Characterization of the Stilbenedisulfonate Binding Site on Band 3†

Lawrence M. Schopfer and James M. Salhany*

Veterans Administration Medical Center and Departments of Internal Medicine and of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska 68198-5290

Received January 17, 1995; Revised Manuscript Received May 3, 1995®

ABSTRACT: Stilbenedisulfonates are potent inhibitors of Band 3 mediated anion exchange. They bind tightly to the protein and form a 1-to-1 reversible complex. Those stilbenedisulfonates which contain isothocyanato groups such as DIDS (4,4′-diisothiocyanato-2,2′-stilbenedisulfonate) and H_2DIDS (4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate) can also react rapidly with lysine residues within the binding pocket to yield an irreversible covalent adduct. The reactive lysine residue is known as lysine-A, and is thought to have an unusually low pK_a. In this report, we characterize the kinetics of DIDS adduct formation with respect to the effect of substrate anions, competitive inhibitory anions, and pH on the rate of covalent adduct formation. We investigate the following: (a) whether stilbenedisulfonates bind to or block access of substrate anions to the transport site; (b) whether the rapidity of the covalent reaction of DIDS at neutral pH is due to a low pK_a for lysine-A within the binding pocket; and (c) whether once bound, DIDS and H_2DIDS isothiocyanato groups are accessible to reagents. For this latter experiment, we have utilized a newly discovered reaction of the DIDS isothiocyanato groups with azide to test for accessibility. Our results show that substrate anions, DIDS, and Band 3 form a ternary complex. Significantly, the binding of large substrate anions, such as iodide, is not weakened by DIDS to any greater extent than is the binding of smaller substrates such as chloride or fluoride. These results are not consistent with a "partial blockade" hypothesis for the relationship between the stilbenedisulfonate and transport sites. Rather, they support an allosteric site−site interaction hypothesis. Our pH dependence results show that the apparent pK_a for the DIDS/lysine-A reaction is greater than 9.26. This is consistent with typical lysine pK_a values, and indicates that lysine-A does not have an unusually low pK_a. Finally, we show that azide can react with the isothiocyanato groups of DIDS and H_2DIDS within their Band 3 complexes, indicating that the stilbenedisulfonate binding site is accessible to solute. These results support a view which suggests that the stilbenedisulfonate site is a superficial inhibitory site on Band 3 which inhibits transport by allosteric interactions within the protein, rather than by either direct or partial blockade of the transport site.

Stilbenedisulfonates are a class of potent inhibitors of various anion transport functions found in a wide variety of cells (Cabantchik & Greger, 1992). While this class of molecules can inhibit the chloride channel function and the chloride cotransport function, they are best known as potent inhibitors of Band 3 anion exchange (Passow, 1986; Jennings, 1989; Salhany, 1990). The general characteristics of the stilbenedisulfonate site have been most extensively studied using two members of the stilbenedisulfonate class of molecules (DIDS[1] and H_2DIDS) which contain lysine-reactive isothiocyanato R-groups. These molecules bind rapidly (Salhany et al., 1993) and tightly (Janas et al., 1989; Salhany et al., 1993) to form a 1-to-1 reversible complex with the Band 3 subunit. After formation of the reversible complex, these stilbenedisulfonates then react via their isothiocyanato groups with lysine(s) residue(s) known as lysine-A to make the final covalent adduct[2] (Passow, 1986) (Scheme 1).

Scheme 1

$$\text{DIDS} + \text{Band 3} \overset{K_d}{\leftrightarrow} \text{reversible complex} \xrightarrow{k_{max}} \text{covalent adduct}$$

The formation of the covalent adduct is rapid at neutral pH. Some studies have suggested that such a rapid rate of reaction is the consequence of an abnormally low pK_a for the reaction of lysine-A with either DIDS (Ship et al., 1977) or H_2DIDS (Ship et al., 1977; Kampmann et al., 1982; Kietz et al., 1991). In addition, it has been shown that the remaining isothiocyanato group of covalently bound H_2DIDS is inaccessible to β-mercaptoethanol (Jennings & Passow, 1979), while, in contrast, the equivalent DIDS adduct has been shown to react with ferritin (Rothstein et al., 1976). Finally, until recently, the stilbenedisulfonate site had been

† This work was supported by the Medical Research Service of the Veterans Administration.

* To whom correspondence should be addressed at the Department of Internal Medicine, University of Nebraska Medical Center, 600 S. 42nd St., Omaha, NE 68198-5290. Phone: 402-559-6281. Fax: 402-559-9004.

® Abstract published in *Advance ACS Abstracts,* June 15, 1995.

[1] Abbreviations: DIDS, 4,4′-diisothiocyanato-2,2′-stilbenendisulfonate; H_2DIDS, 4,4′-diisothiocyanatodihydro-2,2′-stilbenedisulfonate; DAS, 4,4′-diacetamido-2,2′-stilbenedisulfonate; DBDS, 4,4′-dibenzamido-2,2′-stilbenedisulfonate; DNDS, 4,4′-dinitro-2,2′-stilbenedisulfonate; $C_{12}E_8$, polyoxyethylene 8-lauryl ether; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; PMSF phenylmethanesulfonyl fluoride.

[2] Throughout this paper, the term "adduct" will be used to refer to the covalent product of the DIDS/Band 3 reaction, and the term "complex" will be used to refer to the noncovalent product obtained on mixing DIDS with Band 3.

0006-2960/95/0434-8320$09.00/0

viewed as a pure competitive inhibitory site of Band 3 anion exchange (Passow, 1986). However, recent site-directed mutagenesis studies (Wood et al., 1992; Passow et al., 1992) and studies of the effect of chloride on the "off" rates of DBDS and H_2DIDS (Salhany et al., 1994) indicate that the stilbenedisulfonate and chloride transport sites are not purely competitive. Rather, stilbenedisulfonates appear to be linear mixed-type inhibitors (Salhany et al., 1994). Such inhibition lowers the V_{max} partially, and either allosterically lowers substrate affinity at the transport site or lowers the affinity by partially blocking an access channel to the transport site via steric hindrance.

In the present study, we use a modified version of a sensitive fluorescent reaction between DIDS and Band 3 (Schopfer & Salhany, 1992) to measure k_{max} in Scheme 1 directly. Such measurements allow us to test hypotheses concerning (a) the accessibility of substrate anions to the transport site within the DIDS/Band 3 reversible complex and (b) the pK_a of lysine-A. We then introduce a newly discovered reaction between DIDS and azide to show that the isothiocyanato groups of both DIDS and H_2DIDS are accessible. Our DIDS kinetic results suggest that the stilbenedisulfonate site lowers substrate affinity to the transport site allosterically, not by partial steric blockade of an access channel, and that the pK_a of lysine-A is >9.26.

MATERIALS

DIDS, disodium salt (*trans*-form, 80−90% pure), was purchased from Sigma. A stock solution of 12.8 mM was made in water, protected from light, and stored at −20 °C. This stock remained completely stable for at least 6 months, withstanding a minimum of 30 cycles of thawing and refreezing. Stability was indicated by (A) no change in the absorbance at 340 nm and (B) identical determinations for the concentration of isolated Band 3 using either 6-month-old DIDS or freshly prepared DIDS (see the titration protocol for determination of the concentration of Band 3, below). Working stock solutions were prepared daily by dilution into water and were stored on ice, in the dark. The concentration of DIDS was determined by the absorbance at 340 nm using an extinction coefficient of 36 000 M^{-1} cm^{-1} (Eisinger et al., 1982). α-Phosphatidylcholine (type III-B from bovine brain), α-chymotrypsin (type II from bovine pancreas), BSA (fraction V), SDS, PMSF, and $C_{12}E_8$ were purchased from Sigma. H_2DIDS was purchased from Molecular Probes and its concentration determined by the absorbance at 283 nm using an extinction coefficient of 39 000 M^{-1} cm^{-1} (Molecular Probes, 1989). Dithiothreitol (electrophoresis purity) was from Bio-Rad. All other chemicals were of reagent grade and were used without purification.

