Kinetic Evidence for Ternary Complex Formation and Allosteric Interactions in Chloride and Stilbenedisulfonate Binding to Band 3†

James M. Salhany,* Renee L. Sloan, Karen A. Cordes, and Lawrence M. Schopfer

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

Received March 14, 1994; Revised Manuscript Received July 27, 1994®

ABSTRACT: The molecular basis for chloride and stilbenedisulfonate interaction with band 3 was investigated by measuring the kinetics of stilbenedisulfonate release from its complex with the transporter. We found that 150 mM NaCl accelerated the rate of release of DBDS (4,4′-dibenzamidostilbene-2,2′-dibenzamidostilbene-2,2′-disulfonate) and H_2DIDS (4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate) by more than 10-fold at constant ionic strength. The accleration effect saturated as a function of chloride concentration. This is an indication of specific binding within a ternary complex involving stilbenedisulfonate, chloride, and band 3. To see if stilbenedisulfonates block an access channel to the transport site, we studied the effect of rapidly mixing DBDS-saturated resealed ghosts with chloride at constant ionic strength and osmotic pressure. Once again, we observe a large, uniform acceleration in the rate of DBDS release. These findings are not consistent with molecular models where stilbenedisulfonates are proposed to block access to a deeper transport site. We suggest that the intramonomeric stilbenedisulfonate site is not located on the chloride transport pathway but rather interacts with the transport site though heterotropic allosteric site–site interactions. On the basis of our kinetic evidence for ternary complex formation and on transport inhibition evidence in the literature showing a linear dependence of $K_{I\text{-}app}$ on substrate, we suggest that stilbenedisulfonates are linear mixed-type inhibitors of band 3 anion exchange, not pure competitive inhibitors as has been assumed on the basis of analysis of transport inhibition data alone.

Stilbenedisulfonates are potent inhibitors of chloride/bicarbonate exchange, which is a transport function found in several types of biological membranes (Cabantchik & Greger, 1992). This function has been assigned to the anion exchange (AE) family of transporter proteins (Kopito, 1990), of which erythrocyte band 3 (AE1) is the best characterized example (Passow, 1986; Jennings, 1989; Salhany, 1990). Stilbenedisulfonates apparently behave as competitive inhibitors of band 3 anion exchange (Shami et al., 1978; Barzilay & Cabantchik, 1979; Restrepo et al., 1991) despite the fact that they are not substrates of the system and do not inhibit transport from the inner surface of the cell (Knauf, 1979; Passow, 1986).

The molecular basis for apparent competitive inhibition by stilbenedisulfonates is unclear. A review of the available literature yields studies which both support and refute a simple competition model (Figure 1). For example, chloride causes the apparent dissociation constant (K_d) for all the stilbenedisulfonates to increase. Linear relationships between chloride concentration and K_d have been found for DNDS[1] (Frohlich, 1982; Falke & Chan, 1986) and for a spin-labeled stilbenedisulfonate (Schnell et al., 1983). Such a linear dependence supports a direct competition model. In contrast, DBDS showed a hyperbolic dependence of K_d *versus* chloride when studied over a wider chloride concentration range (Dix et al., 1986). Saturation behavior suggests that DBDS and chloride form a ternary complex.

Recent site-directed mutagenesis studies have been interpreted to indicate that the stilbenedisulfonate site and the transport site are not mutually exclusive (Wood et al., 1992; Passow et al., 1992). Two lysine residues were investigated: Lys 593 and Lys 851 (human sequence numbering). Both of these lysines bind PLP (Passow et al., 1992) and offer covalent binding sites for the isothiocyano groups of H_2DIDS (Okubo et al., 1994). In agreement with our earlier work (Salhany et al., 1987), PLP labeling of either lysine was shown to involve functionally equivalent sites with respect to transport inhibition (Passow et al., 1992). The double mutant did not bind PLP covalently, did not form a covalent adduct with H_2DIDS, and showed a 200-fold lower affinity for the reversible binding of DNDS, all with no significant effect on K_m for chloride transport. These results indicate that the stilbenedisulfonate site is not coincident with the chloride binding transport site. It has been suggested that the stilbenedisulfonate binding site either straddles the entrance to an access channel leading to the transport site (Figure 1; Passow et al., 1992; Okubo et al., 1994), or is not part of the transport pathway but interacts allosterically (Figure 1; Salhany & Gaines, 1981; Zaki, 1992; Passow et al., 1992).

One classical means to discriminate between simple competitive and ternary complex binding models is to perform quantitative kinetic measurements of inhibitor binding and release in the absence and presence of substrate. Simple predictions can be tested such as whether inhibitor binding follows one-step or multi-step kinetics and whether addition of substrate slows or accelerates inhibitor binding. Of particular interest is whether addition of substrate accelerates the rate of inhibitor release. We have recently introduced a

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

* To whom correspondence should be sent at the Department of Internal Medicine, University of Nebraska Medical Center, 600 S. 42nd St., Omaha, NE 68198-5290.

® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulfonate; $C_{12}E_8$, poly(oxyethylene-8-lauryl ether); 5P(8), 5 mM sodium phosphate, pH 8.0; PBS, phosphate-buffered saline (150 mM sodium chloride and 5 mM sodium phosphate, pH 8.0); PLP, pyridoxal 5′-phosphate.

