Biochemistry

 Volume 20, Number 18 September 1, 1981

Interactions between Transport Inhibitors at the Anion Binding Sites of the Band 3 Dimer†

Ian G. Macara* and Lewis C. Cantley

ABSTRACT: Evidence is presented that the binding of aromatic disulfonates to the external transport sites of the red cell anion-exchange protein (band 3) can exhibit negative cooperativity. Fluorescence resonance energy transfer has been used to compare the affinities of an aromatic disulfonate 4,4′-bis-(4-nitro-2,1,3-benzoxadiazolyl)dihydrostilbene-2,2′-disulfonate [H_2(NBD)$_2$DS] for "empty" band 3 dimers (in which neither external transport site is occupied) and for "half-filled" dimers (in which one site per dimer is occupied by a covalently attached fluorescent stilbenedisulfonate). H_2(NBD)$_2$DS apparently binds to the external anion transport site since it is a potent inhibitor of [^{35}S]sulfate influx into red cells (K_i = 20–50 nM), binds reversibly to approximately one site per band 3 monomer (1.6×10^6 sites/cell), and is displaced by covalent labeling with a disulfonic stilbene. The affinity of H_2(NBD)$_2$DS for membranes in which 80% of the transport sites are occupied by covalently attached 4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate (BIDS) was approximately 1 order of magnitude lower than that for unmodified membranes. However, when a similar proportion of the transport sites on red cells was blocked by reaction with BIDS, [^{35}S]sulfate was taken up with a lower V_{max} but with a K_m identical with that observed for unmodified cells, suggesting that no subunit interactions are necessary for transport. Therefore, in order to test whether the observed negative cooperativity of aromatic disulfonate binding could be ascribed simply to steric hindrance, the distance between transport sites was measured by fluorescence resonance energy transfer. H_2(NBD)$_2$DS and eosin maleimide were used as acceptors, with BIDS as donor. Transfer efficiencies were determined by donor fluorescence quenching, by acceptor fluorescence enhancement, and from donor lifetime changes. Uncertainties in the distance were estimated from measured depolarization factors. The donor–acceptor distance was found to be only 28–52 Å. Since the probes are large molecules, they could therefore be very close together, and the observed negative cooperativity might be explained by overlapping sites. The results suggest that the subunits of a band 3 dimer transport anions independently but that access to the transport sites may be provided by a cavity between the subunits.

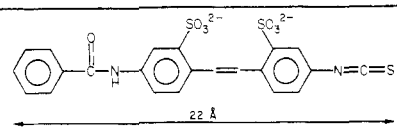
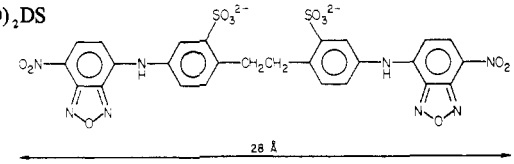
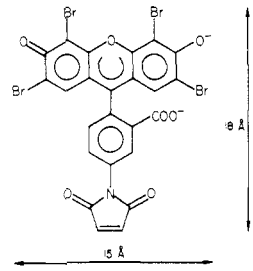
The passive exchange of anions across the plasma membrane of the erythrocyte is catalyzed by a 95 000-dalton integral membrane protein known as band 3 (Knauf, 1979; Cabantchik et al., 1978). The protein appears to exist in the membrane as a stable dimer (Clarke, 1975; Yu & Steck, 1975; Nigg & Cherry, 1979a). There is evidence, less conclusive, that other transport proteins are also oligomeric, in particular the Ca^{2+}-stimulated and (Na^+, K^+)-stimulated ATPases, the mitochondrial Fo proton channel, and bacteriorhodopsin (Hobbs & Albers, 1980). There are three (or more) possible reasons for this feature: (1) formation of the oligomer could drive the insertion of subunits into the membrane and stabilize the resulting membrane–protein complex; (2) the sites or channels responsible for transport could be formed from a hydrophilic interface between subunits; (3) the mechanism of transport might require allosteric interaction between subunits, as in the alternating-sites schemes proposed for the mitochondrial and chloroplast coupling factors (Hackney et al., 1979). A lack of information about the structure of transport systems has prevented a resolution of these alternatives.

Band 3 will accept a wide variety of anions as substrates for exchange, and exchange is potently inhibited from the external side of the membrane by aromatic disulfonates, especially the stilbenedisulfonates (Knauf, 1979). Although these compounds are not transported, they bind at the transport site of the protein, of which there appears to be one per subunit (Lepke et al., 1976; Ship et al., 1977; Jennings & Passow, 1979). A simple and widely accepted model for anion exchange by band 3 is that of a reciprocating carrier which can only change its orientation when loaded (Wieth, 1972; Gunn et al., 1973). In light of observations that equilibrium anion exchange at high concentrations exhibits self-inhibition, however, the model has been expanded to include a second, external "modifier" site (Dalmark, 1976; Gunn & Fröhlich, 1979). The inhibitor NAP-taurine [*N*-(4-azido-2-nitrophenyl)-2-aminoethanesulfonate][1] has been reported to be specific for this

† From the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138. *Received October 31, 1980.* This work was supported by Grant GM 26199 from the National Institutes of Health (L.C.C.). I.G.M. was supported by a grant from the Charles A. King Trust, Boston, MA.

0006-2960/81/0420-5095$01.25/0

Table I: Characteristics of Fluorescent Probes for the External Transport Site of Band 3

name	abbreviation	structure	$\lambda_{absorbance}$ (nm)	E_{max} (mM^{-1} cm^{-1})	$\lambda_{emission}$ (nm)
4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate	BIDS		335[a]	51[a]	435[b]
4,4′-bis(4-nitro-2,1,3-benzoxadiazolyl)dihydrostilbene-2,2′-disulfonate	$H_2(NBD)_2DS$		340 470 (490)[c]	33 (31)[c]	540
eosin 5-maleimide			522[d]	83[e]	550

[a] Values are for BIDS–lysine. [b] From corrected emission spectrum of BIDS-labeled ghosts. [c] Values are for $H_2(NBD)_2DS$ bound to ghosts from difference spectrum. [d] From eosine maleimide-labeled ghosts. [e] Taken from Cherry et al. (1976).

"modifier" site. However, it is evident that the substrate inhibition could alternatively be a consequence of negative cooperativity between band 3 subunits, and the effects of NAP-taurine could be explained similarly, without recourse to a second site. This explanation appears to have been avoided in part because the fractional inhibition of anion exchange is proportional to the fraction of band 3 molecules which have reacted with an isothiocyanostilbenedisulfonate, complete inhibition occurring when 1.2×10^6 molecules/cell have reacted. This number is believed to equal the number of band 3 polypeptides (Lepke et al., 1976; Ship et al., 1977). Recent studies using the fluorescent stilbenedisulfonate 4,4′-dibenzamidostilbene-2,2′-disulfonate (DBDS) indicate, however, the presence at physiological ionic strength of only one high affinity site per dimer (Dix et al., 1979). Certain other stilbenedisulfonates, such as 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS), appear to behave in a similar fashion, giving nonlinear Scatchard plots at high ionic strength (I. G. Macara and L. C. Cantley, unpublished observations). However, no direct evidence for interaction between subunits has so far been presented. We now provide evidence for such interaction by using the technique of fluorescence resonance energy transfer to compare the affinities of anionic probes of the external transport sites and to measure the distance between these sites. The results, taken together with previous distance measurements made in this laboratory (Rao et al., 1979), suggest that independent anion transport sites on a band 3 dimer are accessed from a common cavity between the subunits and that observed interactions between large aromatic disulfonates are a consequence of steric hindrance.

Experimental Procedures

Materials. Eosin 5-maleimide was purchased from Molecular Probes, Inc., and eosin was from Sigma. NBD-chloride and DIDS were from Pierce Chemical Co., and DADS was from Eastman. BIDS was prepared from DADS as described by Rao et al. (1979). $H_2(NBD)_2DS$ was prepared from H_2DADS by reaction with a 2-fold molar excess of NBD-chloride in aqueous solution, made alkaline (about pH 10) with sodium hydroxide, at room temperature for 24 h. The H_2DADS had been produced by reduction of DADS with hydrogen gas over a palladium on charcoal catalyst (Lepke et al., 1976). Reduction was continued to completion as judged by the disappearance of the absorbance maximum at 340 nm, which is characteristic of stilbenedisulfonates. The red-colored NBD adduct was purified by preparative thin-layer chromatography on silica plates (EM laboratories, Inc.) using methanol/chloroform (2:1 v/v) as the solvent. Unreacted NBD-chloride runs ahead of the adduct and unreacted H_2DADS behind the adduct in this system. The band containing $H_2(NBD)_2DS$ was scraped from the plate, dissolved in methanol, and evaporated to dryness. All reactants and products were protected from light and stored over desiccant. However, the only reagents found to be photolabile were the amines DADS and H_2DADS. The purity of the $H_2(NBD)_2DS$ was checked by thin-layer chromatography using the same system as for purification or pyridine/acetic acid/water (4:1:20 v/v/v). The product was also characterized by fluorescence and absorption spectroscopy. Absorption maxima were a 340 and 470 nm, emission was maximal at 540 nm, as expected for an NBD-amine adduct (Ghosh & Whitehouse, 1969), and the excitation and absorption spectra were similar. The extinction coefficient at 470 nm (33 mM^{-1} cm^{-1}) was approximately twice that of NBD-Cl reacted with a monoamine. The structure and spectral characteristics of the compound are given in Table I. Eosin was purified by precipitation from ice-cold acid solution, and its purity was checked by thin-layer

[1] Abbreviations used: DADS, 4,4′-diaminostilbene-2,2′-disulfonate; H_2DADS, 4,4′-diaminodihydrostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; BIDS, 4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; $H_2(NBD)_2DS$, 4,4′-bis-(4-nitro-2,1,3-benzoxadiazolyl)dihydrostilbene-2,2′-disulfonate; NAP-taurine, *N*-(4-azido-2-nitrophenyl)-2-aminoethanesulfonate; $NaDodSO_4$, sodium dodecyl sulfate; 5P8, 5 mM sodium phosphate, pH 8.0; SSH, 100 mM sodium sulfate–20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.4; CS7.4, 28.5 mM sodium citrate–205.3 mM sucrose, pH 7.4.

chromatography using the same systems as for $H_2(NBD)_2DS$.