Unsealed Red Cell Membranes. In-date blood was obtained from the Omaha chapter of the American Red Cross as oversized or undersized units and used to prepare unsealed ghost membranes as described by Steck et al. (1970). Twenty microgram-per-milliter PMSF was included in the lysis buffer to prevent proteolysis. Unsealed red cell membranes were stripped of glyceraldehyde-3-phosphate dehydrogenase by washing with 5 mM sodium phosphate, pH 8, plus 150 mM sodium chloride (Fairbanks et al., 1971). Membranes were then reequilibrated with 5 mM sodium phosphate, pH 8, prior to use.

The concentration of Band 3 in the membranes was determined by titration with DIDS. Specifically, 12 samples were prepared by diluting 50−100 μL aliquots of a packed red cell membrane suspension into 1 mL of 23 mM sodium citrate buffer, pH 7.4, to give a final Band 3 concentration of approximately 1 μM. Then, separate microliter amounts of a DIDS stock solution were added to each sample to give a range of DIDS concentrations from 0.1 to 6 μM. The samples were sealed with Parafilm and incubated at 25 °C in the dark for 25 min, which is sufficient time for complete formation of the DIDS adduct under these conditions (Schopfer & Salhany, 1992). The fluorescent yield of each sample was then measured (excitation at 360 nm and emission at 450 nm) and plotted against the DIDS concentration. The fluorescent yield was linearly dependent on DIDS concentration until all of the Band 3 was reacted. At that point, the slope of the line flattened sharply, and further increases in fluorescence were due to the free DIDS. The concentration of DIDS at the breakpoint in the graph was taken to be equal to the concentrations of Band 3 in the reaction, from which the concentration of Band 3 in the membrane suspension was calculated. Determination of Band 3 concentration by this method was reproducible, from assay to assay, to within 10%. The concentration of Band 3 in unsealed membranes varied by as much as 2-fold from preparation to preparation. The factors causing this variability between preparations were not determined.

Isolation of Intact Band 3 and Integral Domain of Band 3. Red cell membranes were stripped of all exogenous protein by sequential incubation with 2 mM EDTA, pH 8, and 1 M potassium iodide, pH 7.5. Then intact Band 3 was purified in 0.1% $C_{12}E_8$ by aminoethyl-Sepharose column chromatography as described by Casey and Reithmeier (1991). The concentration of purified Band 3 was determined by the absorbance at 280 nm using an extinction coefficient of 93 000 M^{-1} cm^{-1} (Salhany et al., 1994).

The integral domain of Band 3 was isolated as described by Oikawa et al. (1985). The concentration of integral domain was determined by the absorbance at 280 nm using an extinction coefficient of 59 400 M^{-1} cm^{-1}, determined using the methods described by Salhany et al. (1994).

METHODS

DIDS Covalent Adduct Formation. Reaction of DIDS with Band 3 in unsealed ghosts begins with the rapid formation of a noncovalent complex, see Scheme 1 (k_{on} = 1.3 × 10^6 M^{-1} s^{-1} at 25 °C; J. M. Salhany, unpublished observations). The equilibrium dissociation constant for this complex is about 30 nM, at pH 7.3 (Janas et al., 1989). Because of the rapid, high-affinity binding, mixing approximately 1 μM DIDS with a 2-fold excess of Band 3 (at pH 7.2) results in the rapid (half-time of about 0.5 s) conversion of all of the DIDS into its noncovalent complex with Band 3. Subsequent formation of the covalent adduct can be followed as a simple, first-order increase in fluorescence with excitation at 360 nm and emission at 450 nm (Schopfer & Salhany, 1992; Van Dort et al., 1994). Fluorescence was monitored with a Perkin-Elmer fluorescence spectrophotometer, Model 650-40, at 25 °C. Excitation and emission slits were set at 2 nm, the excitation path length was 4 or 10 mm, and the emission path length was 10 mm. *trans*-DIDS undergoes photoisomerization in our fluorescence spectrophotometer (Schopfer & Salhany, 1992). However, noncovalent complex formation of DIDS with Band 3

eliminates all indication of this reaction. Except where stated otherwise, we have used a 2-fold excess of Band 3 over DIDS to ensure that DIDS is in complex with Band 3. Tests for free DIDS in solution were periodically made by doubling the Band 3 concentration (4-fold excess of Band 3 over DIDS) and looking for an increase in the adduct formation rate. Measurement of the rapid reaction rates found at high pH required using a cuvette mixing device (Add-A-Mixer, NSG Precision Cells Inc.) which permits mixing in 3−5 s. A precise description of the buffers and reaction conditions is given in the figure legends. The pH of each sample was measured after the reaction using a Fisher calomel, microprobe, combination glass electrode and a Beckman pH meter, Model 71.

Cross-Linking. Cross-linking of chymotrypsinized Band 3 in red blood cells with diisothiocyanatostilbenedisulfonates was performed, in general, according to the method of Jennings and Passow (1979). Red blood cells were washed in 10 mM sodium phosphate, pH 7.4, containing 150 mM sodium chloride (buffer A), resuspended to 50% hematocrit in the same buffer, and incubated with α-chymotrypsin (1 mg/mL) at 37 °C for 1 h. Then they were washed twice with 5 volumes of buffer A supplemented with 0.5% BSA, and then twice in buffer A alone. Seven samples were prepared by diluting 1 mL aliquots of chymotrypsinized red cells to 10% hematocrit in buffer A (i.e., about 2 μM in Band 3). Twelve micromolar H_2DIDS was added to samples 2, 3, and 4, while 12 μM DIDS was added to samples 5, 6, and 7. All seven samples were incubated at 37 °C, in the dark, for 1 h in order to allow the covalent adduct at lysine-A to form. Then 30 mM sodium azide was added to samples 3 and 6, while 50 mM β-mercaptoethanol was added to samples 4 and 7 and incubation was continued for 25 min in order to inactivate the remaining isothiocyanate group on the stilbenedisulfonate/Band 3 adduct, if possible. The red cells were washed twice with buffer A supplemented with 0.5% BSA and twice with buffer A alone, to remove excess reagents. Each sample was then suspended at 10% hematocrit in 150 mM sodium bicarbonate buffer at pH 9.5 and incubated at 37 °C for 1 h in the dark, in order to induce cross-linking by H_2DIDS, and DIDS if possible. Cells were then collected, chilled to ice temperature, lysed with 40 volumes of ice-cold 5 mM sodium phosphate buffer, pH 8, containing 20 μg/mL PMSF, and washed twice in the same way. One-half milliliter of the resulting white, unsealed membranes was taken from each sample, suspended in 10 mL of 0.1 N sodium hydroxide (pH 13) at 0 °C, and incubated for 10 min in order to induce cross-linking by DIDS, if possible. These samples were then washed twice with 20 mL of ice-cold water. Fifty microliter aliquots of each sample, from both pH 9.5 and pH 13 treated material, were solubilized with 100 μL of 40 mM Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, and 80 mM dithiothreitol at 100 °C for 3 min and then electrophoresed at 10 °C on a 10% SDS−polyacrylamide gel according to Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R250 (Pierce Chemical Co.).

RESULTS

DIDS/Band 3 Covalent Adduct Formation. The reaction of DIDS with Band 3 to form the covalent adduct at lysine-A was readily followed in red cell membrane preparations by the increase in fluorescence (Figure 1). This reaction was

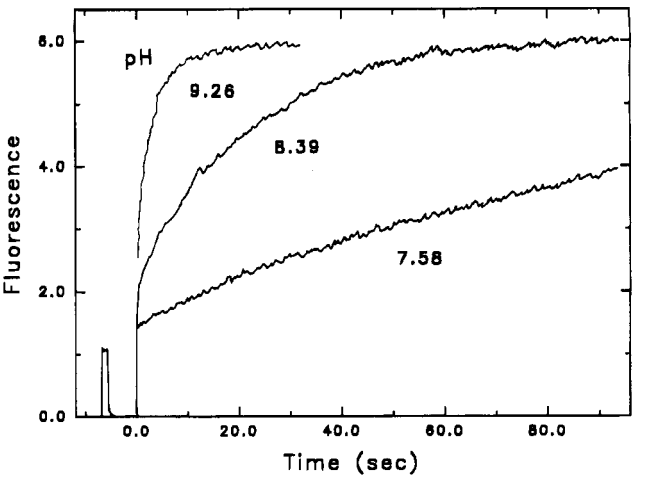

FIGURE 1: Representative time courses for the formation of the DIDS/Band 3 covalent adduct at various pH values. Reaction was initiated by adding 10 μL of 149 μM DIDS (in water) to 2.0 mL of buffer containing unsealed ghost membranes preequilibrated to 25 °C in 10 mM Bis-Tris, 10 mM Tris, and 250 mM sodium gluconate adjusted to the desired pH with acetic acid. This gave a final concentration for DIDS of 0.75 μM and for Band 3 of 1.6 μM. Final measured pH was 9.26 (top trace), 8.39 (middle trace), and 7.58 (bottom trace). The fluorescence was monitored as described under Methods. The fluorescence units are arbitrary.

