing the cells with cold citrate/sucrose, before addition of the [^{32}P]phosphate.

Equilibrium Binding. Equilibrium binding to control and DIDS-treated ghosts was performed as previously reported (Macara & Cantley, 1981). Ghosts were suspended to 2 mg/mL in 28.5 mM sodium citrate and incubated with NAP-taurine (in the dark) at 37 °C for 15 min before centrifugation (40000*g* for 15 min at 4 °C). Supernatants were treated with perchloric acid before measuring the absorbances of the free probes. NAP-taurine was measured at 260 nm (absorptivity = 14.9 mM^{-1} cm^{-1} in 5% perchloric acid).

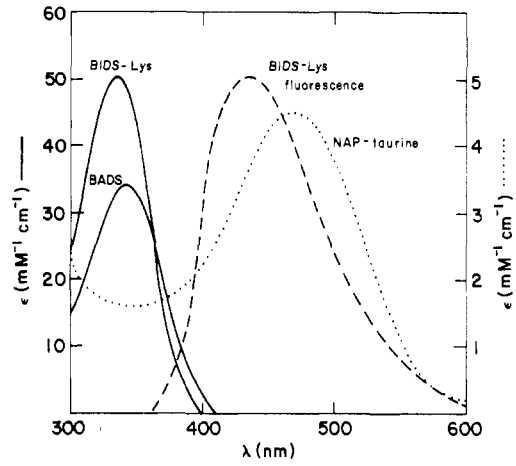

FIGURE 1: Absorption and emission spectra of probes for anion sites on band 3. (—) Absorption spectra of BADS and BIDS-lysine in 28.5 mM sodium citrate, pH 7.4; (···) absorption spectrum of NAP-taurine in 28.5 mM sodium citrate, pH 7.4; (- - -) BIDS-lysine emission spectrum (corrected) in arbitrary units.

Fluorescence Measurements. Fluorescence measurements were performed as described by Rao et al. (1979) and Macara & Cantley (1981) on an SLM 4800 polarization spectrofluorometer interfaced to a PET microcomputer. Titrations were performed in 28.5 mM sodium citrate, pH 7.4. Fluorescence resonance energy transfer was measured by donor quenching. Quenching was corrected in each case for inner-filter absorbance which was determined experimentally by titrating the donor, free in solution, with acceptor as described by Rao et al. (1979). DBDS free in solution (which was fluorescent properties similar to BIDS ghosts) was used in determinations of inner-filter quenching of BIDS ghost fluorescence. Unlabeled ghosts were used to correct for light scattering, which was usually ~5% of the measured fluorescence. The total dilution was always less than 15% and was accounted for in the inner-filter correction.

Results

Competition between Binding of NAP-taurine and BADS. Although the covalent labeling of cells with DIDS decreases subsequent labeling by photoactivated NAP-taurine (Cabantchik et al., 1976), the nature of the interaction between the two probes has not been determined. The effects of NAP-taurine on the equilibrium binding of a stilbenedisulfonate were therefore studied. 4-Benzamido-4′-aminostilbene-2,2′-disulfonate (BADS) was selected as a noncovalent analogue of DIDS, and its ability to quench the fluorescence of nearby tryptophan residues was utilized as a measure of binding to band 3 (Rao et al., 1979). The absorptivity of NAP-taurine at 340 nm is considerably smaller than that of BADS (see Figure 1) and therefore causes less tryptophan fluorescence quenching when bound to band 3. Results are shown in Figure 2. In the absence of NAP-taurine, BADS binds to cell membranes with a K_d of 1 μM.[2] The affinity

[2] At high BADS concentrations, some curvature in the double-reciprocal plots becomes apparent, suggesting negative cooperativity or a second weaker site [see Dix et al. (1979)]. The data at high BADS concentrations were omitted so that only competition at the high-affinity site is investigated.