0006-2960/94/0433-11909$04.50/0

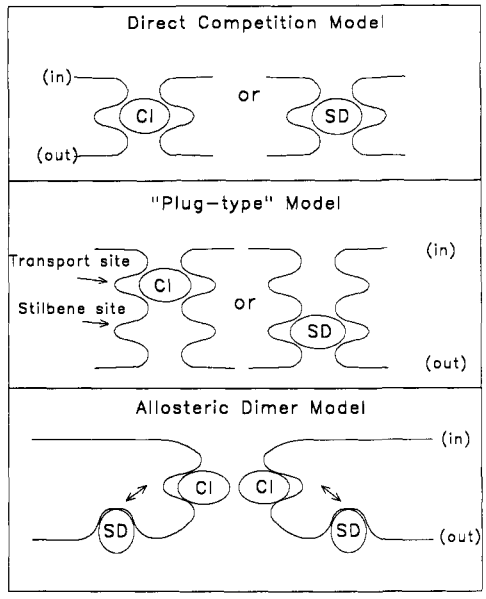


FIGURE 1: Models for competitive inhibition of chloride (Cl) transport by stilbenedisulfonates (SD). Direct competition model: either chloride or stilbenedisulfonate can occupy the transport site on one band 3 monomer. Chloride cannot accelerate the rate of stilbenedisulfonate release in this model. "Plug-type" model: Chloride and stilbenedisulfonate have separate sites within a transport channel in a band 3 monomer that may or may not interact. When stilbenedisulfonate is bound, chloride can still enter its site, but only from the cytoplasmic surface of the membrane. In a sealed system, extracellular chloride cannot reach the transport site when stilbenedisulfonate is bound. Allosteric dimer model: The stilbenedisulfonate site is not on the transport pathway. Rather, sites interact allosterically. In this model, extracellular chloride has access to its binding site even though the stilbenedisulfonate site is occupied. Thus, an allosterically interacting ternary complex can form. It should be noted that although the stoichiometry of covalent stilbenedisulfonate binding has been established as 1:1 with the band 3 monomer (Jennings & Passow, 1979), there is no direct evidence on the stoichiometry of chloride binding to isolated band 3.

stopped-flow fluorescence method to measure the rate of stilbenedisulfonate release from its complex with band 3 (Salhany et al., 1993). In the present paper, we apply this method to test the effect of chloride on that rate. We find that chloride uniformly accelerates the rate of stilbenedisulfonate release from its complex with band 3. The acceleration was observed using isolated band 3, band 3 in unsealed ghosts, and also using band 3 in resealed ghosts where exofacial chloride was introduced in a rapid mixing experiment. Such acceleration in the rate of stilbenedisulfonate release by substrate anions does not support a direct competition model. Rather, it suggests that stilbenedisulfonates, chloride, and band 3 form a ternary complex involving separate but allosterically interacting substrate and inhibitor binding sites. Furthermore, the work with resealed ghosts suggests that stilbenedisulfonates do not block access to the transport site but rather inhibit transport through heterotropic, allosteric site–site interactions as suggested earlier (Salhany & Gaines, 1981; Zaki, 1992; Passow et al., 1992).

EXPERIMENTAL PROCEDURES

Preparations

Buffers. Several different buffers were used in this study, depending on the particular type of sample employed. For the isolated band 3 experiments we have buffer A (150 mM sodium chloride, 5 mM sodium phosphate, 25 μM phosphatidylcholine, and 0.01% $C_{12}E_8$, pH 7.4) and buffer B (23 mM sodium citrate, 5 mM sodium phosphate, 25 μM phosphatidylcholine, and 0.01% $C_{12}E_8$, pH 7.4.). For the unsealed ghosts experiments we have buffer C (150 mM sodium chloride and 5 mM sodium phosphate, pH 7.4) and buffer D (23 mM sodium citrate and 5 mM sodium phosphate, pH 7.4). For the resealed ghosts experiments we have buffer E (150 mM sodium chloride, 5 mM sodium phosphate, and 1 mM $MgSO_4$, pH 7.4) and buffer F (23 mM sodium citrate, 5 mM sodium phosphate, 1 mM $MgSO_4$, and 200 mM sucrose, pH 7.4). For studies on the variations of chloride using unsealed ghosts, buffers C and D were mixed in various ratios to maintain constant ionic strength.

Stilbenedisulfonates. DBDS was synthesized by the method of Kotaki et al. (1971). The criterion for sample purity was observation of a single spot on thin-layer chromatography. H_2DIDS and DIDS were obtained from Molecular Probes (Eugene, OR) and Aldrich (Milwaukee, WI). Stilbenedisulfonate concentrations were determined spectrophotometrically using the following extinctions coefficients: DBDS, 50 000 M^{-1} cm^{-1} at 336 nm (Rao et al., 1979); DIDS, 36 000 M^{-1} cm^{-1} at 340 nm (Eisinger et al., 1982); and H_2DIDS, 39 000 M^{-1} cm^{-1} at 286 nm (Haugland, 1989).

Unsealed Ghosts. Unsealed ghosts were prepared from day-old, underweight units of human whole blood donated by the Omaha chapter of the American Red Cross. Cells were washed in cold PBS and lysed 1:30 in 5P(8). Unsealed ghosts were stripped of cytosolic associated proteins such as hemoglobin and glyeraldehyde-3-phosphate dehydrogenase as described previously (Salhany et al., 1980). The concentration of band 3 in the ghost preparation was determined by substoichiometric titration with DIDS (Schopfer & Salhany, 1992; Van Dort et al., 1994). Ghosts were then washed either in buffer C or D or in various ratios of those buffers to vary chloride while maintaining constant ionic strength.

Mg^{2+}-Resealed Ghosts. Red cells were washed three times in PBS and centrifuged. The packed cells were diluted 1:10 in 5P(8) containing 1 mM $MgSO_4$ and then incubated for 0.5 h at 37 °C. The resealed ghosts were then washed three times in 5P(8) containing 1 mM $MgSO_4$ and twice in buffer F (citrate/sucrose). This method is known to cause rapid lysis and immediate resealing to yield an intracellular content which is proportional to the dilution ratio used (Steck, 1974; Lieber & Steck, 1982a,b). A test for sealing to anions is discussed below.

Isolated Band 3. Band 3 was isolated as described previously (Casey & Reithmeier, 1991; Schopfer & Salhany, 1992). Protein concentration was calculated using a newly determined extinction coefficient for absorbance at 280 nm of 93 000 M^{-1} cm^{-1}. This extinction coefficient was determined by two methods. In the first method, a value of 92 600 M^{-1} cm^{-1} was calculated from the tryptophan, tyrosine, and cystine content of band 3 (Tanner et al., 1988) using model extinction coefficients for these amino acids, as described by Gill and von Hippel (1989) and Perkins (1986). As a control for local environmental influences which might alter the extinction coefficients for these residues in band 3, the absorbance of band 3 in 6 M guanidine hydrochloride was compared to that in buffer B. No difference was seen, even after 5.5 h of incubation, supporting the validity of the calculated extinction coefficient. In the second method, the concentration of tyrosine in a band 3 solution was determined from the change in absorbance which occurred upon ionization to form tyrosinate (Edelhoch, 1967). This concentration together with the tyrosine content of band 3 and the 280-nm absorbance of the starting solution gave an extinction value of 93 500 M^{-1} cm^{-1}.