Preparation of Labeled Ghosts. Ghosts were prepared from recently outdated blood by lysis at 4 °C in 5 mM sodium phosphate, pH 8.0 (5P8), after washing in phosphate-buffered saline and labeling with fluorescent probes as required. Ghosts were washed in 5P8 until white and free of hemoglobin, as judged by the absorbance at 403 nm after solubilization in $NaDodSO_4$. Intact cells were labeled with BIDS [method of Rao et al. (1979)] or eosin maleimide [method of Nigg & Cherry (1979b)] to various stoichiometries. Portions of the BIDS-labeled cells were then reacted with saturating concentrations of the eosin maleimide and vice versa. Stoichiometries were determined as described in Rao et al. (1979) from the absorbances of detergent-solubilized ghosts at 280 [for protein, assuming an absorptivity of 1.09 mL/(mg·cm)], 335 (for BIDS, assuming an absorptivity of 51 mM^{-1} cm^{-1}), and 528 nm (for eosin, assuming an absorptivity of 86 mM^{-1} cm^{-1}; Nigg & Cherry, 1979b).

Equilibrium Binding. Binding of $H_2(NBD)_2DS$ was measured by using both intact cells and unsealed ghosts, by mixing with known concentrations of the probe, and by measuring the concentration remaining in solution after centrifugation (Dix et al., 1979). Intact cells were at 30% hematocrit in isotonic, isoosmotic citrate/sucrose (CS7.4, 28.5 mM sodium citrate–205.3 mM sucrose, pH 7.4). Aliquots of the suspension were immediately centrifuged after addition of $H_2(NBD)_2DS$ (15 s in an Eppendorf microfuge), and the supernatants were treated with 50 μL of 20% $NaDodSO_4$ to denature any hemoglobin present. $H_2(NBD)_2DS$ concentrations in the supernatants were determined from the absorbance at 470 nm, after correction for the small absorbance due to the denatured hemoglobin. The total concentration of $H_2(NBD)_2DS$ was corrected for the volume occupied by the cells, as estimated from the fractional volume available to [^{14}C]sucrose. Equilibrium binding to control and DIDS-treated ghosts was measured as described by Dix et al. (1979). Supernatants were treated with 7% Cl_3CCOOH and centrifuged (5000*g* for 5 min) before measuring the absorbance at 470 nm.

Transport and Inhibition Measurements. The permeability of intact red cells to eosin and $H_2(NBD)_2DS$ was determined by incubating cells at 50% hematocrit in CS7.4 (at 25 or 4 °C) and 170 μM eosin or $H_2(NBD)_2DS$. Aliquots were removed at intervals and treated as described above to measure the free concentrations of the colored anions. Inhibition of anion exchange by $H_2(NBD)_2DS$ was determined from measurement of the initial rate of [^{35}S]sulfate efflux from intact cells [method of Barzilay & Cabantchik (1979)]. Cells were first loaded to equilibrium with either 35 or 100 mM [^{35}S]sulfate and washed in cold sulfate of the same concentration. Efflux was then initiated by adding 0.5 mL of cells at 50% hematocrit to 5.0 mL of 100 mM sodium sulfate–20 mM Hepes, pH 7.4 (SSH), or to SSH diluted with CS7.4 to give 35 mM sulfate containing various concentrations of inhibitor, and the mixture was incubated at 25 °C. Samples were removed at intervals and centrifuged for 15 s in an Eppendorf microfuge, and 0.2 mL of supernatant was counted in Aquasol.

The effect of pretreatment of cells with BIDS on the K_m for sulfate transport was determined from measurement of the initial rates of [^{35}S]sulfate influx, using mixtures of SSH and CS7.4 to maintain isotonicity and osmolarity while varying the sulfate concentration. Cells were pretreated with BIDS as described above to give about 60% inhibition of influx. Influx was initiated by addition of 2.5 mL of SSH/CS7.4 containing [^{35}S]sulfate to 0.5 mL of packed cells prewashed in buffer of the correct sulfate concentration, and the mixture was incubated at 25 °C. Samples (0.5 mL) were removed at intervals, and influx was stopped by addition of 1.0 mL of ice-cold SSH containing 2 μM of the stilbenedisulfonate DBDS followed by centrifugation for 15 s in a microfuge. The pellet was washed in 1 mL of the same solution and then counted for [^{35}S]sulfate after lysis in 10% Cl_3CCOOH. The amount of BIDS reacted with the cells was determined as described by Rao et al. (1979).

Absorption Difference Spectroscopy. A red shift of 20 nm in the visible absorption maximum at 470 nm of $H_2(NBD)_2DS$ was observed upon its binding to red cell ghosts. This shift was utilized to measure the affinity and stoichiometry of $H_2(NBD)_2DS$ binding to the ghosts by a difference spectroscopy titration. Ghosts at a concentration of 1.2 mg/mL in 28.5 mM sodium citrate, pH 7.4, were titrated with $H_2(NBD)_2DS$ by using divided cuvettes. Equal amounts of the probe were added to the compartment containing ghosts in the sample cuvette and to that containing only buffer in the reference cuvette. After each addition, the difference spectrum was recorded on a Cary 219 at 23 °C from 390 to 650 nm. Overall dilution was 2% and was neglected in calculating changes in absorbance.

Resonance Energy Transfer Measurements. Steady-state and lifetime fluorescence measurements were performed largely as described by Rao et al. (1979) on an SLM 4800 polarization spectrofluorometer interfaced to a PET microcomputer which was provided with a data-averaging program. Titrations were performed in 28.5 mM sodium citrate, pH 7.4, by using 0.3 × 0.3 cm microcuvettes thermostated at 25 °C. BIDS-labeled ghosts were usually excited at 360 nm, and the emission was monitored at 427 nm. The efficiencies of energy transfer from the donor to acceptor molecules were determined from donor fluorescence quenching, sensitized emission fluorescence, and donor lifetimes by using the methods and correction factors described in Rao et al. (1979). Efficiency of transfer, E, is related to donor quenching and to donor lifetimes by

$$E = 1 - (X_{DA}/X_D) \quad (1)$$

where X_{DA} and X_D are the fluorescence intensities, or fluorescence lifetimes, of the donor in the presence and absence of acceptor, respectively. For calculation of E from the enhanced emission of the acceptor, eq 4 and 5 of Rao et al. (1979) were used.

The distance at which the efficiency of energy transfer, E, is exactly half is called the critical distance, R_0, and is calculated as described in eq 2:

$$R_0 = (9.79 \times 10^6)(JQn^{-4}K^2)^{1/6}\ \text{Å} \quad (2)$$

J is the overlap integral for donor fluorescence and acceptor absorbance peaks. Q is the quantum yield of the donor, n is the refractive index of the medium (assumed to be 1.4 for water), and K^2 is the dipole–dipole orientation factor. K^2 can vary between 0 and 4. Ignorance of its true value is the major cause of uncertainty in determining R_0 and hence of the true separation of donor and acceptor (Schiller, 1975). The true distance, R, can then be related to E by

$$R = R_0[(1/E) - 1]^{1/6}\ \text{Å} \quad (3)$$

The range of possible values for R_0 can be estimated from depolarization factors calculated as described below.

Fluorescence Anisotropy. The limiting fluorescence anisotropy, r_{om}, of eosin ghosts was determined from a linear extrapolation of the low viscosity portion of a Perrin plot ($1/r$ vs. T/η) as described by Dale & Eisinger (1975), using sucrose

to change the viscosity of the suspension. The r_{om} for BIDS was taken from Rao et al. (1979) to be 0.345. Axial donor or acceptor depolarization factors were calculated as described by Dale et al. (1979):

$$\langle d_{D,A}{}^{x} \rangle = (r_{om}/r_f) \quad (4)$$

where $\langle d_{D,A}{}^{x} \rangle$ is the donor or acceptor axial depolarization factor and r_f is the fundamental anisotropy in the absence of all rotation (i.e., infinite viscosity).

The axial transfer depolarization factor is given by

$$\langle d_T{}^{x} \rangle = \langle d_T \rangle / (\langle d_D{}^{x} \rangle \cdot \langle d_A{}^{x} \rangle) \quad (5)$$

where $\langle d_T \rangle$ is the ratio r_{om}/r_f for acceptor-sensitized emission when exciting the donor with polarized light.