first-order under all conditions employed. The time courses of the reaction at various pH values show that at least 70% of the reaction can be obtained over the pH range used in this study. Background scattering from the membranes amounted to about 18% of the signal and is shown as the spike prior to time zero. Formation of the noncovalent complex (in 50 mM Bis-Tris/acetate buffer, pH 7.2 at 25 °C) caused the fluorescence excitation maximum of DIDS to shift from 340 nm to about 360 nm while the emission maximum remained at about 415 nm (data not shown). The quantum yield of noncovalently bound DIDS did not change from that of free DIDS in solution, which is about 25% that of the DIDS/Band 3 adduct (Van Dort et al., 1994). Thus, fluorescence from the noncovalent complex is scarcely visible when adduct formation is monitored as described under Methods (excitation at 360 nm and emission at 450 nm).

Anion Dependence of DIDS Adduct Formation. Figure 2 illustrates the effect of selected anions on the apparent rate (k_{app}) for the formation of the DIDS/Band 3 covalent adduct. Increasing the concentration of the anion caused an increase in k_{app} which reached a limit at high anion concentration. We analyzed these data as thermodynamic titrations of Band 3 by the anions, using the observed k_{app} as a measure of the amount of anion/Band 3 complex formed. This analysis is patterned after a discussion by Fersht (1977) on the analysis of the pH dependence of reaction rates, from which we adapted the equation:

$$k_{app} = \frac{k_{max}[\text{anion}] + k_{min}K_d}{K_d + [\text{anion}]} \quad (1)$$

where [anion] is the total anion concentration (correction of the total anion concentration for the amount of anion in complex with Band 3 can be omitted since the concentration of Band 3 is much smaller than any anion concentration used), k_{max} is the limiting maximal rate, k_{min} is the extrapo-

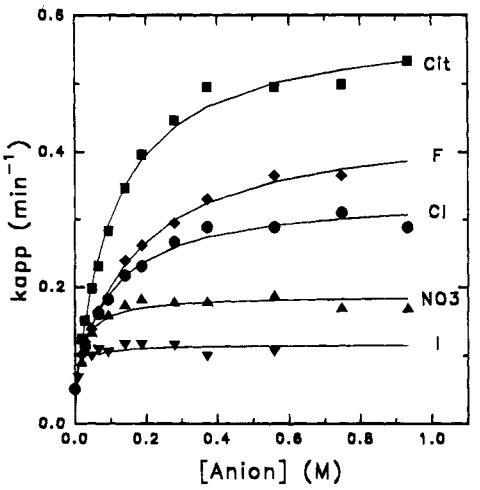

FIGURE 2: Dependence of the DIDS adduct formation rate constant on anion concentration for selected anions. One milliliter of unsealed red cell membranes, equilibrated in the appropriate buffer at 25 °C, was mixed with 7 μL of 128 μM DIDS to give final concentrations of 0.9 μM DIDS and 1.8 μM Band 3. Reactions involving fluoride, chloride, nitrate, or iodide were buffered with 50 mM Bis-Tris/acetate, pH 7.2 (15 mM acetate). Reactions involving citrate required no additional buffering agent. The pH for all reactions remained at 7.2 ± 0.05. All reactions were run to completion. Rates were obtained from semi-log plots of Δ fluorescence (final fluorescence minus fluorescence at time equal t) versus time. Plots of all reactions were linear to greater than 95% completion. Symbols indicate the data; the lines are the fitted result (see text). Some data points near the origin have been omitted from the figure for the sake of clarity.

lated rate at zero anion concentration, and K_d is the dissociation constant for anion binding to the DIDS/Band 3 noncovalent complex. For each anion, the dependence of k_{app} on anion concentration followed the profile of a single, saturable binding process (Figure 2). Values for k_{max}, k_{min}, and K_d were extracted by a nonlinear least-squares fit (SigmaPlot v.5.00, Jandel Corp.) of the data to eq 1.

Both the k_{max} and K_d were dependent on the anion employed (Table 1). The most chaotropic anions such as iodide and thiocyanate caused the least increase in k_{app}, and their effectiveness saturated at relatively lower concentrations. Less chaotropic anions such as fluoride and acetate caused larger increases in k_{app} and saturated at higher concentrations. In all cases, the extrapolated rate at zero anion concentration (k_{min}) was nonzero. For phosphate, citrate, and acetate, k_{min} was about 0.025 min^{-1}. For the remaining anions, k_{min} was equal to k_{app} for the supporting 50 mM Bis-Tris/acetate buffer, or about 0.05 min^{-1}. All of the preceding reactions were performed with a 2-fold excess of Band 3 over DIDS. Essentially identical results were obtained when the ratio of DIDS-to-Band 3 was 1-to-1 (using chloride, sulfate, nitrate, citrate, or acetate) or 1-to-10 (using chloride or sulfate).

We also examined the effect of phosphate on the DIDS adduct formation rate in isolated, intact Band 3 and in the isolated, integral domain of Band 3, both solubilized in 0.01% $C_{12}E_8$ containing 25 μM α-phosphatidylcholine. The rate showed the same sensitivity to phosphate concentration with these preparations as it did with Band 3 in unsealed membranes (data not shown). Thus, the anion effect is due to anion interactions with Band 3 and not to anion interactions involving cytoskeletal proteins or bilayer lipids.

Since we used a wide range of anion concentrations in these studies, one might suspect that the increase in k_{app} was a response to increasing ionic strength. If the increase were simply due to ionic strength, then, for the monovalent anions, the value of k_{app} should be independent of the nature of the anion and dependent only upon its concentration. From Figure 2, it can be seen that this is not the case. If, on the other hand, the increase in k_{app} was only partly due to ionic strength, then the ionic strength contribution could be no greater than the increase seen for the least effective salt, i.e., sodium thiocyanate. With thiocyanate, the increase in k_{app} (k_{max} minus k_{min}) was 0.04 min^{-1}. Thus, the maximum possible ionic strength contribution would be a minor portion of the effect for most anions.

Alternatively, one might suspect that the change in k_{app} was a consequence of the lyotrophic (Voet, 1937) or Hofmeister (1888) properties of anions, i.e., the effect of anions on the structure of water (Collins & Washabaugh, 1985) or the nonspecific binding of anions to the protein amide backbone (Robinson & Jencks, 1965). In fact, Deuticke (1977) has pointed out that the effectiveness of anions at inhibiting chloride transport matched their Hofmeister order. We do see a linear relationship between our K_d values and the Hofmeister number for the monovalent anions as would be expected for a Hofmeister effect (Figure 3, circles). However, linearity breaks down when the polyvalent anions are included in the comparison. Therefore, the increase in k_{app} is not due to the Hofmeister/lyotrophic properties of the anions. As a confirmation of this conclusion, we should point out that if the rate changes were due to a nonspecific property of the anions, such as the Hofmeister effect, one would then expect that the maximum limiting rate would be the same for each anion, with only the effectiveness (K_d) of the anion being variable. Figure 2 shows that this is not the case.