Isolated band 3 was studied either in buffer A or buffer B. Chromatography on a 1- × 30-cm Sephadex G-25 column (Pharmacia) was used to remove chloride. Confirmation of separation of protein from chloride was made by comparing the A_{280} profile with the freezing point depression profile measured with an osmometer.

Analytical Methods

Stopped-Flow Measurements of DBDS Binding. Experimental details follow the general methods published earlier (Salhany et al., 1993).

Stopped-Flow Measurements of the Kinetics of Stilbenedisulfonate Release from Band 3. Stopped-flow fluorescence methods for stilbenedisulfonate replacement reactions on band 3 were introduced previously (Salhany et al., 1993). In the present study, we measure the rate of DBDS release from its complex with band 3 by mixing with excess DIDS. The rate of release was followed by exciting the sample at 335 nm and measuring emission through a 415-nm cutoff filter.

We induced H_2DIDS release in an analogous fashion and measured the release rates by following changes in protein fluorescence quenching [see Salhany et al. (1993)] by exciting the sample at 280 nm and observing the emission through a 315-nm cutoff filter (Durrum 315 SW-CO). It was necessary to mix ghosts with H_2DIDS just prior to the initiation of the reaction to prevent significant formation of the covalent adduct and loss of signal. Fortunately, the rate of H_2DIDS covalent adduct formation is quite slow (0.02 min^{-1} at pH 7.4; Salhany et al., 1993) and poses no significant problem in our experiments.

Dithionite Transport. Dithionite transport measurements were performed as described previously (Salhany & Swanson, 1978; Salhany & Gaines, 1981; Salhany & Cordes, 1992).

Data Analysis. Data were analyzed using either Enzfitter (Leatherbarrow, 1987) or Sigma Plot (Jandel Scientific, San Rafael, CA).

RESULTS

(1) Preliminary Considerations and Control Experiments: (a) Relationship between DBDS and DIDS Binding Sites on Isolated Band 3. It is generally assumed that all members of a series of inhibitors bind to the same site on a given protein. We used isolated band 3 to test this assumption for the binding of DBDS and DIDS. In the first experiment, isolated band 3 was reacted for 30 min with a 1.6-fold excess of DIDS in chloride-free buffer B, resulting in 99% covalent labeling of the band 3 monomer population based on a previously described assay (Schopfer & Salhany, 1992). Subsequent DBDS binding to band 3 was eliminated by this covalent binding of DIDS (Figure 2A). In the second experiment, band 3 was labeled with various substoichiometric amounts of DIDS. The amount of subsequent DBDS binding was determined from the relative amplitude of the fluorescence change which accompanies formation of the band 3 DBDS complex (Figure 2A). The relationship between the loss in signal due to DBDS–band 3 complex formation and the fraction of DIDS bound to the band 3 monomer population is linear (Figure 2B). Since DIDS binds to band 3 with a 1:1 monomer stoichiometry (Jennings & Passow, 1979), the results in Figure 2B would be most consistent with the view that DBDS also binds to band 3 with a 1:1 monomer stoichiometry.

(b) Control Experiments for Stilbenedisulfonate Release Rate Measurements. In contrast to DBDS (Figure 2A), the reversible binding of DIDS does not produce a change in fluorescence when the sample is excited at 335 nm (unpublished observations). Thus, replacement of DBDS by DIDS results in a decrease in fluorescence intensity for sample excitation at 335 nm with emission > 415 nm. However, once DIDS is bound to band 3, there is an increase in fluorescence intensity at these wavelengths due to formation of the covalent adduct with band 3 (Schopfer & Salhany, 1992). The adduct formation rate in citrate (k = 0.01 s^{-1} at 25 °C) is about 5-fold slower than the rate of DBDS release in citrate (k = 0.05 s^{-1}). In 150 mM sodium chloride, the DBDS release rate increases ~17-fold (see below) while the adduct formation rate increases only about 2-fold. Thus, the baseline for the DBDS/DIDS replacement reaction is well determined in our experiments. All release rate measurements show excellent exponential behavior to within the last 10% of the reaction.

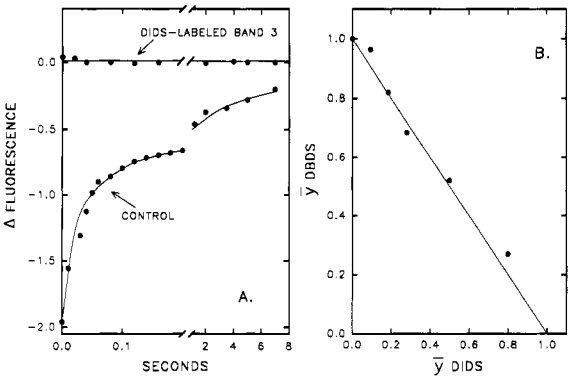


FIGURE 2: Fluorescence change associated with DBDS binding to control and DIDS-labeled isolated band 3. Band 3 was isolated and chloride was removed as described in Experimental Procedures. The protein was in buffer B (citrate). (A) Time course of the reactions. The reactions were initiated in the stopped-flow spectrometer by mixing 5 μM band 3 or DIDS/band 3 covalent adduct with 5 μM DBDS at 23 °C. The excitation wavelength was 335 nm and the reaction was followed through a 415-nm cutoff filter. (B) Plot of the fractional change in the amplitude of the fluorescence associated with DBDS binding ($\bar{y}$ DBDS = $\Delta F_{obs}/\Delta F_0$) measured at constant DBDS concentration (2.2 μM after mixing) versus the fraction of DIDS bound to the band 3 monomer population ($\bar{y}$ DIDS). The concentration of band 3 was 1.3 μM after mixing. The fractional saturation of the band 3 monomer population with DIDS was determined as described by Van Dort et al. (1994).

Several important control experiments were performed to prove that the rate-limiting step in our experiments is DBDS release. (1) We studied the dependence of the reactions on the concentrations of DIDS, using 10 μM DBDS at 25 °C (Figure 3A). According to theory (Gibson & Roughton, 1955; Antonini & Brunori, 1971), a double-reciprocal plot of the observed DBDS release rate *versus* DIDS concentration should give a straight line whose y-intercept is the reciprocal of the release rate constant. Varying the DIDS concentration from 1.4 to 5.7 μM gave such a plot (Figure 3A), from which a limiting rate of 0.050 s^{-1} was determined. Thus, the kinetics of our reaction are consistent with a process in which released DBDS is completely replaced by DIDS. (2) The measured rate constant for the release of DBDS in chloride-free buffer at 23 °C was 0.047 s^{-1} (Figure 4A). We found that in the presence of 3 μM DBDS neither the rate constant nor the extent of the reaction varied significantly for DIDS between 3 and 6.5 μM, either in the absence or presence of chloride. Under these conditions the release of DBDS from its complex with band 3 is always rate-limiting. Therefore, we have chosen to use 3 μM DBDS and 6.5 μM DIDS in most of the following experiments.