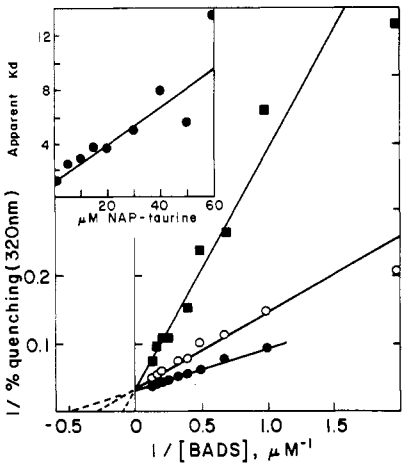

FIGURE 2: Competition between binding of BADS and NAP-taurine to red cell membranes. Quenching of intrinsic membrane tryptophan fluorescence by BADS was used to monitor binding, with excitation at 295 nm and emission detected at 335 nm. Membranes at 0.23 mg of protein/mL in 29 mM sodium citrate, pH 7.4, 25.0 °C, were titrated with BADS in the absence (●) or presence (○, ■) of NAP-taurine. Data were corrected for light scattering and inner-filtering quenching as described under Experimental Procedures. Typical data are shown in a double-reciprocal plot [for 0 (●), 15 (○), and 50 (■) μM NAP-taurine]. Apparent K_d values for BADS binding were calculated from the original data by a nonlinear least-squares procedure. The inset shows the variation in apparent K_d with NAP-taurine concentration. The line was drawn through the data with assumption of a true K_d for BADS binding of 1.3 μM and a K_i for NAP-taurine of 8.6 μM. The correlation coefficient, by least-squares analysis, was 0.883.

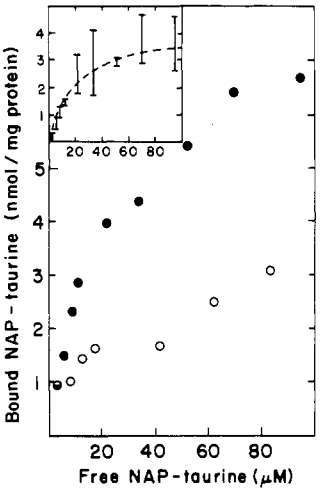

FIGURE 3: Equilibrium binding of NAP-taurine to erythroycte membranes. Membranes were prepared either from untreated (●) cells or from cells labeled to saturation with a large excess of DIDS (○). Binding was estimated from the concentration of free NAP-taurine (determined from its absorbance at 260 nm) remaining in the supernatant after centrifugation of a suspension of membranes in 29 mM sodium citrate, pH 7.4. Solutions containing NAP-taurine were protected from light. Data points are means from two separate experiments. The inset shows the difference in binding between untreated and DIDS-treated cells. Error bars give values ±1 standard deviation, calculated from the unaveraged data shown in the main figure (n = 4). The line through the data in the inset was drawn on the basis of a single class of binding sites with K_d = 15 μM and a stoichiometry of 3.8 nmol/mg. The estimated stoichiometry of NAP-taurine binding based on a nonlinear least-squares fit is 2.5–4.5 nmol/mg of protein. The estimated K_d = 10–20 μM.

is higher than that reported previously [10 μM; Rao et al. (1979)] because the titration was performed at a higher ionic strength [150 vs. 25 mM; see Dix et al. (1979) for the effect of ionic strength on DBDS binding]. NAP-taurine decreased the apparent K_d for BADS in a competitive manner, with a K_i of 9 μM (Figure 2, inset). This value is close to that reported by Knauf et al. (1978a) for the noncompetitive inhibition of Cl^- exchange in red cells and to that reported in the present study for inhibition of [^{32}P]phosphate influx (see below). It was noted that only freshly prepared membranes exhibited competition between NAP-taurine and BADS binding; binding of BADS to membranes stored at 4 °C for more than 3–4 days, or frozen and thawed, was apparently independent of the total NAP-taurine concentration, up to at least 100 μM. The cause of this change in behavior is not understood, but it is possible that the frozen membranes no longer bind NAP-taurine. These results indicate that NAP-taurine and BADS compete for binding to band 3 on freshly prepared red cell ghosts.