Since the common, nonspecific properties of anions have been shown to be of little importance in the effect of anions on the DIDS/Band 3 adduct formation reactions, it follows that the anion effect involves a specific interaction of the anion with Band 3. Support for a specific effect is given in Figure 3, where we have correlated our K_d values (circles, also see Table 1) with Michealis constant (K_m) and inhibition constant (K_i) values for anions interacting with Band 3 (squares, also see Table 1). The K_m and K_i values were taken from experiments in the literature for which the anion concentrations on both sides of the membrane were equal and were varied together. The temperatures in these experiments varied from 0 to 25 °C (see Table 1). However, a change in temperature has been shown not to affect the K_m value of anion binding to Band 3 (Gunn, 1978; Dissing et al., 1981; Frolich, 1982; Glibowicka et al., 1988). The pH in these experiments varied from 7.2 to 8.0. However, where comparison could be made, no significant effect of pH on the K_m values was seen (see Table 1). Figure 3 shows that both the K_d and K_m/K_i values follow the same pattern. This similarity in pattern argues strongly that the K_d, K_m, and K_i values all arise from anions interacting with the same site on Band 3. Since K_m and K_i values reflect anion binding to the anion transport site, it follows that the effect of anions on the rate of adduct formation must also arise from the binding of the anion to the anion transport site. In addition,

Table 1: Effect of Anions on DIDS Adduct Formation[a]

experimental				literature				
anion[b]	k_{max}[c] (min^{-1})	K_d[c] (mM)	Hofmeister[d] no. (M)	K_m[f] (mM)	K_i[f] (mM)	temp (°C)	pH	ref for K_m and K_i
thiocyanate	0.09[g]	20[g]	5.55[e]	3		4	7.4	Dissing et al. (1981)
nitrate	0.19 ± 0.11	28 ± 7	5.42		7	21	8.0	Galanter & Labotka (1991)
					7	20	7.6	Schnell et al. (1983)
iodide	0.12 ± 0.007	16 ± 6	5.20[e]		10	0	7.2	Dalmark (1976)
					15	3	8.0	Falke et al. (1984)
bromide	0.22 ± 0.005	49 ± 6	4.65[e]	19		0	7.8	Gunn & Frolich (1979)
					32	0	7.2	Dalmark (1976)
nitrite	0.43 ± 0.025	106 ± 22	4.15[e]					
chloride	0.33 ± 0.007	99 ± 11	3.63	50		0	7.8	Gasbjerg & Brahm (1991)
				67		0	7.2	Dalmark (1976)
					80	3	8.0	Falke et al. (1984)
					30	20	7.6	Schnell et al. (1983)
fluoride	0.46 ± 0.010	183 ± 15	1.85[e]		88	0	7.2	Dalmark (1976)
					129	3	8.0	Falke et al. (1984)
acetate	0.64 ± 0.060	283 ± 55	1.69					
phosphate	0.53 ± 0.013	153 ± 19	0.82	65		25	7.2	Schnell et al. (1981)
tartrate	0.47 ± 0.018	107 ± 20	0.79					
sulfate	0.51 ± 0.013	97 ± 12	0.80		72	20	7.2	Kaufman et al. (1986)
				44		20	7.6	Schnell et al. (1983)
citrate	0.59 ± 0.014	105 ± 12	0.56					
gluconate	0.44 ± 0.008	180 ± 15						

[a] Reactions were run at 25 °C. With citrate and phosphate, no additional buffering agent was required. With acetate, Bis-Tris was included as the primary buffer. With thiocyanate, nitrate, iodide, nitrite, chloride, fluoride, bromide, sulfate, tartrate, or gluconate, 50 mM Bis-Tris/acetate buffer (15 mM acetate) was used to maintain pH. The pH remained at 7.2 ± 0.05 for all reactions. All reactions employed 0.9 μM DIDS and 1.8 μM Band 3. Reactions were followed fluorometrically as described under Methods and the rates calculated from semi-log plots as described in the legend to Figure 2. [b] All anions were used as their sodium salts. [c] Values and error limits were determined by fitting data as described in the text. [d] Values were taken from Hofmeister (1888) and recast in terms of molarity as in Robinson and Jencks (1965). [e] Values were translated from the lyotrophic series (Voet, 1937). [f] Michaelis constant (K_m) and inhibition constant (K_i) values were taken from experiments where the anion concentrations on both sides of the membrane were equal and were varied together. [g] Values were estimated from a visual inspection of the data.

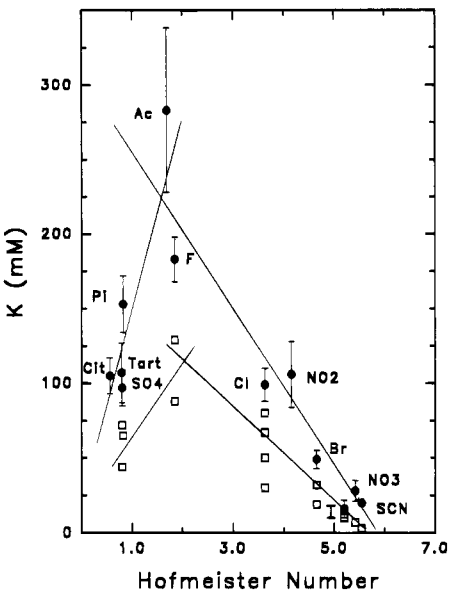

FIGURE 3: Comparison of the affinity of anions for binding to the anion transport site on Band 3 with the effectiveness of anions at accelerating the rate of DIDS adduct formation. Circles, the apparent dissociation constants (K_d) for the effect of anions on the rate of DIDS adduct formation. K_d values were measured as described in the text using conditions described in footnote *a* of Table 1. Squares, Michaelis constants (K_m) or inhibition constants (K_i) for anion binding to the anion transport site. These values were selected from the literature based on criteria which have been described in the text and in footnote *f* in Table 1. Values for K_d, K_m, and K_i are listed in Table 1. The *x*-axis is the Hofmeister number for the anions, given in molarity (see Table 1, footnotes *d* and *e*).

since DIDS must also be bound to Band 3 in order for adduct formation to proceed, a ternary complex between DIDS, anion, and Band 3 must exist.

Our K_d values for a given anion are about 2-fold larger than the affinity constants for that anion in the absence of DIDS (Figure 3). Two-fold is therefore the difference in affinity between anion binding to Band 3 in the native state and anion binding to the DIDS/Band 3 noncovalent complex. Conversely, the affinity of DIDS for the stilbenedisulfonate site will be decreased by only 2-fold in the presence of the anions. It is therefore not surprising that DIDS was not displaced from Band 3 by the anions under our assay conditions.

It is interesting to note that even anions which are traditionally used as noninteracting "spectator" ions, such as citrate and gluconate, caused a marked increase in k_{app}, with K_d values similar to those of the other anions (Table 1). This supports the proposal by Jennings (1989) that there are no "spectator" ions.

pK_a of Lysine-A. The rate of DIDS reaction with lysine-A on Band 3 is sensitive both to pH (Ship et al., 1977) and to anion concentration. In order to separate the pH dependence of the apparent rate (k_{app}) from the anion effect, we used a buffer containing 250 mM sodium gluconate to buffer the anion concentration, with 10 mM Bis-Tris/acetate and 10 mM Tris/acetate to buffer pH. With this buffer system, the changes in acetate concentration which accompanied the changes in pH were insignificant relative to the total anion concentration, and therefore did not contribute to the observed change in rate. Figure 4 shows that with this buffer system, k_{app} for DIDS adduct formation increases continuously from pH 6.16 to pH 9.26. The plot of log(k_{app}) versus pH is linear with a slope of 0.9, consistent with k_{app} responding to a single deprotonation (Fersht, 1977). The most likely candidate for the deprotonation is the ε-amino group of lysine-A (Passow, 1986). Since there is no

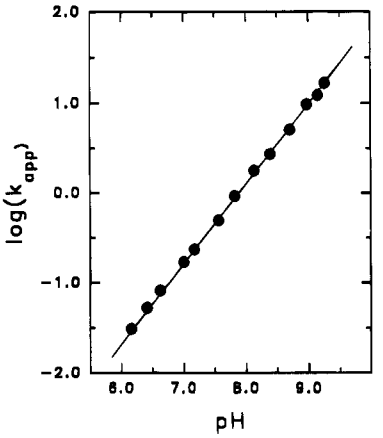

FIGURE 4: Dependence of the rate of DIDS adduct formation on pH. Reaction conditions are described in the legend to Figure 1. Rates were measured as described in the legend to Figure 2.

deviation in the plot, a value for the pK_a of lysine-A cannot be estimated. However, it is possible to set a lower limit for the pK_a at 9.26. Above pH 9.26, the reaction was too fast to be measured accurately under these conditions. Doubling the concentration of Band 3 in the assay had no significant effect on the measured rate at either pH 6.44 or pH 9.17.