These factors were used to determine the limits of the orientation factor, K^2, and hence the uncertainty in the distance between the donor and acceptors, from the plot given in Figure 5 of Dale et al. (1979).

Data Fitting. The affinity of $H_2(NBD)_2DS$ for band 3 is so high that it is not possible to assume in titrating red cell ghosts with the probe that the total concentration of $H_2(NBD)_2DS$ is equal to the free concentration. Titration data were therefore routinely fitted by a nonlinear least-squares procedure to the following equations, which assume the existence of a single class of independent sites. (Although we show later that the sites are not independent but can exhibit negative cooperativity, the assumption is valid to a first approximation at low $H_2(NBD)_2DS$ concentrations.)

The concentration of bound ligand was determined from

$$L_B = 0.5(B_0 + L_0 + K - \sqrt{(B_0 + L_0 + K)^2 - 4B_0L_0}) \quad (6)$$

where B_0 and L_0 are the total concentrations of band 3 and $H_2(NBD)_2DS$, respectively, and K is the dissociation constant. The free ligand concentration, L, and fractional occupation of sites, P, are given in eq 7 and 8:

$$L = L_0 - L_B \quad (7)$$

$$P = L/(L + K) \quad (8)$$

Results

$H_2(NBD)_2DS$ Binding to Band 3. The direct demonstration of interaction between transport sites of the band 3 dimer requires the determination of the affinity of a probe to dimers in which both sites are unoccupied and those in which only one site is unoccupied. This has been accomplished by measuring the affinity of a noncovalent inhibitor of anion exchange to band 3 before and after labeling half of the band 3 dimer with an irreversible inhibitor. A new probe, $H_2(NBD)_2DS$ (see Table I), has been prepared and used as a reversibly binding spectral probe of the anion transport site. The probe was expected to bind exclusively to the external transport site of band 3 since it resembles H_2DIDS, a potent competitive inhibitor of anion exchange which acts as an affinity label for the external transport site (Lepke et al., 1976, Shami et al., 1978). That it does so was confirmed as follows: (a) the $H_2(NBD)_2DS$ was found to be impermeant to intact red cells and to bind to approximately one site per band 3 monomer (1.6×16^6/cell) (Figure 1A); (b) it inhibited [^{35}S]sulfate influx with a K_i of 20–50 nM (Figure 1B); (c) $H_2(NBD)_2DS$ binding was prevented by prior reaction with DIDS (Figure 2A).

The affinity of $H_2(NBD)_2DS$ for band 3 was determined by equilibrium binding to red cell ghosts (Figure 2A), donor quenching of intrinsic tryptophan fluorescence (Figure 2B), and absorption difference spectroscopy (Figure 2C). Good agreement was achieved for the dissociation constants (50–80 nM) obtained by the three methods (Table II). The results were also in close agreement with the value of K_i for inhibition by the probe of [^{35}S]sulfate influx into intact cells (Figure 1B) and with the stoichiometry of covalent labeling of band 3 with

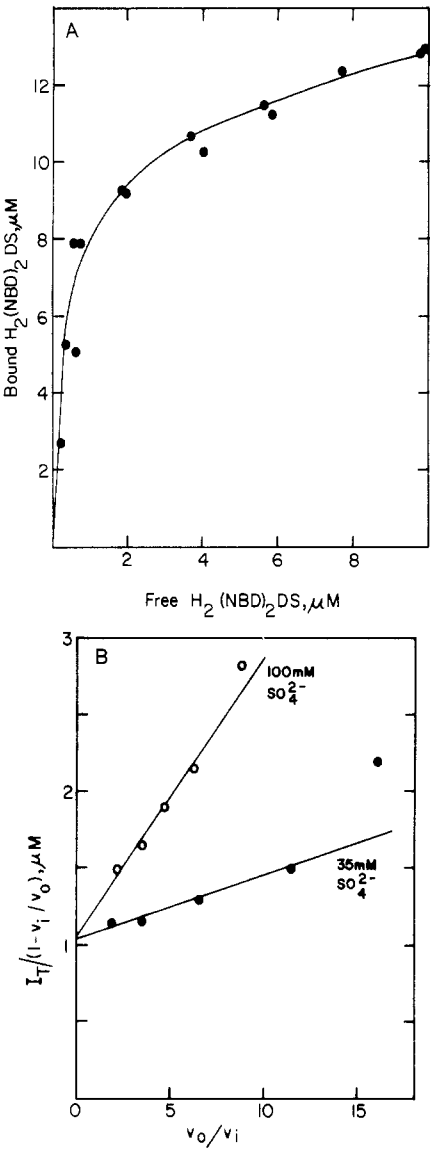


FIGURE 1: (A) Equilibrium binding of $H_2(NBD)_2DS$ to intact red cells. $H_2(NBD)_2DS$ was added to cells at 30% hematocrit in CS7.4, and the free $H_2(NBD)_2DS$ concentration in the supernatant was determined after centrigugation (5000g for 5 min) from its absorbance at 470 nm. The solid line was drawn by assuming a single class of sites with stoichiometry n and dissociation constant K plus nonsaturable binding with partition coefficient c: $v = nL/(K + L) + cL$, where $v = \mu M$ bound $H_2(NBD)_2DS$, and L is the free ligand concentration. The constants assumed were $n = 10.7\ \mu M$ (equivalent to 1.6×10^6 sites/cell), $K = 370$ nM, and $c = 0.27$. Data points are the means of two separate experiments. (B) Inhibition of equilibrium [^{35}S]sulfate exchange out of red cells by $H_2(NBD)_2DS$. Cells were at 10% hematocrit in isotonic, isoosmotic CS7.4/SSH containing either 35 (●) or 100 mM (○) sulfate at 25 °C. Rates of efflux were measured as described under Experimental Procedures. Data were plotted according to the method of Henderson (1972) for tightly binding inhibitors. For noncompetitive inhibition, the slopes should be independent of substrate concentration. For competitive inhibition, $I_T/(1 - v_i/v_0) = K_i(1 + S/K_m)(v_0/v_i) + E_T$ where I_T and E_T = total inhibitor and transporter concentrations, respectively, S is the initial substrate concentration, K_m is the Michaelis constant for the substrate in the absence of inhibitor, v_0 is the initial rate in the absence of inhibitor, and v_i is the rate in the presence of inhibitor. K_m was determined from a similar experiment in the absence of inhibitor to be 40 mM. when $S = 35$ mM, $K_i = 20$ nM, while at $S = 100$ mM, $K_i = 47$ nM. the band 3 concentration, E_T, was determined from the intercept to be 1.1 μM (equivalent to 1.2×10^6 sites/cell).