The effect of prior reaction of cells with a stilbenedisulfonate (DIDS) on subsequent binding of NAP-taurine to ghosts was determined by direct equilibrium binding measurements. The results are shown in Figure 3. The stoichiometry of specific NAP-taurine binding was somewhat obscured by a nonsaturable component. However, the difference between NAP-taurine binding to control ghosts and DIDS-reacted ghosts showed saturable binding with a K_d of ~15 μM and stoichiometry of ~3.8 nmol/mg (Figure 3, inset). This stoichiometry is somewhat higher than that determined for BIDS [~2.5 nmol/mg; Macara & Cantley (1981)] but is similar to that observed for eosin maleimide [~3.5 nmol/mg; Macara & Cantley (1981)] to band 3. The K_d for NAP-taurine is similar to the constant for competitive inhibition of BADS binding in Figure 2. It should also be pointed out that even in the presence of DIDS, the NAP-taurine appears to exhibit a saturable binding component (Figure 3). The results in Figures 2 and 3 suggest that NAP-taurine competes with disulfonic stilbenes for binding to one site per band 3 monomer.

The External NAP-taurine Site and Stilbenedisulfonate Site on Adjacent Subunits Do Not Interact. The following experiment was performed to determine if the affinity for NAP-taurine on one half of the band 3 dimer is affected by binding a stilbenedisulfonate to the other half (as would be expected if the NAP-taurine modifier site is the transport site of an interacting subunit). The K_i for NAP-taurine inhibition of [^{32}P]phosphate uptake was measured on control red cells and on red cells in which ~80% of the band 3 monomers were covalently labeled with BIDS (Figure 4). Although only about 20% as much [^{32}P]phosphate transport was observed for the BIDS labeled cells, the K_i for NAP-taurine inhibition was ~10 μM in both cases. This value is about half that determined by Knauf et al. (1978b). The discrepancy probably arises from the absence of external chloride in our experiments, a condition which produces an increase in intracellular pH and an increase in the proportion of outward-facing sites able to bind NAP-taurine (Knauf et al., 1980). Previous experiments have shown that at 80% labeling with BIDS, most of the band 3 dimers have at least one subunit labeled (Macara & Cantley, 1981). An additional control was done to show that inhibition of [^{32}P]phosphate influx by internal NAP-taurine was much less potent than that by external NAP-taurine. Thus, NAP-taurine and stilbenedisulfonates appear to show simple competition on each subunit of the band 3 dimer with no interaction between sites on adjacent subunits.

An Additional High-Affinity NAP-taurine Site on the Cytoplasmic Side of Band 3. For understanding more clearly the relative locations of the NAP-taurine and stilbenedisulfonate binding sites, fluorescence resonance energy transfer was measured between BIDS and NAP-taurine. Figure 5 is a Scatchard-like plot showing the quenching of BIDS fluorescence by NAP-taurine added to BIDS-labeled ghosts.

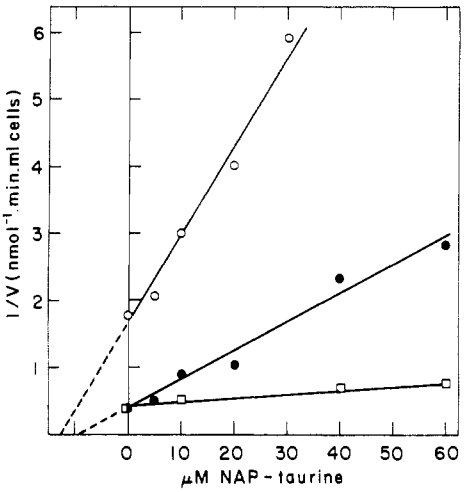

FIGURE 4: Effect of BIDS labeling on K_i for inhibition of [^{32}P]-phosphate influx into red cells by NAP-taurine. Initial rates were measured on cells at 40% hematocrit in isotonic, isoosmotic sodium citrate/sucrose, pH 7.4, at 4 °C, as described under Experimental Procedures. Cells were either untreated (●) or labeled to 80% saturation with BIDS (○). The concentration of sodium phosphate in the suspension was 4.0 mM in each case. Influx was also measured by using unlabeled cells which were preloaded with NAP-taurine and then washed free of external NAP-taurine (□). Points are means of duplicate measurements. Lines were drawn through the data with assumption of K_i values for NAP-taurine of 9.7 (●) or 11.4 (○) μM, as estimated by a least-squares fit to the data [correlation coefficients: 0.989 (●); 0.986 (○)]. [The K_m for phosphate influx is about 80 mM (Ho & Guidotti, 1975). Since the substrate concentration used is much lower than this value, the K_i can be assumed to equal the value of the intercept on the abscissa, to a good approximation.]