To obtain an independent confirmation for the effect of chloride on the DBDS release reaction, we studied the H_2-DIDS/DIDS replacement reaction (Salhany et al., 1993) in

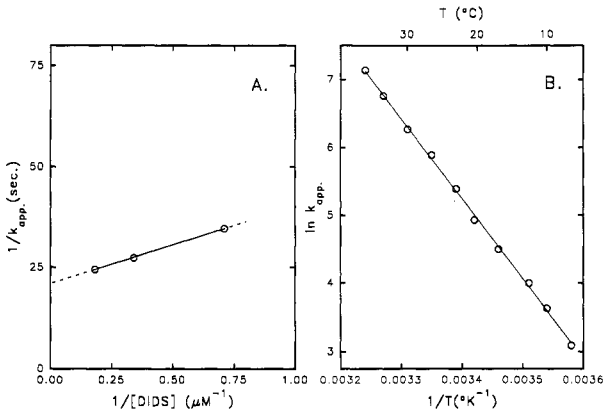


FIGURE 3: Effect of DIDS concentration and temperature on the rate of DBDS release from its complex with band 3 in unsealed ghosts (see Figure 4 for the primary time course data). Unsealed ghosts were prepared in citrate (buffer D) as described in Experimental Procedures. (A) Double-reciprocal plot of k_{obs} versus DIDS concentration for the DBDS/DIDS replacement reaction. Reactions were performed at constant DBDS (10 μM) and varying DIDS (1.4–5.7 μM) concentrations. Temperature was 25 °C. The y-axis intercept yields a value for the rate constant of 0.05 s^{-1} (Antonini & Brunori, 1971). (B) Temperature dependence of the rate constant for DBDS release (k_{app}) expressed in units of hours^{-1}. The reactions were performed in chloride-free buffer (buffer D) using unsealed ghosts, 2 μM DBDS, and 5.6 μM DIDS. Doubling the DIDS concentration had no effect on the measured rate at either 6.3 or 25.5 °C. A linear Arrhenius plot is observed with an activation energy of 22 kcal/mol.

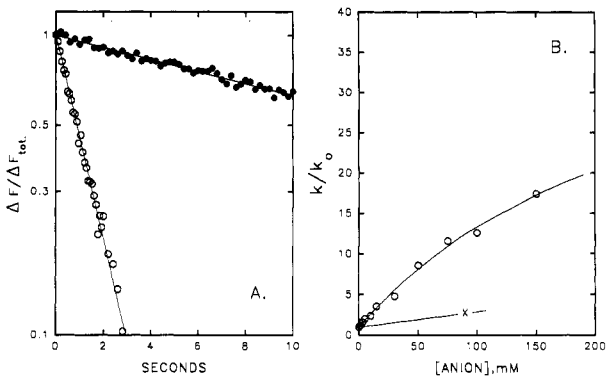


FIGURE 4: Effect of chloride on the kinetics of DBDS release from band 3 in unsealed ghosts. (A) Time course of the DBDS/DIDS replacement reaction measured in the absence (filled circles) and presence (open circles) of chloride. Ghosts were washed in either buffer C or D as described in the text and diluted to yield a concentration of band 3 of about 1.8 μM with 6 μM DBDS present. These samples were then mixed in the stopped-flow with 13 μM DIDS in the same respective buffers to initiate the reaction. Replacement of DBDS by DIDS leads to a reduction in the fluorescence when measured by excitation at 335 nm. The temperature was 23 °C. DBDS release follows monophasic kinetics in both buffer systems. The lines drawn for each condition were based on a fit to the equation $y = \exp(-kt)$; the value of k for citrate (closed circles) is 0.047 s^{-1}, while that for chloride (open circles) is 0.79 s^{-1}. (B) Chloride concentration dependence of the kinetics of DBDS release from band 3 in unsealed ghosts. The ratio (k/k_0) is for the observed rate constant of the DBDS/DIDS replacement reaction at various chloride concentrations (k) and the rate constant at zero chloride (k_0). This ratio is plotted versus chloride concentration at constant ionic strength. Ionic strength was maintained constant by substituting sodium citrate for sodium chloride based on a calculated ionic strength equal to 150 mM sodium chloride. All buffers contained 5 mM sodium phosphate and the pH was 7.4. Unsealed ghosts were prepared as described in Experimental Procedures. Ghosts were split into 2-mL samples and washed once in each respective chloride/citrate buffer. Band 3 and stilbenedisulfonate concentrations were as in panel A. The reactions were performed at 23 °C as described above. The data could be best fit by a hyperbolic equation of the form $y = [a(Cl)/K_{app} + (Cl)] + 1$, where $a = 45 \pm 7$ and $K_{app} = 266 \pm 59$ mM. The point (×) corresponds to the relative DBDS release rate measured in 90 mM sodium sulfate and 5 mM sodium phosphate, pH 6, under conditions otherwise identical with those described above. The rate constant was about 3-fold larger than the zero sulfate condition.

unsealed ghosts. Protein fluorescence quenching was used to follow this reaction (excitation 280 nm, emission > 315 nm through a cutoff filter). Using these excitation and emission wavelengths, reversible binding of DIDS quenches band 3 fluorescence to a greater extent than does reversible binding of H_2DIDS [see Salhany et al. (1993)]. There is no significant additional spectral change associated with formation of the DIDS covalent adduct. Thus, replacement of H_2DIDS by DIDS is associated with a decrease in protein fluorescence.

As an additional control, we studied the temperature dependence of the DBDS release rate. The results shown in Figure 3B indicate that the DBDS release rate is strongly temperature-dependent with an activation energy of 22 kcal/mol.