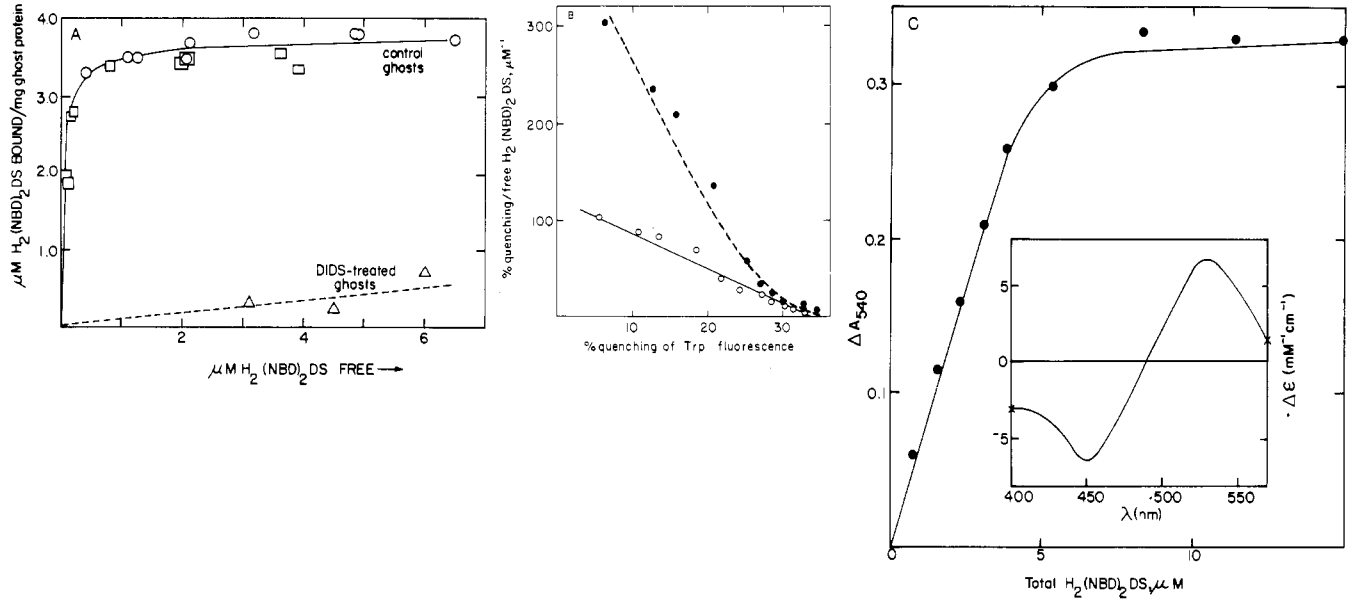


FIGURE 2: (A) Equilibrium binding of $H_2(NBD)_2DS$ to erythrocyte membranes. Control (○, □) or DIDS-treated (△) membranes were suspended to 3 mg of protein/mL in 28.5 mM sodium citrate, pH 7.4. $H_2(NBD)_2DS$ was added and incubated at 25 ° for 30 min, and then the membranes were centrifuged at 10000*g* for 15 min. Supernatants were treated as described in Figure 1A. Points are means of duplicate measurements. Data from two separate control experiments (○, □) are shown. The solid line through the data was drawn by assuming a single class of sites with K_d = 78 nM and stoichiometry = 3.7 nmol/mg of membrane protein. (B) Quenching of membrane tryptophan fluorescence by $H_2(NBD)_2DS$. Membranes were suspended to 0.2 mg/mL in 28.5 mM (●) or 2.9 mM (○) sodium citrate, pH 7.4, and excited at 294 nm. Emission was monitored at 334 nm as a function of $H_2(NBD)_2DS$ concentration. Data were corrected for inner-filter quenching as described in Rao et al. 1979. The solid line through the data was drawn by assuming a single class of noninteracting sites with K_d = 270 nM and maximum quenching of 33% (low citrate). The free $H_2(NBD)_2DS$ was calculated from total $H_2(NBD_2)DS$ and total band 3 monomer concentration as described undr Experimental procedures. At high citrate concentration, the data did not fit well to a single class of noninteracting sites. The dashed line was drawn by assuming sequential, negative cooperative binding, according to the equation $P = 2L(Q_1K_2 + Q_2L)/(K_1K_2 + 2K_2L + L^2)$ where P = fractional occupation of sites, L = free $H_2(NBD)_2DS$ concentration, K_1 and K_2 = intrinsic dissociation constants, and Q_1 and Q_2 = percent quenching at saturation of first and second sites. K_1 = 50 nM, K_2 = 270 nM, Q_1 = 21.5%, and Q_2 = 17%. (C) Absorption difference titration of $H_2(NBD)_2DS$ binding to erythrocyte membranes. Membranes were suspended to 1.2 mg of protein/mL in 28.5 mM sodium citrate, pH 7.4, at 23 °C. They were titrated with $H_2(NBD)_2DS$ as described under Experimental Procedures. Inset shows difference spectrum at a total $H_2(NBD)_2DS$ concentration of 2.7 μM. The solid line through the data was drawn by assuming a single class of sites with K_d = 80 nM and maximum absorbance difference of 0.31. The free $H_2(NBD)_2DS$ was calculated from the total $H_2(NBD)_2DS$ and total concentration of band 3 monomers as described under Experimental Procedures.

Table II: Binding of $H_2(NBD)_2DS$ to Erythrocyte Membranes

method	% occupancy of band 3 by BIDS[a]	dissociation constant, K_D[b] (nm)	stoichiometry[b] (nmol bound/mg of membrane protein)
inhibition of [^{35}S]-sulfate efflux (intact cells)[c]		20–50[e]	
equilibrium binding[d]		78	3.7
donor quenching[d] (tryptophan fluorescence)		(76), 50[g]	3.5[f]
absorption difference titration[d]		80	3.6[f]
donor quenching[d] (BIDS fluorescence)	8, 13, 84	270, 290, 720	3.4[f]
donor quenching[d] (tryptophan fluorescence)	80	860	

[a] 100% occupancy assumed equal to 2.5 nmol of BIDS/mg of ghost protein (see Figure 6). [b] K_D and stoichiometries of $H_2(NBD)_2DS$ binding were determined by a nonlinear least-squares procedure (see Experimental Procedures). [c] Isotonic, isoosmotic citrate–sucrose (CS7.4/SSH), pH 7.4, 25 °C. [d] 28.5 mM sodium citrate, pH 7.4, 25 °C. [e] Inhibition constant; competitive inhibition (see Figure 1B). [f] These stoichiometries were obtained from a nonlinear least-squares fit to eq 8–10 (Experimental Procedures), allowing both the K_D and the stoichiometry (B_0 in eq 8) to vary. [g] K_D determined by a nonlinear least-squares procedure using a sequential interacting sites model, the equation for which is given in Figure 2B. The value in parentheses was determined by assuming a single site and using only the data obtained at low $H_2(NBD)_2DS$ concentrations.

eosin maleimide (3.4 nmol/mg of protein; Figure 6A). Eosin maleimide has been shown previously to react almost exclusively with band 3 in intact red cells (Nigg & Cherry, 1979b).

Although the constants in Table II were determined by assuming a single class of noninteracting sites, it is apparent that the tryptophan quenching data at high ionic strength (Figure 2B) could be better fit by a two-site model or a negative cooperativity model. In addition, the fact that the two sets of transport inhibition data, for 35 and 100 mM substrate concentration, could not be fit by assuming a single competitive inhibition constant also suggests a possible negative cooperativity in $H_2(NBD)_2DS$ binding. Such apparent negative cooperativity has previously been observed for the binding of the stilbenedisulfonate DBDS to red cell ghosts at high ionic strength (Dix et al., 1979). The apparent cooperativity disappears at low ionic strength for both DBDS (Rao et al., 1979) and $H_2(NBD)_2DS$ (Figure 2B). It was not possible to obtain sufficiently accurate data at low $H_2(NBD)_2DS$ concentrations in the equilibrium binding experiment of Figure 2A to resolve two binding affinities.

$H_2(NBD)_2DS$ Binding to Band 3 Dimer after Covalent BIDS Attachment to One Subunit. The stilbenedisulfonate BIDS (see Table I) binds covalently to approximately one site per band 3 monomer and irreversibly inhibits anion transport (Rao et al., 1979). The fluorescence of this probe overlaps the absorbance of $H_2(NBD)_2DS$ (see Table I) such that at a favorable distance (<70 Å) and favorable transition dipole orientation, significant resonance energy transfer can occur between these probes. BIDS was reacted with intact red cells

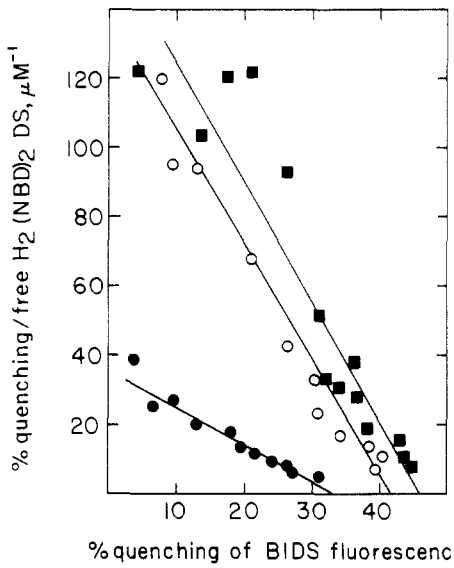


FIGURE 3: Quenching of BIDS–ghost fluorescence by $H_2(NBD)_2DS$. Ghosts were covalently labeled to 0.2 (■), 0.3 (○), or 2.1 (●) nmol of BIDS/mg of ghost protein. These stoichiometries correspond to 8%, 13%, and 84% saturation of the BIDS reactive site. Titration was as described in Figure 2B (in 28.5 mM citrate) but with excitation of the BIDS moiety at 364 nm and emission monitored at 427 nm. The solid lines were drawn assuming a single class of sites with K_d = 270 nM and maximum quenching, E_{max} = 46% (8% labeled); K_d = 290 nM and E_{max} = 41% (13% labeled); K_d = 720 nM and E_{max} = 32% (84% labeled). The free $H_2(NBD)_2DS$ concentration was calculated from the total $H_2(NBD)_2DS$ and total concentration of unlabeled band 3 monomers as described under Experimental Procedures.