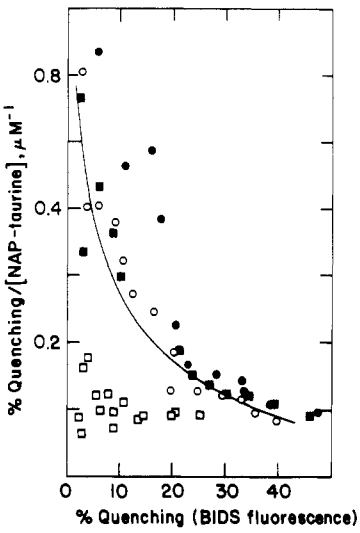

FIGURE 5: Quenching of BIDS fluorescence by NAP-taurine in membranes labeled to various stoichiometries with BIDS. Titrations were performed as described in Figure 2 but with exciting light of 360 nm, emission being detected at 427 nm. Membranes had been prepared by labeling with 0.4 (●), 0.63 (○), and 1.75 (■) nmol of BIDS/mg of protein (16, 25, and 70% saturation, respectively). Membranes labeled to 25% saturation were also titrated in the presence of 5% Triton X-100 (□). The data were analyzed by assuming two independent classes of sites, binding being described by the equation $E = Q_1L/(K_1 + L) + Q_2L/(K_2 + L)$, where E is the percent quenching of BIDS fluorescence due to bound NAP-taurine, L is the concentration (μM) of free NAP-taurine, K_1 and K_2 are the dissocition constants, and Q_1 and Q_2 are the percent quenching of BIDS fluorescence extrapolated to saturating NAP-taurine concentration. The line through the data was drawn with the assumption that K_1 = 13 μM, Q_1 = 9% and K_2 = 260 μM, Q_2 = 52%. These values were obtained by a nonlinear least-squares procedure using data from the membrane labeled to 25 and 50% saturation with BIDS. From the value of Q_1, the maximum possible separation of BIDS and NAP-taurine (bound at the high-affinity site) can be calculated[4] to be 51 Å.

Although the quenching shows a saturable component with a K_d of ~15 μM, the percent maximal quenching is independent of whether band 3 has been labeled to only 10 or to 80% of maximal stoichiometry. This result is surprising since at 10% BIDS labeling, most BIDS-labeled monomers should have an adjacent subunit available for NAP-taurine binding while at 80% BIDS labeling, most band 3 dimers will have both subunits occupied by BIDS (assuming random labeling).[3] This results suggests that NAP-taurine is not quenching BIDS fluorescence by binding to the external modifier site on the other subunit of the dimer since binding at this site would be considerably reduced on ghosts saturated to 80% with BIDS. The NAP-taurine binding which persists on DIDS-saturated red cell ghosts (see Figure 3) may be responsible for quenching the BIDS fluorescence. NAP-taurine had little effect on BIDS fluorescence after solubilizing ghosts in Triton X-100.