(2) Effect of Preequilibration with Chloride on the Rate of Stilbenedisulfonate Release from Band 3 in Unsealed Ghosts and from Isolated Band 3. There is a large acceleration in the rate of DBDS release from band 3 in unsealed ghosts when chloride is present (Figure 4A; chloride, open circles; citrate, closed circles). To assess the nature of the chloride dependence, unsealed ghosts were washed in buffers containing various ratios of chloride and citrate at constant ionic strength and pH. These ghosts (which contain 6 μM DBDS) were then mixed in the stopped-flow spectrometer with 13 μM DIDS in the same respective buffers. Single-exponential decay processes were observed at all chloride concentrations. Figure 4B shows a plot of the ratio of k_{obs}/k_0 *versus* chloride concentration which reveals saturation behavior. The line drawn through the data is based on a fit to a simple hyperbolic function (see legend to Figure 4), giving an apparent half-effect constant of 266 mM. The curve saturates at a maximum ratio of 45.

Evidence supporting the specific nature of the chloride effect is shown in Figure 4B, where sulfate was tested for its effect on the kinetics. At pH 6, where sulfate binding is expected to be maximal (Kaufmann et al., 1986), we observed only a 2.7-fold increase in the rate of DBDS release from unsealed ghosts in 90 mM sulfate (Figure 4B). In contrast, 90 mM chloride accelerates the release rate by ~12-fold, fully 4-fold greater than 90 mM sulfate. Furthermore, addition of 150 mM citrate (pH 7.4) to ghosts caused only a 3-fold increase in the DBDS off rate compared to 5 mM phosphate, pH 7.4 (data not shown), while 150 mM chloride increases the rate 17-fold (Figure 4B). These results show that even the excessively high ionic strength experienced by ghosts in 150 mM citrate cannot reproduce the very large acceleration experienced when chloride is present. The results with citrate support the findings of Hautmann and Schnell (1985), who saw no effect of citrate on chloride transport between 0 and 40 mM (within the range of our replacement experiments in Figure 4B). There is a very slight inhibition of chloride transport by citrate, but this effect occurs only at very high citrate concentrations (K_i = 125 mM) (Hautmann & Schnell, 1985), well out of the range of our experiments.

It was important to establish the generality of the observed acceleration in the rate of DBDS release from band 3 by investigating whether chloride could also accelerate the release of the smaller stilbenedisulfonate analogue, H_2DIDS. The H_2DIDS/DIDS replacement reaction for band 3 in unsealed ghosts showed exponential kinetics and was uniformly ac-

celerated by preequilibrating the ghosts in buffer C. The kinetic traces look like those in Figure 4A and are therefore not shown. H_2DIDS release was accelerated 11.5-fold by 150 mM NaCl *versus* a 17.5-fold acceleration for DBDS (Figure 4A). The absolute values for the H_2DIDS release rate constants (k_0 = 0.02 s^{-1} in buffer D *versus* k = 0.23 s^{-1} in buffer C, T = 23 °C) were significantly slower than for DBDS (Figure 4A).

Because unsealed ghosts are a heterogeneous system with the potential for nonspecific stilbenedisulfonate and chloride binding sites, it seemed crucial to show that the effect of chloride on the kinetics of DBDS release was specific for band 3. To accomplish this, we prepared isolated band 3 (Schopfer & Salhany, 1992) and measured DBDS release rates in the absence and presence of chloride at constant ionic strength. Once again, kinetic time courses looked like those in Figure 4A and are therefore not shown. While the absolute values of the rate constants were significantly larger for isolated band 3 (k_0 = 0.3 s^{-1} in buffer B *versus* k = 3.3 s^{-1} in buffer A, T = 23 °C) than for band 3 in unsealed ghosts (Figure 4A), the size of the chloride-induced acceleration was still about 10-fold. Thus, it is clear that the acceleration due to chloride seen in band 3 in unsealed ghosts is reproduced using the isolated transporter. It is worth pointing out here that the H_2DIDS release rate constant for isolated band 3 was also about 3-fold larger [see Salhany et al. (1993)] than the H_2-DIDS release constant for band 3 in unsealed ghosts (see above). The specific cause of these slight but significant kinetic differences between isolated and membrane-bound band 3 remains to be determined.

(3) Effect of Rapid Mixing with Chloride on the DBDS Release Kinetics from Unsealed and Mg^{2+}-Resealed Ghosts. To discriminate between "plug-type" and other allosteric mechanisms (Figure 1), it is necessary to use resealed ghosts which are sealed to simple anion diffusion (see Discussion). It was necessary to first perform these experiments with unsealed ghosts to show that rapid mixing with chloride in the stopped-flow spectrometer gave the same acceleration in DBDS release as was seen with preequilibrating unsealed ghosts in chloride buffer. Mixing chloride-free, DBDS-saturated unsealed ghosts with 150 mM chloride buffer containing 13 μM DIDS rapidly accelerated the rate of DBDS release (Figure 5A). The rate constant increased about 8-fold for a final chloride concentration of 75 mM. This agrees reasonably well with the results in Figure 4B, where unsealed ghosts were preequilibrated with DBDS and chloride and then mixed with DIDS. Mixing resealed ghosts suspended in buffer F (citrate/sucrose + 1 mM $MgSO_4$) with the ionically and osmotically equivalent buffer E (chloride plus 1 mM $MgSO_4$) also accelerated the rate of DBDS release from its complex with band 3 about 8-fold (Figure 5B). It should be noted that there was no evidence either for lags in the acceleration process or for biphasic kinetics when resealed ghosts were used. The time courses were purely exponential to >90% of the reaction.