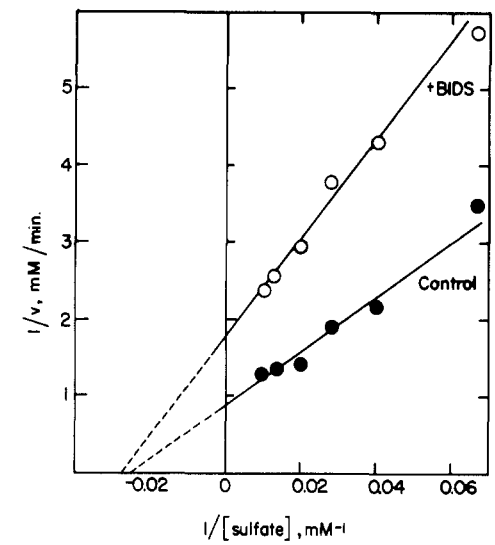


FIGURE 4: Inhibition of [^{35}S]sulfate influx into red cells by BIDS. Cells were labeled with BIDS to 47% saturation (1.2 nmol/mg of protein). [^{35}S]Sulfate (15–100 mM) in isotonic, isoosmotic CS7.4/SSH was added to packed cells to give a hematocrit of 17%. The amount of [^{35}S]sulfate accumulated by the cells was measured at intervals as described under Experimental Procedures, and the initial rates were estimated by drawing tangents to time courses for influx. Saturation curves were fit to the original data by a nonlinear least-squares procedure. K_m for control cells (●) = 40 mM; K_m for BIDS-labeled cells (○) = 37 mM. Percent inhibition of influx by BIDS = 60%.

at subsaturating concentrations, and membranes were prepared after thoroughly washing away noncovalently bound BIDS (Experimental Procedures). $H_2(NBD)_2DS$ binding to these ghosts was then determined by monitoring quenching of BIDS fluorescence (Figure 3). Since $H_2(NBD)_2DS$ can only quench BIDS fluorescence by binding close by, this experiment selects for $H_2(NBD)_2DS$ binding to subunits which are associated with a BIDS-labeled subunit (inner filter absorbance was small and corrected for). The affinity of $H_2(NBD)_2DS$ for BIDS-labeled membranes was found to be approximately 1 order of magnitude weaker than that for unlabeled membranes (see Table II). Under the assumption that no association of dimers occurs in the membrane, the apparent affinity will be independent of the BIDS stoichiometry since the mean separation between dimers will be too great to allow significant energy transfer between donors and acceptors on different dimers (mean separation is 150 Å assuming 6×10^5 dimers per cell). However, if a significant fraction of band 3 molecules exists as tetramers or higher oligomers, then transfer between dimers becomes significant, and the affinity will appear to decrease with increasing BIDS stoichiometry (see Appendix). Such a decrease in affinity was, in fact, observed (Figure 3 and Table II). As expected, when the BIDS stoichiometry approached saturation, less transfer to $H_2(NBD)_2DS$ was observed since most band 3 dimers have both sites occupied by BIDS and no sites available for $H_2(NBD)_2DS$.

For confirmation that BIDS does indeed decrease the affinity for $H_2(NBD)_2DS$ of band 3, the affinity was also measured by tryptophan fluorescence quenching, as described in Figure 2B, but using ghosts labeled to 2 nmol/mg of protein with BIDS. The apparent K_d was found to be 860 nM (Table II), which is 1 order of magnitude lower than that measured by the same technique in the absence of BIDS (about 50 nM).

These results demonstrate that binding a stilbenedisulfonate to one-half of a dimer significantly lowers the affinity of the remaining site. They also suggest that the nonlinearity of Scatchard plots for the binding of DBDS at high ionic strength (Dix et al., 1979) is the result of homotropic interactions.

Effect of BIDS on K_m of [^{35}S]Sulfate Influx. Since occupation of one subunit of the band 3 dimer by BIDS decreases the affinity of the other for $H_2(NBD)_2DS$, it was of interest to determine if BIDS also decreases the affinity of an adjacent subunit for a small transportable anion such as sulfate. Intact cells were therefore labeled covalently with subsaturating concentrations of BIDS, unreacted BIDS was removed, and the K_m for [^{35}S]sulfate influx was compared with that using untreated cells. The results are shown in Figure 4. The BIDS inhibited influx by 62%. The K_m for influx into untreated cells was found to be 41 mM. This is in very good agreement with the values determined in previous studies using different techniques (Schnell et al., 1977; Barzilay & Cabantchik, 1979). The K_m for influx into BIDS-labeled cells was found to be 37 mM. No significant change in K_m was therefore produced by reaction with BIDS. This result could be either because binding BIDS to one-half of the dimer has no effect on binding of small anions to the other transport site or because the occupation of one subunit by BIDS completely blocks transport by both subunits in the band 3 dimer. The stoichiometry of labeling was therefore estimated from the absorbance at 335 nm of detergent-solubilized ghosts prepared from BIDS-treated cells (Rao et al., 1979) and compared with the percent inhibition of [^{35}S]sulfate influx. The BIDS reaction with intact red cells saturates at 2.5 nmol/mg of membrane protein (Figure 5). The results were as follows: at a stoichiometry of 1.2 nmol/mg of protein (47% saturation), inhibition was 62%; at 2.2 nmol/mg of protein (90% saturation), inhibition was 85%. Although the error in estimating the BIDS stoichiometry from the absorbance at 335 nm is large, it appears that inhibition is proportional to the fraction of band 3 subunits occupied by BIDS rather than that of band 3 dimers. It is also clear that BIDS is not preferentially reacting with two subunits of the same dimer since $H_2(NB-$

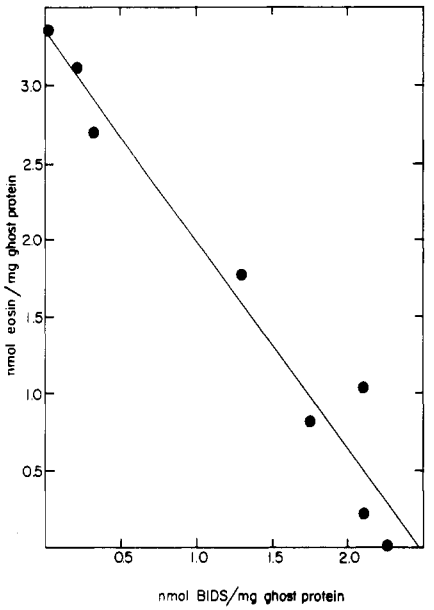


FIGURE 5: Relationship between reaction of eosin maleimide and of BIDS with red cells. Cells were labeled with BIDS to several different extents and were then labeled to saturation with eosin maleimide or vice versa. Stoichiometries were determined from the absorbances of the labels on detergent-solubilized, hemoglobin-free ghosts, as described under Experimental Procedures.

D)$_2$DS can quench BIDS fluorescence by binding at the other subunit when BIDS is present at subsaturating concentrations (Figure 3). These results are in agreement with those of Ship et al. (1977) for the inhibition of [^{35}S]sulfate efflux by DIDS. Apparently, labeling one subunit with BIDS does not affect the K_m for sulfate transport by the other subunit. The observed interaction between BIDS and H_2(NBD)$_2$DS might therefore be a consequence of the large size of the probes and their close proximity to each other rather than being the result of an induced conformational change in the protein. It is therefore imperative to know the distance between the external transport sites of the band 3 dimer.

Distance between Transport Sites. We have determined the distance between the external transport sites by fluorescence resonance energy transfer, using both H_2(NBD)$_2$DS and eosin 5-maleimide as acceptors and BIDS as fluorescent donor. H_2(NBD)$_2$DS has been shown above to bind to the transport site of band 3. The reaction of eosin maleimide with erythrocytes has been characterized by Nigg & Cherry (1979b). The stoichiometry of reaction was 1.3×10^6 sites/cell, and inhibition of [^{35}S]sulfate efflux was shown to be proportional to the fractional occupation of these sites by the label. At least 80% of the label was bound to band 3 on electrophoresis. We now demonstrate that BIDS labeling and eosin maleimide labeling are mutually exclusive (Figure 5), confirming that eosin maleimide reacts at the external transport site of band 3. The maximal stoichiometry of labeling by eosin maleimide (3.3 nmol/mg) is rather higher than that for BIDS (2.5 nmol/mg) but agrees well with that for the new probe, H_2-(NBD)$_2$DS (Table II). This difference may be the result of an underestimate of the molar absorptivities for eosin maleimide and H_2(NBD)$_2$DS. Alternatively, the maximum observed BIDS stoichiometry may be lower than the true band 3 stoichiometry. This situation could arise either through extreme negative cooperativity of BIDS binding to band 3 (as is observed for binding of the similar probe, DBDS; Dix et al., 1979) or through self-inhibition of the reaction of BIDS with band 3. The latter possibility arises because although the symmetry of the two sulfonate groups on BIDS allows it to