For clarification of the location of the NAP-taurine site which caused quenching of BIDS fluorescence, the quenching experiments were repeated in right-side-out and inside-out vesicles. The data presented in Figure 6A show that the high-affinity site (K_d ~15 μM) at which NAP-taurine quenches BIDS fluorescence is on the outside of inside-out vesicles (i.e., on the cytoplasmic side). Although NAP-taurine quenched BIDS fluorescence of right-side-out vesicles, the apparent K_d for this effect was considerably higher than that observed for inside-out vesicles. The data in Figure 6B show that the fluorescence of *N*-pyrenemaleimide [which binds exclusively to the cytoplasmic side of red cell ghosts; Rao et al. (1979)] is also quenched by NAP-taurine binding to a high-affinity site (K_d ~ 20 μM). This site is abolished by treatment with trypsin. The data in Figure 6C show that the high-affinity NAP-taurine site is still present after all peripheral proteins are stripped by treatment with NaOH/EDTA but that mild trypsin digestion (conditions which remove the 40 000-dalton cytoplasmic domain of band 3) again eliminates the site. These results all indicate that a high-affinity NAP-taurine binding site exists on the cytoplasmic-facing side of band 3 (or an associated protein), that this site is close enough to the stilbenedisulfonate binding site to cause fluorescence quenching, and that mild tryptic digestion or Triton X-100 solubilization eliminates the site. Since 20 μM NAP-taurine in the cytoplasm of red cells has little effect on anion exchange (see Figure 3), this high-affinity cytoplasmic NAP-taurine site has no clear role in transport.

Discussion

In this paper we have shown that NAP-taurine and the stilbenedisulfonate BADS compete for equilibrium binding to the band 3 protein of red cell membranes. The NAP-taurine inhibition constant for competition with BADS is very similar to the noncompetitive inhibition constant for inhibition of anion exchange in red cells by NAP-taurine (Knauf et al., 1978a). Moreover, the stoichiometry of the stilbenedisulfonate-protected NAP-taurine binding site is approximately the same as the stilbenedisulfonate binding stoichiometry (ca. one site per band 3 monomer). We have also shown that covalently binding BIDS to one half of a band 3 dimer does not affect NAP-taurine binding to the adjacent subunit. Finally, we have shown that a second high-affinity NAP-taurine site exists on the cytoplasmic surface of band 3 and that binding to this site

[3] In a previous paper we showed that this assumption is at least partially valid by looking at interactions between two stilbenedisulfonates (Macara & Cantley, 1981).