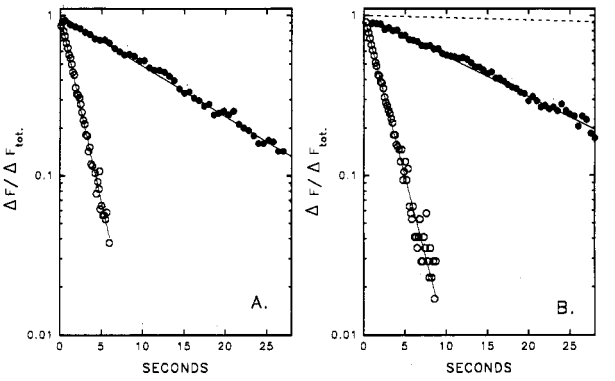


FIGURE 5: Effect on the kinetics of DBDS release of rapidly mixing chloride-free unsealed (A) and resealed (B) ghosts with chloride. (A) Unsealed ghosts were prepared in buffer D (citrate) as described in Experimental Procedures. DBDS was added to give a before-mixing concentration of 6 μM, with ghosts at a concentration equivalent to 1.5 μM band 3. This suspension was mixed in the stopped-flow spectrometer either with chloride-free buffer D containing 13 μM DIDS (closed circles) or with isoionic buffer C (chloride) containing chloride and 13 μM DIDS (open circles). The temperature was 23 °C. The release rate constant for unsealed ghosts in buffer D (A, closed circles) was k = 0.06 s^{-1}. The value increased to k = 0.5 s^{-1} after mixing to a final chloride concentration of 75 mM. (B) Magnesium-resealed ghosts were prepared in chloride-free buffer F as described in Experimental Procedures. Buffer F is both isoionic and isoosmotic with 150 mM sodium chloride. The before-mixing concentration of ghosts was about equivalent to 0.4 μM band 3, with DBDS at 6 μM. This suspension was mixed in the stopped-flow spectrometer with 13 μM DIDS in either the same buffer (buffer F, citrate/sucrose/Mg^{2+}, closed circles) or buffer E (chloride plus 1 mM $MgSO_4$, open circles). The rate constant in the absence of chloride was k = 0.05 s^{-1}, while with chloride present k = 0.43 s^{-1}. See the Discussion section of the text for the significance of the dashed line in panel B.

DISCUSSION

The results of this paper suggest (a) that stilbenedisulfonates and chloride can be bound simultaneously to band 3 to form a ternary complex, (b) that the intramonomeric stilbenedisulfonate site is not on the pathway of approach to the transport site for extracellular chloride, and (c) that allosteric interactions occur within the ternary complex to account for the large acceleration in the rate of stilbenedisulfonate release caused by chloride binding (Figures 4 and 5).

The primary observation indicating formation of a ternary complex between band 3, chloride, and stilbenedisulfonates is the chloride-dependent acceleration in stilbenedisulfonate release at constant ionic strength (Figure 4A). Formation of a ternary complex predicts saturation of the apparent release rate as a function of chloride concentration, and saturation does occur (Figure 4 B). The half-effect constant for the chloride dependence was 266 ± 59 mM. We have found that 150 mM sodium chloride lowers DBDS affinity about 4-fold (unpublished observations). Using the principle of microscopic reversibility and taking the overall K_d for chloride binding to be 80 ± 30 mM (Falke et al., 1984), one would predict that the apparent half-effect constant should be about 320 ± 120 mM. This value agrees with our measured half-effect constant to within the errors of the respective determinations. Falke et al. (1984) indicated that the K_d value for chloride binding determined by NMR line broadening agrees with the K_m for chloride transport determined in the equilibrium exchange experiment. These comparisons support the view that the chloride binding site responsible for the acceleration of stilbenedisulfonate release from band 3 is the transport site. The acceleration probably does not involve chloride binding to the site responsible for partial substrate inhibition of band 3 anion exchange (Cass & Dalmark, 1973; Gasbjerg & Brahm, 1991). The chloride self-inhibition site is located on the inner surface of the membrane (Knauf & Mann, 1986), while acceleration can be produced by mixing DBDS-saturated resealed ghosts with chloride (Figure 5B). Thus, acceleration in DBDS release is seen even when the substrate inhibition site is inaccessible to chloride.

The previous impression from chloride NMR line-broadening studies (Falke & Chan, 1986), from work with spin-labeled stilbenedisulfonates (Schnell et al., 1983), and from work on the direct binding of stilbenedisulfonates to red cells (Frohlich, 1982) was that chloride and stilbenedisulfonates compete for

the same binding site on band 3. In all three studies, this conclusion was made based on observation of a linear dependence for K_d on chloride concentration. Our demonstration of ternary complex formation using DBDS agrees with the equilibrium binding results of Dix et al. (1986) showing saturation behavior for the dependence of the DBDS K_d on chloride concentration. The experiments of Dix et al. (1986) covered a wider chloride concentration range (0–600 mM) than was used in the above studies of Falke and Chan (1986) (0–400 mM) and Frohlich (1982) (0–90 mM) using DNDS. Thus, it seems likely that the impression of competitive binding is a consequence of the limited chloride concentration range used in most studies. Finally, it is also apparent from our work with H_2DIDS that chloride-induced acceleration in inhibitor release, and thus ternary complex formation, is not unique to the use of DBDS.

Ternary complex formation between band 3, chloride, and stilbenedisulfonates finds support from certain NMR results in the literature. For example, neither DNDS reversible binding nor covalent binding of DIDS completely inhibits chloride NMR line-broadening (Falke et al., 1984; Falke & Chan, 1986; Shami et al., 1977). Shami et al. (1977) used solubilized band 3 in their NMR experiments, making it unlikely that non-band 3 sites on the red cell membrane were involved. Furthermore, Falke and Chan (1986) found that covalent binding of DIDS did not block chloride binding to all of the band 3 sites. Rather, it lowered the chloride affinity of those sites. They interpreted this behavior in terms of "partial blockade". We take these results as evidence for ternary complex formation with allosteric site–site interactions to explain the lowered chloride affinity.

Ternary complex formation also finds support in site-directed mutagenesis studies (Wood et al., 1992; Passow et al., 1992) as mentioned in the introduction. To accommodate their results with earlier findings of apparent competitive inhibition of transport (Shami et al., 1978; Barsilay & Cabantchik, 1979), the authors suggest that stilbenedisulfonates may plug an access channel to a deeper transport site (Figure 1; Passow et al., 1992). Our results with resealed ghosts challenge the "plug-type" model.

Rapid mixing of resealed ghosts with chloride accelerated the rate of DBDS release from band 3 in a manner which was virtually identical to the open system. There was no evidence for kinetic heterogeneity after such rapid mixing with chloride. Heterogeneity would be expected if the sample contained a mixture of unsealed ghosts (with accelerated DBDS release) and sealed ghosts (with an unaffected DBDS release rate). In our experiments, resealed ghosts begin the reaction in chloride-free buffer with 6 μM DBDS. This is enough DBDS to fully saturate >99% of the band 3 monomers present (unpublished observations). Thus, chloride could not rapidly enter the cell *via* the band 3 transport system without prior dissociation of DBDS.