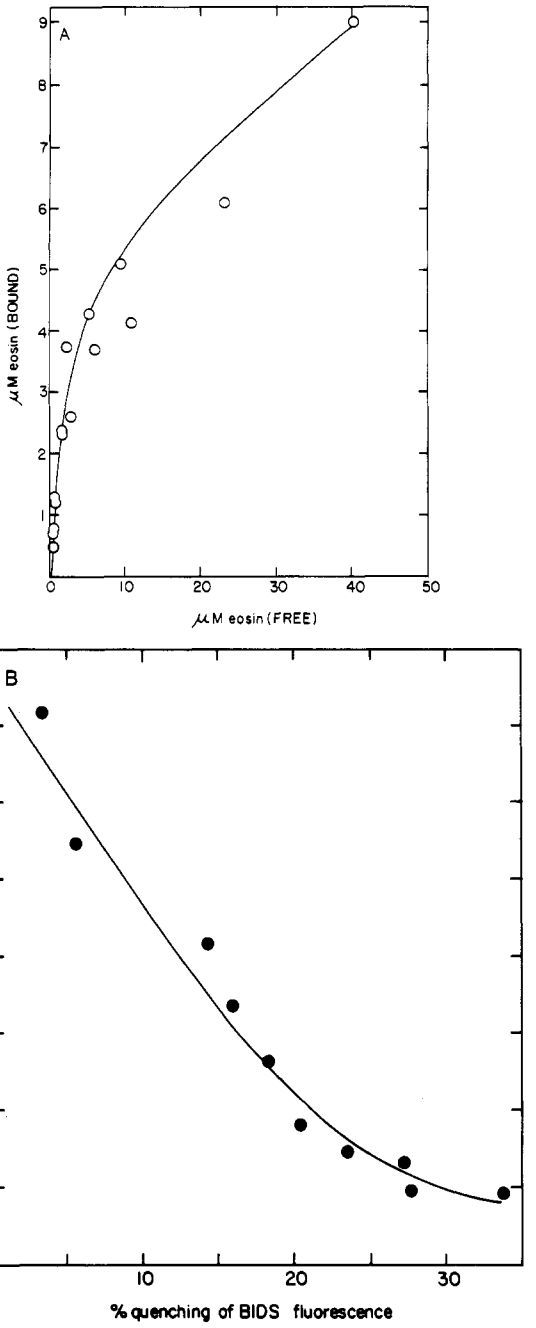


FIGURE 6: (A) Equilibrium binding of eosin to red cells. Binding was measured as for H_2(NBD)$_2$DS binding described in Figure 1A but at 4 °C. The solid line was drawn through the data by assuming a single class of noninteracting sites with a K_d of 3.3 μM and maximum stoichiometry of 1.2×10^6 sites/cell plus nonsaturable partition coefficient of 0.086. (B) Donor quenching of BIDS-labeled ghosts by eosin. Quenching of BIDS-labeled ghosts (0.3 nmol of BIDS/mg of protein) was measured as described in Figure 3. The solid line was drawn as in (A), assuming K_d = 3 μM and the maximal efficiency of specific quenching, E_{max} = 23% plus additional nonspecific quenching.

bind to band 3 in two opposite orientations, it is very likely that in only one orientation will reaction with the transport site lysine be able to occur. Self-inhibition of this type has previously been observed for the reaction of SITS with band 3 (P. Knauf, personal communication).

When free eosin was used, it was found that a high-affinity binding site could be detected on intact red cells (at 4 °C) with a K_d of about 3 μM and a stoichiometry of 1.2×10^6 sites/cell (Figure 6A). Donor quenching of BIDS fluorescence by eosin also revealed a class of binding sites on red cell ghosts with a K_d of 3 μM (Figure 6B). These results provide further

Table III: Energy Transfer Parameters: Donor (BIDS) and Acceptors at External Transport Sites of Band 3 Dimer

donor (BIDS) fractional saturation[a]	acceptor	fractional saturation[a]	method	transfer efficiency, E
0.08	eosin 5-malemide	1.24	donor quenching	0.23
0.13	eosin 5-malemide	1.08	donor lifetime	0.33
0.13	eosin 5-malemide	1.08	donor quenching	0.17
0.52	eosin 5-malemide	0.72	acceptor emission enhancement	0.31
0.72	eosin 5-malemide	0.32	acceptor emission enhancement	0.14
0.84	eosin 5-malemide	0.08	acceptor emission enhancement	0.05
0.84	eosin 5-malemide	0.44	donor quenching	0.13
0.32	eosin	?	donor quenching	0.23
0.08	$H_2(NBD)_2DS$		donor quenching	0.46
0.13	$H_2(NBD)_2DS$		donor quenching	0.41
0.84	$H_2(NBD)_2DS$		donor quenching	0.32

donor	$d_D^{x\,b}$	acceptor	$d_A^{x\,b}$	$d_T^{x\,b}$	critical distance, R_0[c] (Å)	E_{lim}[d]	D–A separation, R (Å)
BIDS	0.93	eosin 5-malemide	0.88	0.003	26–45	0.3	29–52
BIDS	0.93	$H_2(NBD)_2DS$	?	?	28–46[e]	0.5	28–46

[a] A fractional saturation of 1.0 is assumed equal to 2.5 nmol/mg of protein. [b] Axial depolarization factors, calculated according to Dale et al. (1979) from measured fluorescence anisotropies. [c] R_0 is the distance at which the energy transfer from donor to acceptor is 0.5. The extreme values reflect the uncertainty in K^2 as determined from d_T^x. Quadrupole interactions were ignored in calculating R_0. Given the dimensions of the acceptors used (Table I), the error in R so introduced can be calculated (Curie, 1963) to be less than 7% even at the minimum possible value of R. This error is much smaller than the error due to uncertainty in the value of K^2. The presence of interacting tetramers could also lead to a small error in R_0 and a maximum underestimate in R of about 5 Å (see Appendix). [d] The efficiency of transfer when every donor occupied site has an acceptor on the other half of the dimer. [e] Determination of the anisotropy of bound $H_2(NBD)_2DS$ was complicated by the contributions due to scattered light from the membranes and to free $H_2(NBD)_2DS$. The uncertainty in orientation factor, K^2, was therefore assumed to be the same as that for the BIDS–eosin malemide sample.

evidence that eosin and its derivatives interact with the external transport site of band 3. However, it was discovered that eosin is rapidly taken up by red cells at room temperature via a pathway insensitive to DIDS (probably diffusion across the membrane in uncharged form). Eosin was not, therefore, used for further experiments.

Resonance energy transfer efficiencies were determined by donor fluorescence quenching, by acceptor fluorescence enhancement, and from donor lifetime decreases for ghost labeled to several different stoichiometries with BIDS and/or eosin maleimide. The results are summarized in Table III. As expected, the highest efficiency of donor quenching (23%) or donor lifetime decrease (33%) or acceptor enhancement (31%) occurs at low donor stoichiometries where each donor is likely to have an adjacent acceptor. As the donor concentration is increased and acceptor decreased, the efficiency of transfer decreases. Transfer efficiencies were not significantly decreased by solubilization of the labeled ghosts in Triton X-100, confirming previous reports (Clarke, 1975; Yu & Steck, 1975) that the band 3 dimer remains associated in this detergent. Despite the long lifetime of the eosin triplet state (2.5 ms; Nigg & Cherry, 1979b) compared to that of the BIDS singlet state (0.7 ns; Rao et al., 1979), no saturation of the eosin was observed with the light intensities used in these experiments (data not shown). In the limit of saturating acceptor, the efficiency of transfer for the BIDS–eosin maleimide couple appears to be about 0.3 and for BIDS–$H_2(NBD)_2DS$ about 0.5.

Axial depolarization factors (Table III) were calculated from the measured anisotropies of the bound fluorophores by the method of Dale et al. (1979). A transfer depolarization factor was also measured for the BIDS–eosin maleimide pair, and Figure 5 from Dale et al. (1979) was used to determine the limiting values of the orientation factor, K^2. From these values, the BIDS–eosin maleimide separation was calculated to be 29–52 Å. Accurate measurements of the viscosity-dependent anisotropy of reversibly bound $H_2(NBD)_2DS$ were complicated by contributions to the observed anisotropy from light scattered by the membranes and from that of the free $H_2(NBD)_2DS$. Assuming the depolarization factor is similar in magnitude to that for the eosin maleimide gives a possible range for R of 28–49 Å.

Discussion

The occupation of an anion transport site on one subunit of the band 3 dimer by BIDS, a stilbenedisulfonate, has been shown to decrease the affinity of the transport site on the other subunit for a similar aromatic disulfonate, $H_2(NBD)_2DS$. However, partial saturation of band 3 with covalently bound BIDS had no detectable effect upon the K_m for [^{35}S]sulfate influx into red cells via band 3. Although the error in estimating the stoichiometry of BIDS labeling of band 3 was large, it appears that the fractional inhibition of sulfate influx is proportional to the fractional labeling of band 3 subunits and that labeling of one subunit does not therefore block transport by the dimer. This result suggests that the observed negative cooperativity is a function of the large sizes of the aromatic sulfonates used and that no interaction between subunits occurs upon the binding of small, transportable anions such as sulfate.

The distance between the transport sites might therefore be expected to be of the same order as the size of the aromatic disulfonates (20–30 Å). By fluorescence resonance energy transfer measurements, the distance was found to be 30–50 Å. The range is quite large because the fluorophores [BIDS, eosin maleimide, and $H_2(NBD)_2DS$] are rigidly bound to the protein, and their dipole transition moments are relatively motionless with respect to one another during the lifetime of the donor excited state. If the dipole transition moments of donor and acceptor were close to parallel, the distance would be nearly 50 Å; if they were close to perpendicular (with one transition dipole perpendicular to the vector joining the two probes), the distance would be 30 Å or less. [The transfer depolarization factor of 0.003 eliminates the possibility that $K^2 = 0$; see Dale et al. (1979).] It can be shown that if the eosin maleimide transition dipole moment is in the plane of the membrane and the anionic groups of both the donor (BIDS) and acceptor are located at equivalent sites on the two halves of a symmetric dimer, then the transition dipole moment of the BIDS must be normal to the membrane. This arrangement would result in a small value for K^2 and hence for

the donor–acceptor separation, R. The results of Nigg & Cherry (1980) are consistent with, but do not require, such an arrangement. Attempts to measure the orientations of the transition dipole moments with respect to the plane of the membrane are now in progress. The minimum donor–acceptor separation (30 Å) is similar to the distance between the centers of BIDS and $H_2(NBD)_2DS$ if they were laid end to end. Therefore, the fluorophores may be very close to one another when bound to band 3 and could possibly be occupying overlapping binding sites.