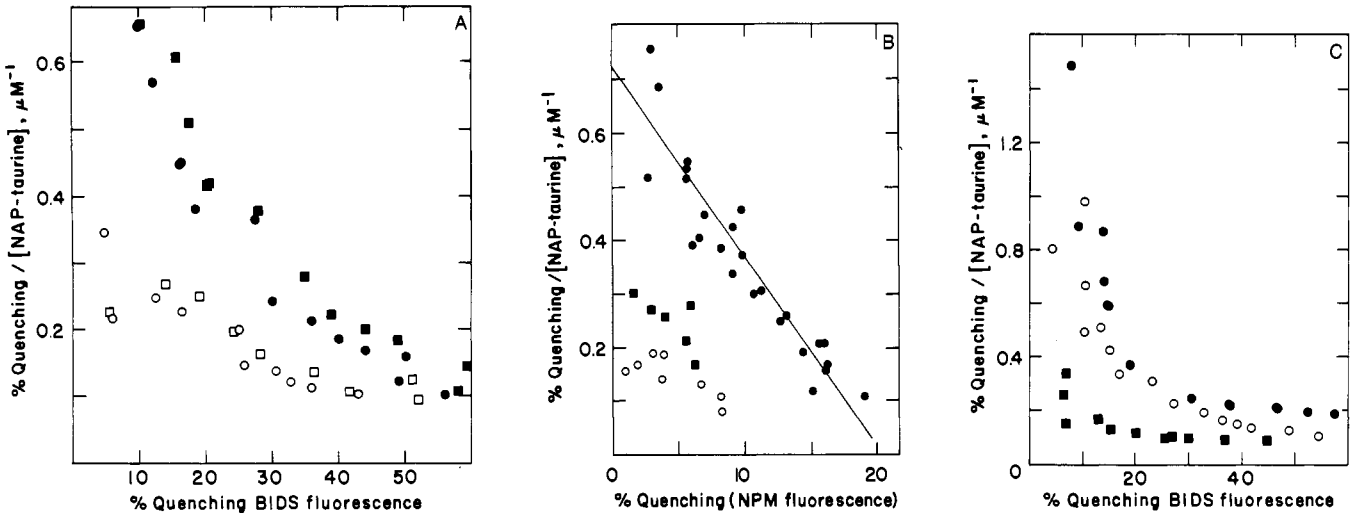

FIGURE 6: (A) Quenching of BIDS fluorescence in labeled IO (●, ■) and RO (○, □) vesicles by NAP-taurine. Vesicles were prepared by the method of Steck & Kant (1974). Purity (estimated as described under Experimental Procedures) of IO vesicles was 90–93%; purity of RO vesicles was 78–89%. Titrations were performed as described in Figure 2, except that 2.9 mM sodium citrate, pH 7.0, was used to prevent vesicles from shrinking, and the suspensions were maintained at 25 (■, □), or 5 °C (●, ○). Membrane protein concentration was 0.08 mg/mL. Data were not corrected for contamination by vesicles of incorrect orientation. Similar results were obtained for two separate preparations of vesicles. (B) Quenching of NPM-labeled membranes by NAP-taurine. NPM-labeled membranes were prepared as described under Experimental Procedures. About half of the NPM was bound to band 3, as judged by 343-nm absorbance scans of $NaDodSO_4$–polyacrylamide gels after electrophoresis (Rao et al., 1979). Titrations were performed as described in Figure 2 but with excitation at 345 nm and emission at 396 nm. Quenching by intact membranes (●) was compared with that after treatment with trypsin (100 μg/mL trypsin for 1 h at 0 °C). Both the trypsinized membranes (○) and supernatant containing the cleavage products (■) were titrated with NAP-taurine. The maximum possible separation of the NAP-taurine binding site and the nearest NPM chromophore can be calculated[4] from the data (●) to be 17 Å. The line was drawn through the data with assumption of a K_d for NAP-taurine binding of 20 μM. (C) Effect of trypsinization and of removal of peripheral proteins on quenching of BIDS fluorescence of labeled membranes by NAP-taurine. Membranes were untreated (●), stripped of peripheral proteins in 10 mM NaOH and 0.1 mM EDTA (○), or stripped and then treated with trypsin as described above (■). Titrations were as described in Figure 2.

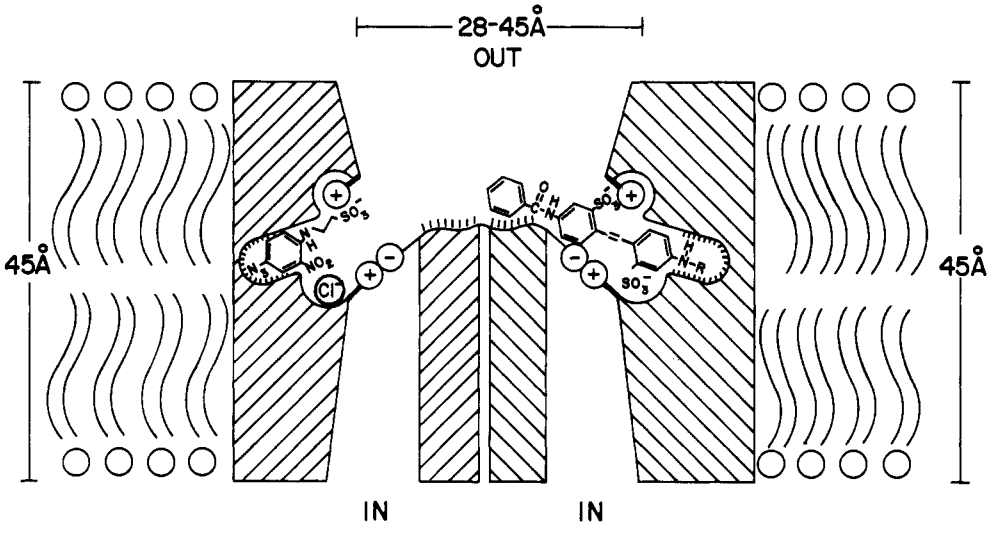

FIGURE 7: Proposed model of the band 3 dimer. The figure is drawn roughly to scale, the distance between external transport sites being taken from Macara & Cantley (1981). These sites are arranged such that two large stilbenedisulfonate molecules could interact when bound and, thus, exhibit negative cooperativity. Two anion sites are present on each subunit, access to each being controlled by a negatively charged gate. The BIDS molecule is shown blocking both sites. NAP-taurine is shown blocking only the outer site, so that the inner site remains accessible to transportable anions such as Cl^-. NAP-taurine and stilbenedisulfonates cannot bind simultaneously to the same subunit, and when on adjacent subunits they are too far apart to interact.

causes quenching of BIDS fluorescence, but has no detectable effect on anion exchange.