We also examined the pH dependence of DIDS adduct formation with isolated, intact Band 3 and isolated, integral domain of Band 3. Reactions were performed at 25 °C in 10 mM Bis-Tris/acetate, 10 mM Tris/acetate buffer containing 250 mM sodium gluconate, 0.01% $C_{12}E_8$, and 25 μM α-phosphatidylcholine (added to stabilize the isolated protein; Schopfer & Salhany, 1992). Both preparations gave log(k_{app}) versus pH profiles essentially identical to that shown in Figure 4 for the unsealed red cell membranes (data not shown).

Accessibility of DIDS in the DIDS/Band 3 Noncovalent Complex. In the course of our studies on the effect of anions on the rate of DIDS adduct formation, we found that azide was anomalous. Instead of the simple, first-order rise in fluorescence we normally saw for DIDS adduct formation (see Figure 1), the fluorescence rose, then fell, and then rose again. The third phase was dependent upon irradiation, while the rates of the first two phases were dependent on azide concentration. We traced this change of behavior in the presence of azide to a reaction of azide with DIDS.

The existence of a reaction between DIDS and azide was indicated by a time-dependent change in absorbance when azide was added to a buffered solution of DIDS. The spectrum of DIDS (Figure 5, solid line: peak at 340 nm, extinction 36 000 M^{-1} cm^{-1}) isosbestically shifted to longer wavelength (Figure 5, dashed line: peak at 350 nm, extinction 31 200 M^{-1} cm^{-1}) after azide was added. We presume this reaction to be a nucleophilic addition of azide to the isothiocyanate moieties of DIDS, analogous to the reaction of thiols and amines with the isothiocyanates of DIDS. The reaction followed a single, pseudo-first-order time course. A plot of the apparent rate versus azide concentration was linear from 1.5 to 35 mM azide, passed through the origin, and yielded a second-order rate constant of 44 M^{-1} min^{-1} (data not shown).

The reaction of azide with DIDS quenched the intrinsic DIDS fluorescence. Addition of isolated band 3 to the DIDS/azide product resulted in a return of the fluorescence. The

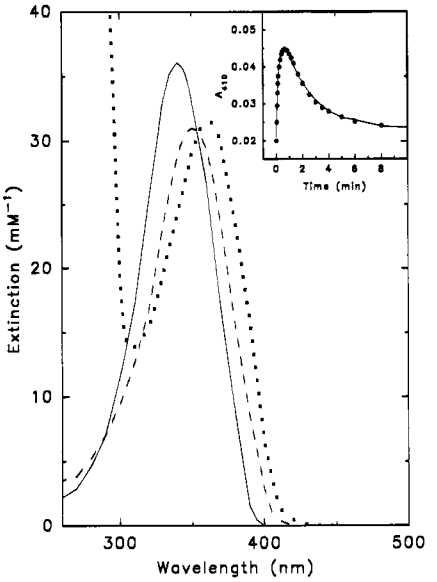

FIGURE 5: Spectra of DIDS and the DIDS/azide reaction products. (Solid line) DIDS in 20 mM sodium phosphate buffer, pH 7.4. (Dashed line) The product from the reaction of DIDS with sodium azide: DIDS (24 μM) in 1 mL of 20 mM sodium phosphate buffer, pH 7.4, was mixed with 33 μL of 0.3 M sodium azide to give a final azide concentration of 9.6 mM. (Dotted line) The final product from the reaction of Band 3-bound DIDS with azide: isolated Band 3 (22 μM) in 1 mL of 50 mM sodium phosphate, pH 7.2, containing 0.08% $C_{12}E_8$, 200 μM α-phosphatidylcholine, 80 mM sodium chloride, and 30 mM sodium azide was mixed with 10 μL of 1.90 mM DIDS, to give a final DIDS concentration of 19 μM. All spectra were recorded at 25 °C, on a Hitachi Model 100-60 dual-beam spectrophotometer. Note: The extinction values for the DIDS−azide products were calculated relative to that of DIDS (extinction at 340 nm equal to 36 000 M^{-1} cm^{-1}, Eisinger et al., 1982). Insert: The time course for the reaction of Band 3-bound DIDS with sodium azide. Isolated Band 3 (18 μM) was equilibrated with 15 mM sodium azide in 1 mL of 50 mM sodium phosphate buffer, pH 7.2, containing 0.08% $C_{12}E_8$, 200 μM α-phosphatidylcholine, and 80 mM sodium chloride, at 25 °C. Reaction was initiated by adding 8 μL of 1.98 mM DIDS, to give a final DIDS concentration of 16 μM. The absorbance at 410 nm (A_{410}) is indicated by the solid circles. All reactions were followed to completion. The line is a nonlinear least-squares fit (SigmaPlot v 5.00, Jandel Corp.) of the time course to the sum of two exponentials: $A_{410} = A_1e^{-k_f t} + A_2e^{-k_s t} + A_3$ where k_f and k_s are the fast and slow rates, respectively, as discussed in the text.

fluorescence intensity, the excitation maximum, and the emission maximum of the complex were qualitatively the same as the fluorescence properties of the second species we saw during the reaction of azide with DIDS in the presence of Band 3. However, the photochemical third phase made fluorescence comparisons imprecise. The photochemical reaction also complicated analysis of the kinetics. We therefore turned to absorbance spectroscopy where the photochemical reaction did not occur.

A time-dependent change in the absorbance of DIDS was found when DIDS was mixed with isolated Band 3 in the presence of azide. This reaction did not represent the formation of the DIDS/Band 3 covalent adduct (see below). The spectrum of the final product (Figure 5, dotted line) had a peak at 362 nm with an extinction of 32 100 M^{-1} cm^{-1}. The same spectrum was obtained when the preformed DIDS/azide product was added to isolated Band 3. This spectrum is very similar to that of the DIDS/Band 3 noncovalent complex (peak at 359 nm with an extinction of 36 000 M^{-1} cm^{-1}, data not shown). However, formation of the DIDS/

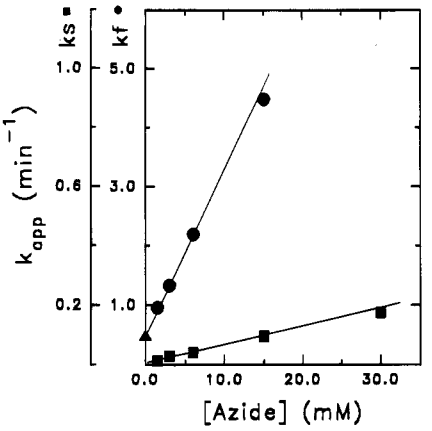

FIGURE 6: Azide concentration dependence of the measured rates for the reaction of azide with DIDS bound to Band 3. Reactions were performed and rates extracted as described in the legend to Figure 5. Azide concentration was varied from 1.5 to 35 mM. Circles, fast phase; squares, slow phase; triangle, the measured rate for the reaction of DIDS with Band 3 in the absence of azide under the same conditions.

Band 3 complex is completed in the 3−5 s required for mixing (J. M. Salhany, unpublished observations), and can therefore not account for the time-dependent spectral change we are following in Figure 5. The contribution of the DIDS/Band 3 complex to the time course of the azide reaction can be seen in the insert to Figure 5, where the 410 nm absorbance is raised to 0.02 at the beginning of the reaction. The similarity between the spectra of the starting DIDS/Band 3 complex and the final DIDS/azide/Band 3 complex resulted in small absorbance changes and restricted the wavelengths at which useful data could be collected. After preliminary testing, we have chosen to follow the kinetics of this reaction at 410 nm.

The time course of the reaction at 410 nm was biphasic (Figure 5, insert). There was a fast rise in absorbance followed by a slower decline. The rates for both phases were comparable to those seen for the first two phases fluorometrically. There was a second-order dependence on azide concentration for each rate (Figure 6). This second-order dependence demonstrates that two separate reactions are occurring between azide and DIDS. We take this to mean that each of the DIDS isothiocyanates reacts with azide at a different rate when DIDS is bound to Band 3.

The apparent rate for the fast phase (k_f) showed a linear dependence on azide concentration, which extrapolated to a nonzero intercept on the y-axis (Figure 6, circles). The slope of the line gives a second-order rate constant for k_f of 270 M^{-1} min^{-1}. This rate is 6-fold larger than the rate for DIDS reacting with azide free in solution and therefore must represent a reaction of azide with DIDS, while it is bound to Band 3. The extrapolated intercept value is equal to the rate of DIDS adduct formation with Band 3 in the absence of azide (Figure 6, triangle). This result indicates that azide competes with lysine-A for reaction with DIDS. This competition could be direct, with lysine-A and azide reacting with the same isothiocyanate on DIDS, or the competition could be indirect, with the position of the DIDS/azide product being shifted in the stilbenedisulfonate binding pocket such that lysine-A can no longer react, and vice versa.