A possible objection to the use of $H_2(NBD)_2DS$ as an energy-transfer acceptor in these studies is that the molecule will behave as two independent acceptors of absorptivity 15.5 mM^{-1} cm^{-1}, separated by 20 Å, rather than as a single acceptor of absorptivity 31 mM^{-1} cm^{-1} (see Table I). However, calculation of the critical distance, R, for the BIDS/$H_2(NBD)_2DS$ couple using the lower absorptivity decreases R_0 by only 12%. Moreover, the estimated distance from the center of the BIDS molecule to the center of the $H_2(NBD)_2DS$ molecule when bound to band 3 is nearly independent of whether both ends of the $H_2(NBD)_2DS$ are assumed equidistant from BIDS or one end is 20 Å closer than the other [see Rao et al. (1979) for multiple acceptor calculations]. The error in treating $H_2(NBD)_2DS$ as a single acceptor is therefore negligible. The error arising from neglect of quadrupole interactions in the determination of R is also negligibly small even at the minimum estimated distance (26 Å) despite the large sizes of the acceptors used (see footnote *c* of Table III).

The measured affinities of BIDS-labeled band 3 for $H_2(NBD)_2DS$ deserve comment since they exhibit a significant decrease as the stoichiometry of BIDS labeling is raised (Figure 3). This effect suggests that associations of dimers of band 3 may exist in the red cell membrane. At low BIDS stoichiometries, such oligomers would possess a relatively high proportion of "empty" subunits adjacent to, but on separate dimers from, the BIDS subunit. These empty dimers would possess sites at a similar distance from, but of higher affinity than, the empty site on the BIDS-labeled dimer and hence would raise the apparent affinity for $H_2(NBD)_2DS$. The effect would decrease, as observed, with increasing BIDS labeling. There is recent electron microscopic evidence for the presence of band 3 tetramers in the erythrocyte membrane (Weinstein et al., 1980) and evidence from analytical ultracentrifugation for self-association into tetramers of isolated band 3 in aqueous solution (Schubert & Dorst, 1979). This point is discussed in detail in the Appendix.

Our results support the hypothesis of Ship et al. (1977) and other workers that band 3 monomers function as independent transport units. They further suggest that the apparent negative cooperativity of binding observed for certain stilbenedisulfonates (Dix et al., 1979) is a consequence of steric hindrance due to the proximity of the external transporting sites on the subunits of the band 3 dimer. Such an arrangement when considered together with the information that the stilbenedisulfonate sites are apparently within the bilayer (Rao et al., 1979) suggests that the transport sites of the band 3 dimer are accessed by a common cavity extending into the bilayer between the subunits.

Appendix

A detailed evaluation of the energy transfer between stilbenedisulfonate binding sites on the band 3 molecule should include the possibility that a significant fraction of the protein is present as a tetramer. Although it is difficult to determine the oligomeric state of band 3 in vivo, the density and distribution of transmembrane particles observed by electron microscopy make it unlikely that any oligomeric states higher than tetramers are present at a significant concentration in red cell membranes (Weinstein et al., 1980). The following discussion will first consider the simple case in which four identical stilbenedisulfonate binding sites are equidistant from the C_4 symmetry axis of a tetramer and no interactions occur between the binding sites. We will then consider the case of negative cooperative interactions.

At very low donor concentrations (<10% saturation), we need only consider cases in which a single donor is bound per tetramer. This leaves three additional sites for the acceptor stilbenedisulfonate to bind. For a symmetrical tetramer, two of the acceptor binding sites (on adjacent subunits) will be at the same distance, R, from the donor (and will have the same K^2 value), while the third acceptor site on the diagonal subunit will be slightly further away ($2^{1/2}R$) and will have a different value of K^2. The dependence of energy-transfer efficiency on the degree of acceptor site saturation is most nonlinear when all three acceptor sites contribute similarly to donor quenching (Rao et al., 1979). To consider this limiting case then, we will assume that the ratio R_0/R (where R_0 is the critical transfer distance) is the same for all three acceptor sites. The efficiency of energy transfer (from steady-state fluorescence quenching measurements), E, under these conditions can be described as a function of acceptor site occupation, s, by (Rao et al., 1979)

$$E = \left(\frac{(R_0/R)^6}{1+(R_0/R)^6}\right)\left(\frac{s(3-s)^2}{9}\right) + \left(\frac{2(R_0/R)^6}{1+2(R_0/R)^6}\right)\left(\frac{s^2(3-s)}{9}\right) + \left(\frac{3(R_0/R)^6}{1+3(R_0/R)^6}\right)\left(\frac{s^3}{27}\right) \quad \text{(A-1)}$$

If all three acceptor sites have identical intrinsic dissociation constants, K, then s will vary with the acceptor concentration, L, by

$$s = 3L/(K+L) \quad \text{(A-2)}$$

At saturating L, the last term in eq A-1 will dominate and $E = 3(R_0/R)^6/[1+3(R_0/R)^6]$. Taking the maximum efficiency of donor quenching to be 0.46 (from the data in Figure 3), one can calculate that $(R_0/R)^6 = 0.284$. The solid line in Figure A-1 is a theoretical plot of E/L vs. E using this value in eq A-1 to solve for E at varying L, assuming $K = 75$ nM (eq A-2). The plot shows only slight curvature, with 50% maximal quenching occurring when $L = 50$ nM. Thus, in the case where the maximal quenching is 0.46 (or less), the concentration of acceptor which causes 50% maximal quenching is quite close to the K observed from direct binding experiments. Notice that this value must always be lower than that observed by direct binding experiments. If only one or two of the acceptors were close enough to allow significant energy transfer, then the curve shown in Figure A-1 would be more nearly linear and the acceptor concentration giving 50% quenching would be even closer to the true K_d. In any event, the fact that a higher apparent K_d for $H_2(NBD)_2DS$ binding is observed from quenching of BIDS fluorescence than is observed from direct binding experiments (Table II) clearly cannot be explained by multiple acceptor interactions in which all binding sites have the same instrinsic K_d.

We consider next the case where tetramers behave as two negative cooperative dimers (with no interactions between the two dimers). In the case where a single donor is bound, then

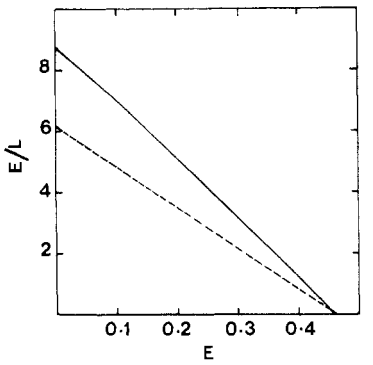


FIGURE A-1: Comparison of energy-transfer efficiencies (E) as a function of acceptor concentration (L) for one (---) or three (—) acceptor sites equidistant constant, K = 75 nM). The solid line was drawn by using eq A-1 and A-2 with K = 75 nM and $(R_0/R)^6$ = 0.284. The dashed line was drawn by using the euation $E = [L/(K + L)][(R_0/R)^6/[1 + (R_0/R)^6]]$ with K = 75 nM and $(R_0/R)^6$ = 0.852. Note that the apparent dissociation constant in the presence of multiple acceptor sites is 33% less than the true intrinsic dissociation constant.

one acceptor site (on the same dimer as the donor) will have an intrinsic dissociation constant, K_2, and the other two acceptor sites will show negative cooperativity with intrinsic constants K_1 and K_2. We will further assume for this model that the acceptor site on the adjacent subunit of the interacting dimer is closer to the donor than the sites on the other dimer. The efficiency of transfer will then vary with acceptor concentration according to

$$E = AB\left[\frac{X}{1+X} + \left(\frac{X+Y}{1+X+Y}\right)\times \left(\frac{2L}{K_1}\right) + \left(\frac{X+2Y}{1+X+2Y}\right)\left(\frac{L^2}{K_1K_2}\right)\right] + CB\left[\left(\frac{Y}{1+Y}\right)\left(\frac{2L}{K_1}\right) + \left(\frac{2Y}{1+2Y}\right)\left(\frac{L^2}{K_1K_2}\right)\right] \quad \text{(A-3)}$$

where $A = L/(K_2 + L)$, $B = 1/[1 + 2L/K_1 + L^2/(K_1K_2)]$, $C = K_2/(K_2 + L)$, $X = (R_0/R)^6$, and $Y = (R_0'/R')^6$. In this equation, R is the distance from donor to acceptor on the same dimer, and R' is the distance to either acceptor on the other dimer.

For this same model, it is of interest to determine what happens when 75% of the tetramer sites are reacted with donor. For simplicity in this discussion, we will assume extreme negative cooperativity in BIDS reactions such that the only species of significant concentration is that with three donors bound.[2] In this case each donor will have only one acceptor site available, with an intrinsic dissociation constant of K_2. The efficiency of transfer (ignoring any self-transfer between donors) will then be described by

$$E = \frac{1}{3}\left(\frac{X}{1+X} + \frac{2Y}{1+Y}\right)\left(\frac{L}{K_2+L}\right) \quad \text{(A-4)}$$

where X and Y are defined as in eq A-3.

The solid lines in Figure A-2 were drawn by using eq A-3 and A-4 assuming K_1 = 75 nM, K_2 = 655 nM, X = 0.97, and Y = 0.1 (determined by simultaneous nonlinear least-squares fit to both sets of data from Figure 3). Taking these values

[2] Actually, a significant concentration of the doubly labeled and quadruply labeled species might be present with a distribution dependent on the negative cooperativity of the reaction. If this distribution were known, these cases could be averaged in by assuming no quenching of the quadruply labeled species and quenching from acceptor at two identical low affinity sites for the doubly labeled species (see Cantley & Hammes, 1976).