In a previous publication we investigated an apparent negative cooperativity for stilbenedisulfonate association with the band 3 dimer and determined that when one subunit was covalently reacted with BIDS, the other subunit bound a noncovalent stilbenedisulfonate with an affinity approximately 1 order of magnitude lower than when both subunits were unoccupied (Macara & Cantley, 1981). However, labeling of one subunit with BIDS did not appear to affect transport of sulfate by the other subunit. The distance between the two disulfonic stilbene binding sites on the dimer was found to be between 28 and 50 Å, and we suggested that the ends of the large disulfonic stilbenes may be close enough to interact on the dimer. We now show that the binding of NAP-taurine, as measured kinetically by inhibition of [^{32}P]phosphate flux, does not interact with stilbenedisulfonate binding on an adjacent subunit. This observation is consistent with the relatively small size of NAP-taurine compared to that of the stilbenedisulfonates.

Since NAP-taurine and BIDS can bind to adjacent subunits in the band 3 dimer, the distance between them should in

principle be obtained by fluorescence resonance energy transfer measurements. The overlap of the BIDS emission spectrum and the NAP-taurine absorption spectrum (Figure 1) is such that R_0 = 35.8 Å (assuming the orientation factor, K^2, = 4).[4] If the distance between the NAP-taurine site and the BIDS site on adjacent subunits is similar to the distance between two disulfonic stilbenes [28–50 Å; Macara & Cantley (1981)], then an efficiency of transfer between 15 and 81% is expected. Therefore, unless the donor and acceptor transition dipoles are in an extremely unfavorably orientation (i.e., $K^2 \sim 0$), energy transfer should be detectable. Although we did detect quenching of BIDS fluorescence by NAP-taurine, the site responsible for quenching did not appear to be the external modifier site. The site causing quenching was approachable from the cytoplasmic side of the membrane and was not affected by increasing the BIDS reaction stoichiometry. The quenching from this cytoplasmic site prevented an accurate determination of the distance between the modifier site and disulfonic stilbene site of adjacent subunits. In any event these sites are probably more than 25 Å apart or greater quenching would have occurred (ignoring the unlikely possibility that K^2 = 0).

The competition between NAP-taurine and stilbenedisulfonate binding to the external side of band 3 is of interest in view of the different types of inhibition of anion transport displayed by the two probes. The most likely explanation of the data is that the sites for the two probes overlap, and one possible arrangement is shown in Figure 7. Two positively charged sites are envisaged, both of which interact with a single stilbenedisulfonate molecule. NAP-taurine, on the other hand, interacts with only one site, when presented from the external side of the membrane. The structure of stilbenedisulfonates is such that the two sites can be only about 10 Å apart. The distance between sites on adjacent subunits is 28–50 Å (Macara & Cantley, 1981), and in Figure 7 they have been located such that stilbenesulfonates occupying both subunits would be in contact with one another, whereas NAP-taurine is too small to make contact. This arrangement of sites explains the kinetic data for inhibition of anion exchange by NAP-taurine and stilbenedisulfonates, the competition between binding of the two types of probe, and the negative cooperativity of stilbenedisulfonate binding to band 3. It also suggests a mechanism for anion exchange by band 3, in which the two positively charged sites function as transport sites, access to each being controlled by an anionic gate. Such a mechanism is illustrated in Figure 8. The movement of the gate is triggered by the binding of the anion which screens the adjacent positive charge and weakens the ionic bond which holds the gate in position. The gate then swings toward the other positive group, which closes access from the outside and allows access to the cytoplasmic side of the membrane. Transport in the opposite direction then occurs by a symmetrical reaction