The slow phase (k_s) also showed a linear dependence on azide concentration (Figure 6, squares). This plot extrapolated to the origin, yielding a second-order rate constant of 31 M^{-1} min^{-1}. Since this rate is not faster than the free solution reaction rate, it is not evident that the slow-phase reaction occurs with the fast-phase product still bound to Band 3. If we assume, however, that the equilibrium dissociation constant for Band 3 and the fast-phase product is at or below 1 μM, as is common for stilbenedisulfonate binding to Band 3, then there would be too little fast-phase product free in solution to support a reaction at the observed rate of 31 M^{-1} min^{-1}. Therefore, the slow phase would also represent a reaction of azide with stilbenedisulfonate, noncovalently bound to Band 3.

In order to ensure that the spectral changes we were following represented the reaction of azide with DIDS and not an azide-induced perturbation of DIDS/Band 3 adduct formation, we denatured the protein at the end of the azide reaction (30 mM azide) using 1% SDS. Greater than 95% of the DIDS/azide products could be separated from the SDS-denatured protein by ultrafiltration using a Centricon-30, 30 000 MW cutoff filter (Amicon). When the DIDS/Band 3 covalent adduct was allowed to form in the absence of azide, none of the stilbenedisulfonate could be separated from the denatured protein in this manner.

Denaturation with SDS also provides a means of discriminating Band 3 bound DIDS from the Band 3 bound DIDS/azide product. One percent SDS caused the absorbance maxima of both to revert to their free solution positions. Although the absorbance maxima are very similar for the Band 3 complexes, as we have indicated above, they are separated by 10 nm in 1% SDS. Adding 1% SDS to the DIDS/Band 3 noncovalent complex resulted in an absorbance shift from 359 nm to 340 nm. Adding 1% SDS to the final product from the reaction of DIDS, azide, and Band 3 resulted in an absorbance shift from 362 nm to 350 nm. This supports our contention that the time-dependent absorbance changes we see when azide is mixed with DIDS and Band 3 are due to a reaction of azide with DIDS in the DIDS/Band 3 noncovalent complex and not the consequence of a simple perturbation of that complex.

Inhibition of the Cross-Linking of Chymotrypsinized Band 3. Jennings and Passow (1979) have shown that when intact red blood cells are treated with chymotrypsin, Band 3 is cut and the resulting fragments can be cross-linked back together by treatment with H_2DIDS. The cross-linking process can be separated into two steps by first treating with H_2DIDS at neutral pH to form the covalent adduct at lysine-539 and then raising the pH to 9.5 in order to promote the cross-linking at lysine-851 (Okubo et al., 1994). Jennings and Passow (1979) found that attempting to block the cross-linking by treatment of the neutral sample with the isothiocyanate-reactive reagent β-mercaptoethanol was ineffective. We have used the same cross-linking strategy to test whether azide blocks cross-linking. In addition, we have used DIDS as the cross-linker, following the pH 13 strategy of Kang et al. (1992), and examined the effect of azide on this process.

Figure 7 is an SDS−PAGE separation of the fragments of chymotrypsinized Band 3 after (1) exo-facial treatment of red blood cells with chymotrypsin and (2) various cross-linking strategies (see Methods for sample preparation). Lane 1 shows the 60 000 MW fragment of Band 3 that resulted from cleavage of the 95 000 MW intact Band 3; the 35 000 MW fragment is not detectable. Lanes 2 and 4 reproduce the original observations of Jennings and Passow (1979) showing that H_2DIDS could cross-link chymotrypsinized

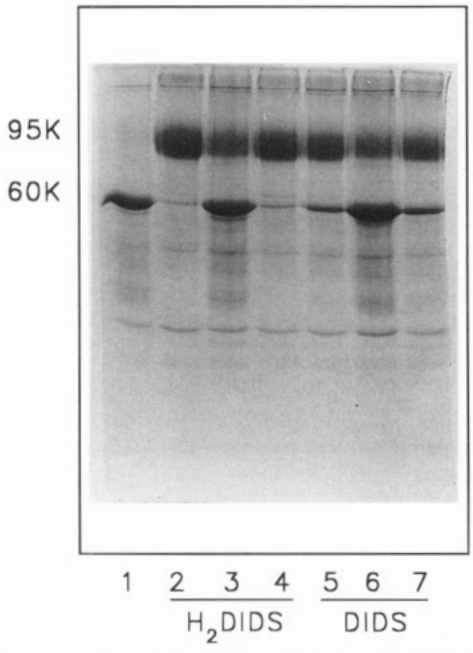

FIGURE 7: Comparison of the ability of DIDS and H_2DIDS to cross-link chymotrypsinized Band 3. Samples were prepared, cross-linked at pH 13, and electrophoresed on a 10% SDS−polyacrylamide gel as described under Methods. Lane 1, no cross-linking; lane 2, H_2DIDS only; lane 3, H_2DIDS plus 30 mM sodium azide; lane 4, H_2DIDS plus 50 mM β-mercaptoethanol; lane 5, DIDS only; lane 6, DIDS plus 30 mM sodium azide; lane 7, DIDS plus 50 mM β-mercaptoethanol.

Band 3 back to its 95 000 MW size (lane 2) and that β-mercaptoethanol was ineffective at preventing the cross-linking (lane 4). Lane 3 shows that treatment of the H_2-DIDS/Band 3 adduct with azide prior to cross-linking largely inhibited cross-linking. Though it is clear that only partial inhibition has been accomplished, the reaction with azide was allowed to run for only 25 min in order to parallel the β-mercaptoethanol conditions used by Jennings and Passow (1979). A longer reaction time would likely produce greater inhibition. Regardless of the extent of inhibition, this result indicates that the unreacted isothiocyanate in the H_2DIDS/Band 3 covalent adduct was accessible to azide. Lanes 5−7 are experiments with DIDS in place of H_2DIDS which parallel the experiments in lanes 2−4. Lane 5 shows that DIDS could cross-link chymotrypsinized Band 3, at pH 13, confirming the findings of Kang et al. (1992). Lane 6 shows that azide prevented cross-linking, indicating that the second isothiocyanate in the DIDS/Band 3 covalent adduct was also accessible to azide. Lane 7 shows that β-mercaptoethanol did not prevent cross-linking. Thus, we can conclude (1) that both DIDS and H_2DIDS are capable of cross-linking Band 3, (2) that the second isothiocyanate moiety of either the DIDS/Band 3 or the H_2DIDS/Band 3 adduct is accessible, and (3) that β-mercaptoethanol is not a suitable probe to test for accessibility of both stilbenedisulfonate isothiocyanato groups.

The samples shown in Figure 7 were incubated at pH 13 to promote cross-linking. When samples which had only been exposed to pH 9.5 were used, those containing DIDS were not cross-linked while those containing H_2DIDS or H_2-DIDS and β-mercaptoethanol were cross-linked. Azide was as effective at preventing cross-linking by H_2DIDS for the pH 9.5 samples as it was for the pH 13 samples (data not shown).

DISCUSSION

The results of this paper offer considerable insight into the structure of the stilbenedisulfonate transport inhibitory site and the relationship of this site to the active site, or to the putative transport access channel. We have found that the DIDS-reactive lysine in this site has an apparently normal pK_a (>9.26) and that once bound the isothiocyanato groups of both H_2DIDS and DIDS are accessible to azide in the solvent. In addition, our anion-dependent study of the adduct formation reaction supports an allosteric point of view, rather than a partial steric hindrance hypothesis.

In particular, we have found that the rate of adduct formation with lysine-A is accelerated by the presence of anions. All 13 anions tested caused this acceleration. The dependence of the rate on anion concentration fit a simple saturation profile (Figure 2). Both the anion-induced acceleration of DIDS adduct formation and the saturating dependence of the rate on anion concentration indicate that a ternary complex was formed involving DIDS, anion, and Band 3. Formation of a ternary complex requires separate binding sites for both DIDS and anion. The relative effectiveness (K_d) for the various anions at accelerating the rate of DIDS adduct formation follows the same pattern as the affinity (K_m or K_i) of anions for the anion transport site (Figure 3). This strongly suggests that the anion binding site in the ternary complex is the anion transport site. Since it can be safely assumed that DIDS binds to the stilbenedisulfonate binding site, it follows that the anion transport site and the stilbenedisulfonate binding site are separate.