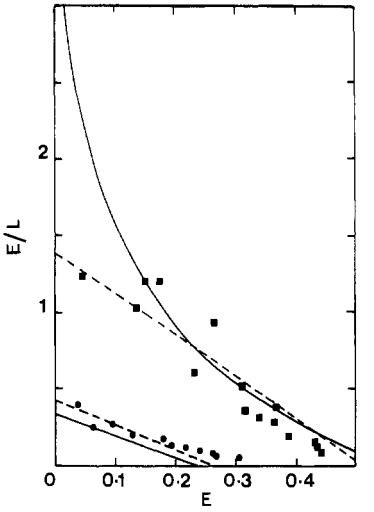


FIGURE A-2: Effect of energy transfer between pairs of dimers on efficiency of donor fluorescence quenching at different donor stoichiometries. The data are taken from Figure 3 for quenching by $H_2(NBD)_2DS$ of BIDS fluorescence at 0.2 (■) and 2.1 (●) nmol of BIDS/mg of membrane protein. The solid lines were drawn by using eq A-3 and A-4 which describe the case in which the dimer pairs do not interact, but the subunits of each dimer show negative cooperativity (K_1 = 75 nM and K_2 = 655 nM). The dashed lines were drawn by assuming the limiting case in which $K_1 = K_2$ = 550 nM, and the acceptor sites are all equidistant from the donor. This case describes a tetramer in which binding of one ligand lowers the affinity at all three reamining sites (to K = 550 nM), and no additional interactions occur between the remaining sites. Parameters were determined by a nonlinear least-squares procedure, fitting both sets of data simultaneously to the appropriate equations.

of X and Y gives R = 28–46 Å and R' = 40–67 Å with the assumption that R_0 = 28–46 Å; see Table III). The dashed lines in Figure A-2 represent the limiting case in which $K_1 = K_2$ and $X = Y$ (in this case, eq A-3 reduces to eq A-1). The best fit to the data was obtained by assuming $K_1 = K_2$ = 550 nM and $X = Y$ = 0.35 ($R = R'$ = 33–54 Å). Band 3 can be interpreted in this model as being a negatively interacting tetramer, so that the presence of donor on one subunit decreases the affinity for acceptor of all three adjacent subunits to K = 550 nM. Note that despite the fact that at 75% saturation with donor no more than a quarter of the band 3 dimers can have a subunit available for acceptor, the maximum efficiency of donor quenching will be ~50% of that at very low percent saturation.

Although it is not possible with the present data to distinguish between the two models (interacting dimers vs. interacting tetramers), it is clear that the observed changes in slope of the plots of E/L vs. E with varying donor concentration (Figures 3 and A-2) can be explained at least qualitatively by the assumption that energy transfer occurs between sites on adjacent dimers.

Acknowledgments

We wish to thank Scot Kuo for having synthesized H_2DADS and for providing many of the computer programs used in this study.

References

Barzilay, M., & Cabantchik, Z. I. (1979) *Membr. Biochem. 2*, 282.

Cabantchik, Z. I., Knauf, P. A., & Rothstein, A. (1978) *Biochim. Biophys. Acta 515*, 239.

Cantley, L. C., & Hammes, G. G. (1976) *Biochemistry 15*, 1.

Clarke, S. (1975) *J. Biol. Chem. 250*, 5459.

Curie, D. (1963) in *Luminescence in Crystals*, pp 220–226, Wiley, New York.
Dale, R. E., & Eisinger, J. (1975) *Biochem. Fluoresc. Concepts 1*, 115.
Dale, R. E., Eisinger, J., & Blumberg, W. E. (1979) *Biophys. J. 26*, 161.
Dalmark, M. (1976) *J. Gen. Physiol. 67*, 223.
Dix, J. A., Verkman, A. S., Solomon, A. K., & Cantley, L. C. (1979) *Nature (London) 282*, 520.
Ghosh, P. B., & Whitehouse, M. W. (1969) *Biochem. J. 108*, 155.
Gunn, R. B., & Fröhlich, O. (1979) *J. Gen. Physiol. 74*, 351.
Gunn, R. B. M., Tosteson, D. C., & Wieth, J. O. (1973) *J. Gen. Physiol. 61*, 185.
Hackney, D. D., Rosen, G., & Boyer, P. D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3646.
Hobbs, A. S., & Albers, R. W. (1980) *Annu. Rev. Biophys. Bioeng. 9*, 259.
Jennings, M. L., & Passow, H. (1979) *Biochim. Biophys. Acta 554*, 498.
Knauf, P. A. (1979) *Curr. Top. Membr. Transp. 12*, 249.
Lepke, S., Fasold, H., Pring, M., & Passow, H. (1976) *J. Membr. Biol. 29*, 147.
Nigg, E., & Cherry, R. J. (1979a) *Nature (London) 277*, 493.
Nigg, E., & Cherry, R. J. (1979b) *Biochemistry 18*, 3457.
Nigg, E., & Cherry, R. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4702.
Rao, A., Martin, P., Reithmeier, R. A. F., & Cantley, L. C. (1979) *Biochemistry 18*, 4505.
Schiller, P. W. (1975) *Biochem. Fluoresc. Concepts 1*, 285.
Schnell, K. F., Gerhardt, S., & Schoppe-Fredenberg, A. (1977) *J. Membr. Biol. 30*, 319.
Schubert, D., & Dorst, D. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 1605.
Shami, Y., Rothstein, A., & Knauf, P. A. (1978) *Biochim. Biophys. Acta 508*, 357.
Ship, S., Shami, Y., Breuer, W., & Rothstein, A. (1977) *J. Membr. Biol. 33*, 311.
Weinstein, R. S., Khodadad, J. K., & Steck, T. L. (1980) in *Membrane Transport in Erythrocytes* (Lassen, U. V., Ussing, H. H., & Wieth, J. O., Eds.) p 35, Munksgaard, Copenhagen.
Wieth, J. O. (1972) in *Oxygen Affinity of Hemoglobin and Red Cell Acid-Base Status* (Rorth, M., & Astrup, P., Eds.) p 265, Munksgaard, Copenhagen.
Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9170.

Interaction of 2-Aminobicyclo[3.2.1]octane-2-carboxylic Acid with the Amino Acid Transport Systems of the Sarcoma 37 Murine Ascites Tumor Cell†

Mark S. Elliott, Richard H. Matthews,* John P. Minton, and Robert Zand

ABSTRACT: The relatively broad and overlapping specificities of amino acid transport systems have made the synthesis of analogues specific to single transport systems desirable. The analogue in general use as a specific substrate for transport system L has been 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH). The affinity of BCH for the binding site of system L has been shown to be less than that of the natural substrate, leucine. Earlier studies from this laboratory suggested that higher homologues in a series could have greater affinity for system L. A higher homologue of BCH, 2-aminobicyclo[3.2.1]octane-2-carboxylic acid (ABOCA), has been synthesized and studied as a substrate and competitor for amino acid transport systems of the sarcoma 37 (S37) ascites cell. ABOCA inhibited the transport system dominant in the low concentration region for histidine uptake (system L) but had no effect on the uptake of labeled *N*-methyl-α-aminoisobutyric acid (MeAIB). MeAIB had no effect on labeled ABOCA uptake in S37 cells. ABOCA inhibited the uptakes of labeled leucine and labeled BCH competitively. Leucine, histidine, and BCH inhibited the uptake of labeled ABOCA competitively. Typical L system substrates demonstrated exchange effects with labeled ABOCA. The b isomer of ABOCA demonstrated slightly greater affinity for system L than did the a isomer. We conclude that ABOCA is an analogue restricted to interaction with amino acid transport system L, that it has greater affinity for system L than does BCH, and that its selection for system L is determined principally by an apolar interaction with steric considerations secondary.

It is now well established that amino acid transport into mammalian cells occurs by multiple transport systems that possess broad and overlapping specificities. Tenenhouse & Quastel (1960) first suggested the multiplicity of amino acid transport systems, and Ahmed & Scholefield (1962) showed that competitive inhibition could be used systematically to discriminate between the individual systems. This was applied to the Ehrlich ascites cell, and two systems known as A (alanine preferring) and L (leucine preferring) were described (Oxender & Christensen, 1963). The occurrence and properties of the A and L systems as they appear in the sarcoma 37 ascites cell have been studied in this laboratory (Matthews et al., 1970, 1975, 1977; Matthews, 1972; Matthews & Zand, 1977, 1979).

The functional isolation of individual transport systems has been a practical problem of importance for progress in the area of amino acid transport, and the problem has been addressed in part by the synthesis of analogues possessing more restricted specificity than that of natural substrates. In the case of

† From the Departments of Physiological Chemistry and Surgery, The Ohio State University, Columbus, Ohio 43210, and the Biophysics Research Division and the Department of Biological Chemistry, Institute of Science and Technology, University of Michigan, Ann Arbor, Michigan. *Received February 12, 1981.*
* Correspondence should be addressed to this author at the Department of Physiological Chemistry, The Ohio State University.

0006-2960/81/0420-5105$01.25/0