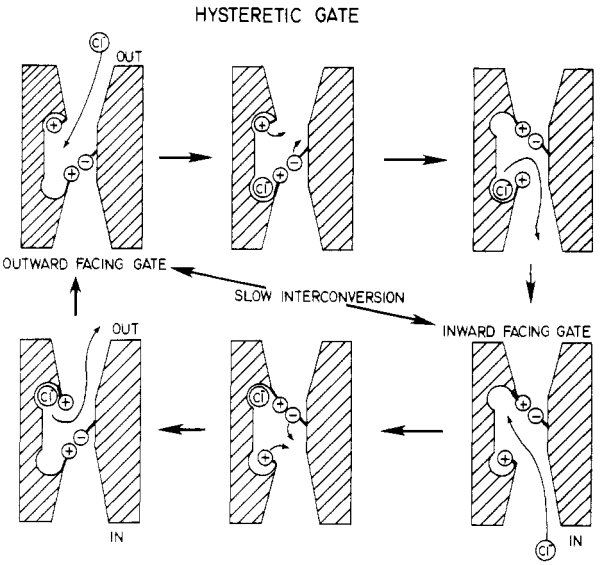

FIGURE 8: Proposed mechanism for anion exchange by band 3 based upon the transport site structure shown in Figure 7. Only a single subunit is shown. External anions can bind at the inner transport site, triggering the movement of the negatively charged gate away from the bound anion. This movement exposes the bound anion to the internal medium and allows access to the outer transport site. Substrate inhibition at high anion concentration is caused by the simultaneous binding of anions to both the inner and outer sites, as shown in Figure 7 for the binding of Cl^- and NAP-taurine. The symmetry of the sites in the diagram is not meant to imply any actual similarity either in the structures of the sites or in their accessibilities to anions.

process. The gate does not "swing" unless an anion binds to neutralize the ionic bond. Substrate inhibition occurs when anions bind to both positive charges simultaneously. NAP-taurine inhibits by preferentially binding to the positively charged group at the top of the diagram when the gate is in the outwardly facing conformation (see Figure 7). Thus, Cl^- can still bind to the transport site when added from the outside of the cell, but NAP-taurine prevents the conformational change necessary for transport. Interestingly, Knauf et al. (1980) have recently demonstrated that the inhibitory potency of external NAP-taurine is increased by imposing a chloride gradient across the red cell membrane that increases the proportion of outward-facing transport sites. This observation implies that the external NAP-taurine site is only available to outwardly facing sites, as predicted by the scheme in Figure 8. The model provides an explanation for the otherwise puzzling similarities in the topological characteristics of the "modifier" and "transport" sites on the one hand (Knauf et al., 1978b, 1980) and their differences in kinetic behavior on the other hand (Knauf et al., 1978a; Shami et al., 1978). Although it bears some similarities to two other models, recently described by Knauf et al. (1980) and by Passow et al. (1980), these both utilize a separate modifier site that has no role in transport, and they provide no a priori reason for the observed proximity between the two types of binding site.

[4] The distance between donor and acceptor at which quenching of donor fluorescence is exactly half is called the critical distance, R_0, and is described by the equation $R_0 = (9.79 \times 10^3)(JQn^{-4}K^2)$ Å. J is the overlap integral for the donor fluorescence and acceptor absorbance peaks, Q is the quantum yield of the donor in the absence of acceptor, n is the refractive index of the solvent, and K^2 is the dipole–dipole orientation factor. The donor–acceptor separation, R, is calculated by using the equation $R = R_0 (1/E - 1)^{1/6}$, where E is the efficiency of energy transfer to the acceptor, at saturating acceptor concentration. K^2 can vary between 0 and 4, and ignorance of its true value is the major cause of uncertainty in determining R_0 (Schiller, 1975). In the present study, K^2 is assumed to be 4. The calculated distances given in the test are therefore the maximum possible distances between donor and acceptor. The true distances may be considerably shorter. R_0 for BIDS/NAP-taurine = 35.8 Å; R_0 for NPM/NAP-taurine = 26.6 Å.

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