While band 3 transport may be blocked by DBDS, alternate chloride transport pathways needs to be considered. For example, it is known that osmotic hemolysis and resealing produces a single diffusion hole in the red cell membrane (Lieber & Steck, 1981a,b). This hole could allow chloride to diffuse into the cell and promote DBDS release by binding to sites at the inner surface of band 3. The rate constant for diffusion through a hole can be determined by using the following equation (Lieber & Steck, 1981a):

$$k = \frac{DA'(V_g^{-1} + V_m^{-1})}{\Delta X} \quad (1)$$

where D is the diffusion coefficient of chloride (148.3×10^{-7} cm^2/s) (*CRC Handbook of Chemistry and Physics*, 1990) V_g is the volume of the ghost (87×10^{-12} cm^3) (Gunn & Frohlich, 1989), and ΔX is the membrane diffusion path (5×10^{-7} cm) (Lieber & Steck, 1982a). Under the conditions of the flux experiments, ghost volume is much smaller than the medium volume (V_m) and the reciprocal of the latter value can be neglected. A' is defined as

$$A' = n\pi(r - a)^2 \quad (2)$$

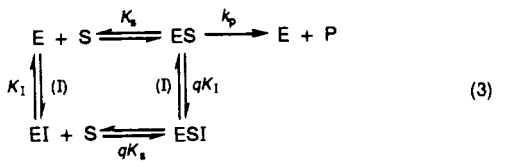
where n = number of holes (n = 1; Lieber & Steck, 1982a), r = radius of the hole (r = 9 Å; see below), and a = radius of chloride, taken from the crystal structure of NaCl to be 1.8 Å (Pauling, 1960). Using these numbers and eqs 1 and 2, we obtained a value of 0.006 s^{-1} for k. This value is about 8-fold slower than the rate of DBDS release in chloride-free buffer (dashed line in Figure 5B). At saturating chloride, the rate for DBDS release will be about 360-fold larger than the rate for chloride diffusion through the single osmotic hole present in the membrane of resealed ghosts.

The above calculation assumes that the hole in our resealed ghost preparation is about 9 Å in radius. According to Lieber and Steck (1982a), the very rapid nature of our lysis and resealing step in 1 mM Mg^{2+}, plus incubation at 37 °C, should yield holes with a radius of 7 Å. To be sure that our resealed ghosts were uniformly sealed to anions, we measured dithionite influx into such Mg^{2+}-resealed ghosts. Influx rates were typical of those which are limited by band 3 anion exchange [data not shown, but seen Salhany and Cordes (1992) for typical time courses]. Labeling all copies of band 3 with DIDS inhibited >98% of the transport activity, showing that dithionite is being transported by band 3 exclusively [also see Van Dort et al. (1991)] and that the ghosts are uniformly sealed to dithionite. Based on the known crystal structure of sodium dithionite ($Na_2S_2O_4$) (Cotton & Wilkinson, 1972), we calculate a Stokes radius for dithionite of about 6 Å. These measurements suggest that the diffusion hole in Mg^{2+}-resealed ghosts cannot be much larger than 6 Å. Thus, using 9 Å for the calculations defines an upper limit for chloride transport by diffusion. On the basis of the above considerations, we conclude that the acceleration in DBDS release caused by rapid mixing of chloride with resealed ghosts is due to ternary complex formation at the outer surface of band 3.

Ternary complex formation involving extracellular chloride and stilbenedisulfonate binding suggests that the intramonomeric stilbenedisulfonate site is not on the pathway for the approach of extracellular chloride to the transport site. Rather, these results suggest that the stilbenedisulfonate site and the transport site are adjacent sites which interact allosterically (Salhany & Gaines, 1981). This distinction between an allosteric model and the "plug-type" model is illustrated in Figure 1. As drawn, it is not possible for extracellular chloride to reach a transport site in the "plug-type" model when the stilbenedisulfonate site is occupied. In the allosteric dimer model, the inhibitor lies off the transport pathway but interacts with the transport site through changes in protein conformational state. Certain aspects of such an allosteric dimer concept are tentatively supported by the recent electron microscopic work on 2-D band 3 crystals (Wang et al., 1993). A central depression forming the "thinnest" part of the band 3 dimer integral domain was found to be located at the interface between the subunits. Various biophysical measurements suggest that the two stilbenedisulfonate sites are greater than 20 Å apart (Macara & Cantley, 1981; Wojcicki & Beth, 1993). This large distance would seem to preclude placement

of two stilbenedisulfonate molecules within a central dimeric channel. Ultimately, it will be necessary to have a high-resolution 3-D structure of band 3 with stilbenedisulfonate or substrate bound before the exact location of sites can be determined.

Our evidence for ternary complex formation seems to contradict the impression from transport inhibition studies that stilbenedisulfonates are pure competitive inhibitors (Knauf, 1979; Passow, 1986). This impression of competitive inhibition comes from a comparison of chloride K_m values from H_2DIDS transport inhibition studies (Shami et al., 1978) using the Hunter–Downs analysis (Hunter & Downs, 1945) and from the observation of a linear dependence for the apparent K_I from Dixon plots *versus* sulfate concentration (Barzilay & Cabantchik, 1979). We suggest that these transport kinetic results are compatible with our direct kinetic evidence for ternary complex formation. Linear Dixon replots and ternary complex formation are indicative of a linear mixed-type inhibition model (Segel, 1975). The classical linear mixed-type model is



(3)

where q is the factor by which K_s changes when I occupies the protein. Chloride NMR evidence (Falke et al., 1984) suggests that q is equal to about 5. Using K_s = 80 mM (Falke et al., 1984; Gasbjerg & Brahm, 1991), qK_s = 400 mM. The equation for $K_{I\text{-app}}$ from Dixon plots for a linear mixed-type model is (Segel, 1975)

$$K_{I\text{-app}} = qK_I\left(\frac{[S] + K_s}{[S] + qK_s}\right) \quad (4)$$

For most reported experimental conditions [S] $\ll$ 400 mM = qK_s. Therefore

$$K_{I\text{-app}} = (K_I/K_s)[S] + K_I \quad (5)$$

Replots of $K_{I\text{-app}}$ *versus* [S] from Dixon plots would give the impression that the inhibitor I was a competitive inhibitor, when in fact it is not. It is clear that a linear mixed-type model embraces results in the literature suggesting both apparent competitive inhibition by stilbenedisulfonates and our results and those of Dix et al. (1986) and Passow and co-workers (Wood et al., 1992; Passow et al., 1992) favoring formation of a ternary complex between band 3, stilbenedisulfonates, and chloride.