To further elaborate on the structure of the ternary complex, it can be argued that the stilbenedisulfonate and anion transport sites are both located on the external side of the same Band 3 subunit. Evidence that both sites are on the same subunit comes from the observation that anions are equally effective at DIDS-to-Band 3 ratios of 1-to-1, 1-to-2, and 1-to-10. Were the DIDS and the anion binding to separate subunits and affecting one another via intersubunit interactions, then those interactions would be altered as the saturation of Band 3 by DIDS increased. The most significant evidence that the anion binding site is on the external side of the ternary complex comes from Falke and Chan (1986) and Salhany et al. (1994). Falke and Chan (1986) showed that DIDS, like DNDS, blocks anion binding to the internal side of Band 3. Salhany et al. (1994) found that the acceleration of DBDS release by chloride involves chloride binding to the external surface of Band 3.

Our demonstration of a DIDS/Band 3/anion ternary complex adds to a growing body of evidence which indicates that the stilbenedisulfonate binding site and the anion transport site are separate: (A) The equilibrium dissociation constant for both DBDS (Dix et al., 1986) and DNDS (Aranibar et al., 1994) binding to Band 3 shows a saturating dependence on chloride concentration. (B) Site-directed mutagenesis of lysines in the stilbenedisulfonate site of Band 3 reduces the affinity of Band 3 for DNDS without affecting the affinity of chloride (Wood et al., 1992). (C) Chloride accelerates the rate of DBDS release from Band 3 (Salhany et al., 1994). (D) The inhibition of phosphate transport by DNDS is noncompetitive (Stadler & Schnell, 1990). (E)

Inhibition of chloride transport by DAS is incomplete, reaching a limit at 20% residual transport activity (Kaplan et al., 1976). (F) Chloride accelerates the rate of H_2DIDS release from its noncovalent complex with Band 3 (Salhany et al., 1994). (G) Exofacial proteolysis of Band 3 with papain reduces the affinity for DNDS by 12-fold without affecting the affinity for sulfate (Jennings & Adams, 1981). (H) Dissociation of DNDS from Band 3 shows a saturating dependence on chloride concentration if the chloride is varied asymmetrically (Knauf et al., 1993). (I) Chloride has no significant effect on the initial, second-order rate constant for DBDS (Salhany et al., 1995), or DIDS or H_2DIDS (J. M. Salhany, unpublished results) binding to Band 3.

We envision a ternary complex where the stilbenedisulfonate and anion transport sites are separate and distinct. An alternative ternary complex model has been proposed where the sites are partially overlapping such that the stilbenedisulfonate would sterically impair access of the anion to the transport site (Jennings, 1989; Falke & Chan, 1986). If partial overlap and steric interactions were at work, then one would expect that large anions would be affected by DIDS more than small anions. From Figure 3 and Table 1, it can be seen that the binding of all anions is similarly weakened in presence of DIDS. This observation is at variance with the partial overlap model and supports a model in which the sites are separate and distinct, and where interactions between sites are allosterically mediated.

Interaction between two different ligands binding to separate sites on a protein is by definition heterotropic allosterism. Koshland (1970) has ascribed this phenomenon to a change in protein structure induced by the binding of one ligand, which is transmitted to the binding site of the second ligand. One well-established protein structural change induced by anion binding to the external surface is the recruitment of internally facing anion binding sites to an outside facing orientation (Passow, 1986). However, a similar change occurs when stilbenedisulfonates bind noncovalently to the external surface (Falke & Chan, 1986). Therefore, the recruitment conformational change is not likely to be the source of the increased rate of DIDS adduct formation in the presence of anions.

The existence of a ternary complex between DIDS, Band 3, and anions indicates that transport inhibition by stilbenedisulfonates is not simply due to prevention of the anion/Band 3 complex formation. Although some stilbenedisulfonates do substantially reduce the affinity of Band 3 for anions, transport inhibition must include a blockade of the membrane translocation step as well. The combination of affinity reduction and translocation inhibition makes stilbenedisulfonates mixed-type inhibitors, as has been discussed by Salhany et al. (1994).

On the basis of the pH dependence of the DIDS adduct formation rate (Figure 4), there can be little doubt that the pK_a of lysine-A is greater than 9.26. This result is independent of the environment of the Band 3; i.e., unsealed ghost membranes; isolated, intact Band 3 in $C_{12}E_8$ micelle; or isolated integral domain of Band 3 in $C_{12}E_8$ micelle. A lower limit for the pK_a of lysine-A of 9.26 is substantially higher than the pK_a values reported by others, using indirect assays involving DIDS [Ship et al. (1977) pK_a of 8−9] or H_2DIDS [Kietz et al. (1991), pK_a of 8.2; Kampmann et al. (1982), pK_a of 7.3].

The finding that the pK_a of lysine-A is not anomalously low helps explain why treatment of intact red cells with low levels of pyridoxal phosphate and borohydride (Kawano et al., 1988) or formaldehyde and borohydride (Jennings, 1982) caused preferential labeling of lysine-B. If the pK_a of lysine-A were low, then lysine-A would have been the preferred target, especially for the simple reductive methylation reaction.

It should be noted that the lysine residue which reacts with DIDS may be different from that which reacts with H_2DIDS. The lysine residues which react with H_2DIDS have been identified as lysines-539 and -851 (Okubo et al., 1994), while those which react with DIDS have not been identified for human Band 3. However, the site-directed mutagenesis results of Garcia and Lodish (1989) have shown that mutation of lysine-539 does not inhibit rapid covalent binding of DIDS. Such results suggest that DIDS and H_2DIDS react with different lysines within the binding site of human Band 3. The DIDS reactive lysine could be the nearby lysine residue at position 542 (Garcia & Lodish, 1989). If so, it seems unlikely that lysines-539 and -542 (which probably lie on the same side of transmembrane helix 5) could have widely different pK_a values.

The reactions of azide with the DIDS/Band 3 noncovalent complex (Figure 6) indicate that both ends of DIDS can react with solute. This in turn suggests that both ends of DIDS are exposed, implying a rather open stilbenedisulfonate binding site. Alternatively, it is possible that reaction of the first isothiocyanate with azide affects the position of the resulting stilbenedisulfonate in its binding pocket, causing the other isothiocyanate to become exposed. A similar argument may be applied to the cross-linking results; i.e., formation of the DIDS/lysine-A adduct exposes the second isothiocyanate. This interpretation would be consistent with earlier suggestions that one end of the stilbenedisulfonate is deeply buried in the protein matrix (Jennings & Passow, 1979; Macara et al., 1983; Rao et al., 1979). Regardless, it is tempting to suggest that both ends of DIDS, in the DIDS/Band 3 noncovalent complex, are exposed and accessible to azide.

Cross-linking of chymotrypsinized Band 3 by H_2DIDS was first described by Jennings and Passow (1979). The process could be separated into two steps: (1) formation of the first covalent adduct, with lysine-539, at pH 7.4; then (2) completion of the cross-link, with lysine-851, by raising the pH to 9.5. They demonstrated that treatment of the initial H_2DIDS/Band 3 covalent adduct at neutral pH with the isothiocyanate-reactive reagent β-mercaptoethanol did not prevent subsequent formation of the cross-link at pH 9.5. From this, they concluded that the remaining isothiocyanate on the H_2DIDS/Band 3 adduct was protected from this reagent. This conclusion was at variance with the observation by Rothstein et al. (1976) that in the DIDS/Band 3 covalent adduct the remaining isothiocyanate was available for reaction with ferritin. In order to rationalize the difference, Jennings and Passow proposed that the structure and reactivity of the Band 3 adduct were different when DIDS was bound in place of H_2DIDS. Further, they questioned whether DIDS could form the cross-link with lysine-851. Kang et al. (1992) demonstrated that DIDS could cross-link chymotrypsinized Band 3 if the pH of the cross-linking step was raised to 13.