One characteristic of linear mixed-type inhibitors in Lineweaver–Burk plots is an effect of inhibitor concentration on V_{max} (Segel, 1975). It may be argued that this characteristic is not seen for band 3. But we would point out that substrate inhibition, which is a prominent characteristic of band 3 anion exchange (Cass & Dalmark, 1973; Gasbjerg & Brahm, 1991), should mask any reduction in V_{max} expected from these model considerations. Indeed, careful and extensive transport kinetic data have been reported by Stadler and Schnell (1990) which support a mixed-type mechanism for the inhibition of band 3 anion exchange by DNDS.

In summary, our results suggest that stilbenedisulfonates and chloride bind simultaneously to band 3 to form a ternary complex and that such ternary complex formation occurs at the outer surface of band 3. Once formed, allosteric interactions within the ternary complex are proposed to explain the observed acceleration in the rate of stilbenedisulfonate release. These findings are most consistent with the view that the stilbenedisulfonate site lies off the transport pathway and that stilbenedisulfonate binding inhibits chloride/bicarbonate exchange allosterically.

REFERENCES

Antonini, E., & Brunori, M. (1971) Hemoglobin and Myoglobin in Their Reaction with Ligands, in *Frontiers of Biology*, Vol. 21, pp 197–198, North-Holland Publishing Co., Amsterdam.

Barzilay, M., & Cabantchik, Z. I. (1979) *Membr. Biochem. 2*, 255–281.

Cabantchik, Z. I., & Greger, R. (1992) *Am. J. Physiol. 262*, C803–827.

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

Cass, A., & Dalmark, M. (1973) *Nature (London) New Biol. 244*, 47–49.

Cotton, F. A., & Wilkinson, G. (1972) *Advanced Inorganic Chemistry. A comprehensive text*, p 451, Intersciences Publishers, New York.

CRC Handbook of Chemistry and Physics (1990) 71st ed., CRC Press, Boca Raton, FL.

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

Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.

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

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.

Frohlich, O. (1982) *J. Membr. Biol. 65*, 111–123.

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

Gibson, Q. H., & Roughton, F. J. W. (1955) *Proc. R. Soc. London, B 143*, 310–334.

Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319–326.

Gunn, R. B., & Frohlich, O. (1989) *Methods Enzymol. 177*, 54–56.

Haugland, R. P. (1989) *Molecular Probes Handbook of Fluorescent Probes and Research Chemicals*, p 99, Molecular Probes, Inc., Eugene, OR.

Hautmann, M., & Schnell, K. F. (1985) *Pflugers Arch. 405*, 193–201.

Hunter, A., & Downs, C. E. (1945) *J. Biol. Chem. 157*, 427–432.

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

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

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

Knauf, P. A. (1979) *Curr. Top. Membr. Transp. 12*, 249–363.

Knauf, P. A., & Mann, N. A. (1986) *Am. J. Physiol. 251*, C1–C9.

Kopito, R. R. (1990) *Int. Rev. Cytol. 123*, 177–199.

Kotaki, A., Naoi, M., & Yagi, K. (1971) *Biochim. Biophys. Acta 229*, 547–556.

Leatherbarrow, R. J. (1987) *Enzfitter: A Nonlinear Regression Data Analysis Program for the IBM-PC*, Elsevier Scientific Publishing Co., Amsterdam.

Lieber, M. R., & Steck, T. L. (1982a) *J. Biol. Chem. 257*, 11651–11659.

Lieber, M. R., & Steck, T. L. (1982b) *J. Biol. Chem. 257*, 11660–11666.

Macara, I. G., & Cantley, L. C. (1981) *Biochemistry 20*, 5095–5105.

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

Passow, H. (1986) *Physiol. Biochem. Pharmacol. 103*, 61–203.

Passow, H., Lepke, S., & Wood, P. G. (1992) *Prog. Cell Res. 2*, 85–98.
Pauling, L. (1960) *The Nature of the Chemical Bond and the Structure of Molecules and Crystals*, p 518, Cornell University Press, Ithaca, NY.
Perkins, S. J. (1986) *Eur. J. Biochem. 157*, 169–180.
Rao, A., Martin, P., Reithmeier, R. A. F., & Cantley, L. C. (1979) *Biochemistry 18*, 4505–4516.
Restrepo, D., Cronise, B. L., Snyder, R. B., Spinelli, L. J., & Knauf, P. A. (1991) *Am. J. Physiol. 260*, C535–C544.
Salhany, J. M. (1990) *Erythrocyte Band 3 Protein*, CRC Press, Boca Raton, FL.
Salhany, J. M., & Swanson, J. C. (1978) *Biochemistry 17*, 3354–3362.
Salhany, J. M., & Gaines, E. D. (1981) *J. Biol. Chem. 256*, 11080–11085.
Salhany, J. M., & Cordes, K. A. (1992) *Biochemistry 31*, 7301–7310.
Salhany, J. M., Cordes, K. A., & Gaines, E. D. (1980) *Biochemistry 19*, 1447–1454.
Salhany, J. M., Rauenbuehler, P. B., & Sloan, R. L. (1987) *J. Biol. Chem. 262*, 15965–15973.
Salhany, J. M., Cordes, K. A., & Schopfer, L. M. (1993) *Biochemistry 32*, 7413–7420.
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.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 170–178, John Wiley & Sons, New York.
Shami, Y., Carter, J., Ship, S., & Rothstein, A. (1977) *Biochem. Biophys. Res. Commun. 76*, 429–436.
Shami, Y., Rothstein, A., & Knauf, P. A. (1978) *Biochim. Biophys. Acta 508*, 357–363.
Stadler, F., & Schnell, K. F. (1990) *J. Membr. Biol. 118*, 19–47.
Steck, T. L. (1974) *Methods Membr. Biol. 2*, 245.
Tanner, M. J. A., Martin, P. G., & High, S. (1988) *Biochem. J. 256*, 703–712.
Van Dort, H., Low, P. S., Cordes, K. A., Schopfer, L. M., & Salhany, J. M. (1994) *J. Biol. Chem. 269*, 59–61.
Wang, D. N., Kuhlbrandt, W., Sarabia, V. E., & Reithmeier, R. A. F. (1993) *EMBO J. 12*, 2233–2239.
Wojcicki, W. E., & Beth, A. H. (1993) *Biochemistry 32*, 9454–9464.
Wood, P. G., Muller, H., Sovak, M., & Passow, H. (1992) *J. Membr. Biol. 127*, 139–148.
Zaki, L. (1992) *Prog. Cell Res. 2*, 59–64.