Our success at demonstrating the accessibility of the DIDS binding site using azide prompted us to compare this reagent to β-mercaptoethanol as a probe of accessibility in the DIDS-(H_2DIDS)/Band 3 covalent adduct. We found, as did Jennings and Passow (1979), that β-mercaptoethanol could not prevent cross-linking by H_2DIDS but that the smaller azide could (Figure 7). Thus, formation of the H_2DIDS/Band 3 covalent adduct does not block accessibility of the remaining isothiocyanate to azide. We repeated the cross-linking experiments with DIDS, confirming the results of Kang et al. (1992) that DIDS could cross-link chymotrypsinized Band 3. Furthermore, we showed that cross-linking was not prevented by β-mercaptoethanol but was prevented by azide. Thus, accessibility of the second isothiocyanate in the DIDS/Band 3 covalent adduct appears to be equivalent to that in the H_2DIDS/Band 3 covalent adduct. The only significant difference we have found between DIDS and H_2DIDS in the cross-linking process is the necessity of raising the pH from 9.5 to 13 in order to promote the second step of the reaction in the presence of DIDS. This can be attributed to a change in protein structure which allowed lysine-851 access to the unreacted isothiocyanate of DIDS (Kang et al., 1992). A structural change upon exposure of Band 3 to pH 13 is indicated by its altered susceptibility to trypsin (Kang et al., 1992).

In conclusion, our results support the existence of separate but allosterically linked transport and stilbenedisulfonate binding sites on Band 3. DIDS is still accessible to solute when bound to the stilbenedisulfonate site, and the ϵ-amino group of lysine-A has an apparently normal pK_a.

REFERENCES

Aranibar, N., Ostermeier, C., Legrum, B., Ruterjans, H., & Passow, H. (1994) *Renal Physiol. Biochem. 17*, 187−189.

Cabantchik, Z. I., & Greger, R. (1992) *Am. J. Physiol. 262*, C803−C827.

Casey, J. R., & Reithmeier, R. A. F. (1991) *J. Biol. Chem. 266*, 15726−15737.

Collins, K. D., & Washabaugh, M. W. (1985) *Q. Rev. Biophys. 18*, 323−422.

Dalmark, M. (1976) *J. Gen. Physiol. 67*, 223−234.

Deuticke, B. (1977) *Rev. Physiol., Biochem. Pharmacol. 78*, 1−97.

Dissing, S., Romano, L., & Passow, H. (1981) *J. Membr. Biol. 62*, 219−229.

Dix, A. J., Verkman, A. S., & Soloman, A. K. (1986) *J. Membr. Biol. 89*, 211−223.

Eisinger, J., Flores, J., & Salhany, J. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 408−412.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606−2617.

Falke, J. J., & Chan, S. I. (1986) *Biochemistry 25*, 7888−7894.

Falke, J. J., Pace, R. J., & Chan, S. I. (1984) *J. Biol. Chem. 259*, 6472−6480.

Fersht, A. (1977) in *Enzyme Structure and Mechanism*, pp 134−137, W. H. Freeman & Co., San Francisco.

Frolich, O. (1982) *J. Membr. Biol. 65*, 111−123.

Galanter, W. L., & Labotka, R. J. (1991) *Biochim. Biophys. Acta 1079*, 146−151.

Garcia, A. M., & Lodish, H. F. (1989) *J. Biol. Chem. 264*, 19607−19613.

Gasbjerg, P. K., & Brahm, J. (1991) *J. Gen. Physiol. 97*, 321−349.

Glibowicka, M., Winckler, B., Aranibar, N., Schuster, M., Hanssum, H., Ruterjans, H., & Passow, H. (1988) *Biochim. Biophys. Acta 946*, 345−358.

Gunn, R. B. (1978) in *Membrane Transport Processes* (Hoffman, J. F., Ed.) Vol. 1, pp 61−77, Raven Press, New York.

Gunn, R. B., & Frolich, O. (1979) *J. Gen. Physiol. 74*, 351−374.

Hofmeister, F. (1888) *Arch. Exp. Pathol. Pharmakol. 24*, 245−260.

Janas, T., Bjerrum, P. J., Brahm, J., & Wieth, J. O. (1989) *Am. J. Physiol. 257*, C601−C606.

Jennings, M. L. (1982) *J. Biol. Chem. 257*, 7554−7559.

Jennings, M. L. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 397−430.

Jennings, M. L., & Passow, H. (1979) *Biochim. Biophys. Acta 554*, 498−519.

Jennings, M. L., & Adams, M. F. (1981) *Biochemistry 20*, 7118−7123.

Kampmann, L., Lepke, S., Fasold, H. J., Fritzsch, G., & Passow, H. (1982) *J. Membr. Biol. 70*, 199−216.

Kang, D., Okubo, K., Hamasaki, N., Kuroda, N., & Shimaki, H. (1992) *J. Biol. Chem. 267*, 19211−19217.

Kaplan, J. H., Scorah, K., Fasold, H., & Passow, H. (1976) *FEBS Lett. 62*, 182−185.

Kaufmann, E., Eberl, G., & Schnell, K. F. (1986) *J. Membr. Biol. 91*, 129−146.

Kawano, Y., Okubo, K., Tokunaga, F., Miyata, T., Iwanaga, S., & Hamasaki, N. (1988) *J. Biol. Chem. 263*, 8232−8238.

Kietz, D., Bartel, D., Lepke, S., & Passow, H. (1991) *Biochim. Biophys. Acta 1064*, 81−88.

Knauf, P. A., Ries, E. A., Romanow, L. A., Bahar, S., & Szekeres, E. S. (1993) *Biophys. J. 64*, A307.

Koshland, D. E., Jr. (1970) in *The Enzymes (Student edition)* (Boyer, P. D., Ed.) Vol. 1, pp 342−397, Academic Press, New York.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Macara, I. G., Kuo, S., & Cantley, L. C. (1983) *J. Biol. Chem. 258*, 1785−1792.

Oikawa, K., Lieberman, D. M., & Reithmeier, R. A. F. (1985) *Biochemistry 24*, 2843−2848.

Okubo, K., Kang, D., Hamasaki, N., & Jennings, M. I. (1994) *J. Biol. Chem. 269*, 1918−1926.

Passow, H. (1986) *Rev. Physiol. Biochem. Pharmacol. 103*, 62−203.

Passow, H., Lepke, S., & Wood, P. G. (1992) *Prog. Cell Res. 2*, 85−98.

Rao, A., Martin, P., Reithmeier, R. A. F., & Cantley, L. C. (1979) *Biochemistry 18*, 4505−4516.

Robinson, D. R., & Jencks, W. P. (1965) *J. Am. Chem. Soc. 87*, 2470−2479.

Rothstein, A., Cabantchik, Z. I., & Knauf, P. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 3−10.

Salhany, J. M. (1990) in *Erythrocyte Band 3 Protein*, CRC Press, Boca Raton.

Salhany, J. M., Cordes, K. A., & Schopfer, L. M. (1993) *Biochemistry 32*, 7413−7420.

Salhany, J. M., Sloan, R. L., Cordes, K. A., & Schopfer, L. M. (1994) *Biochemistry 33*, 11909−11916.

Salhany, J. M., Sloan, R. L., Cordes, K. A., & Schopfer, L. M. (1995) *Biophys. J. 68*, A444.

Schnell, K. F., Besl, E., & Mosel, R. V. (1981) *J. Membr. Biol. 61*, 173−192.

Schnell, K. F., Elbe, W., Kasbauer, J., & Kaufmann, E. (1983) *Biochim. Biophys. Acta 732*, 266−275.

Schopfer, L. M., & Salhany, J. M. (1992) *Biochemistry 31*, 12610−12617.

Ship, S., Shami, Y., Breuer, W., & Rothstein, A. (1977) *J. Membr. Biol. 33*, 311−323.

Stadler, F., & Schnell, K. F. (1990) *J. Membr. Biol. 118*, 19−47.

Steck, T. L., Weinstein, R. S., Straus, J. H., & Wallach, D. F. H. (1970) *Science 168*, 255−257.

Van Dort, H. M., Low, P. S., Cordes, K. A., Schopfer, L. M., & Salhany, J. M. (1994) *J. Biol. Chem. 269*, 59−61.

Voet, A. (1937) *Chem. Rev. 20*, 169−179.

Wood, P. G., Muller, H., Sovak, M., & Passow, H. (1992) *J. Membr. Biol. 127*, 139−148.

BI950097F