Functions of Extracellular Lysine Residues in the Human Erythrocyte Anion Transport Protein

MICHAEL L. JENNINGS, RENÉ MONAGHAN, SUSAN M. DOUGLAS, and J. STEPHEN NICKNISH

From the Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242

ABSTRACT The extracellular lysine residues in the human erythrocyte anion transport protein (band 3) have been investigated using chemical modification with the impermeant homobifunctional active ester bis(sulfosuccinimidyl)-suberate (BSSS). This agent forms covalent intra- and intermolecular cross-links in human band 3 in intact cells (Staros and Kakkad. 1983. *J. Membr. Biol.* 74:247). We have found that the intermolecular cross-link has no detectable effect on the anion transport function of band 3. The intramolecular cross-link, however, causes major changes in the characteristics of the anion transport. These functional alterations are caused by the modification of lysine residues at the stilbene disulfonate binding site. BSSS pretreatment at pH 7.4 irreversibly inhibits Cl-Br exchange by at least 90% when the transport is assayed at extracellular pH above 8. In the same BSSS-pretreated cells, however, the Cl-Br exchange rate is activated by lowering the pH of the flux medium (intracellular pH fixed at 7). The flux is maximal at pH 5–6; a further lowering of the extracellular pH inhibits the anion exchange. This acid-activated Cl-Br exchange in the BSSS-treated cells is mediated by band 3, as indicated by phenylglyoxal and phloretin inhibition of the flux. Thus, the BSSS pretreatment has little effect on the maximal Cl-Br exchange flux catalyzed by band 3, but it shifts the alkaline branch of its extracellular pH dependence by ~5 pH units. BSSS also eliminates the self-inhibition of Cl-halide exchange by high extracellular Br or I concentrations. These results indicate that the BSSS-modified lysines do not participate directly in anion translocation, but that one of the lysines normally provides a positive charge that is necessary for substrate anion binding. This positive charge is removed by the BSSS treatment but can be replaced by lowering the extracellular pH. The results also provide insight regarding the halide selectivity of the maximal rate of chloride-halide exchange: the native selectivity (Br ≫ I) is nearly abolished by BSSS treatment, which suggests that the selectivity results from the very strong binding of iodide to an outward-facing modifier site.

Address reprint requests to Dr. Michael L. Jennings, Dept. of Physiology and Biophysics, The University of Iowa, Iowa City, IA 52242.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/85/11/0653/17$1.00

Volume 86 November 1985 653–669

INTRODUCTION

Red blood cell inorganic anion exchange is one of the most thoroughly characterized ion transport processes in nature (see Knauf, 1979). The protein that catalyzes this anion exchange has been identified (Cabantchik and Rothstein, 1974*a*; Ho and Guidotti, 1975; Passow et al., 1975) as the 95,000-dalton integral membrane protein known as band 3 (Fairbanks et al., 1971). The arrangement of the band 3 polypeptide in the membrane is becoming increasingly well understood as a result of in situ proteolysis and chemical modification experiments (e.g., Steck et al., 1976, 1978; Mawby and Findlay, 1982; Brock et al., 1983; Jennings et al., 1984). The protein consists of an NH_2-terminal 43,000-dalton cytoplasmic domain and a COOH-terminal 52,000-dalton membrane domain (Steck et al., 1976, 1978). The cytoplasmic domain is water soluble and is not believed to be necessary for the anion transport function of band 3 (Grinstein et al., 1978; Lepke and Passow, 1976). The membrane domain apparently consists of several membrane-crossing segments (Brock et al., 1983; Jennings and Nicknish, 1984; Ramjeesingh et al., 1984); little is known about the three-dimensional arrangement of these segments.

Beginning with the work of Passow (1969), numerous studies have shown that chemical modification of amino groups causes inhibition of red cell anion transport. Among the amino group–reactive agents that irreversibly inhibit band 3–mediated anion exchange are 2,4-dinitrofluorobenzene (Passow, 1969; Knauf and Rothstein, 1971), maleic anhydride (Obaid et al., 1972), trinitrobenzenesulfonate (Zaki et al., 1971; Knauf and Rothstein, 1971), isothiocyanobenzenesulfonate (Ho and Guidotti, 1975), diiodosulfophenylisothiocyanate (Mawby and Findlay, 1982), pyridoxal phosphate/borohydride (Cabantchik et al., 1975), formaldehyde/borohydride (Jennings, 1982), phenylisothiocyanate (Sigrist et al., 1980; Cacciola et al., 1984), and DIDS/H_2DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate/4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate) (Cabantchik and Rothstein, 1974*a*). At least three different lysine residues have been chemically modified with resultant transport inhibition. These lysines have been localized in the primary structure relative to known in situ proteolytic cleavage sites (Fig. 1). Lys a reacts at pH 7.4 with H_2DIDS (Cabantchik and Rothstein, 1974*b*), other arylisothiocyanates (Drickamer, 1976; Mawby and Findlay, 1982), and 2,4-dinitrofluorobenzene (Rudloff et al., 1983). Lys b reacts with formaldehyde/borohydride (Jennings, 1982) and with H_2DIDS at alkaline pH (Jennings and Passow, 1979). A lysine in the same general region of the primary structure reacts with pyridoxal phosphate/borohydride (Nanri et al., 1983); this lysine may be identical to Lys b. Both Lys a and Lys b are accessible from the extracellular water; when the single bound H_2DIDS molecule reacts with both these lysines, it produces a covalent intramolecular cross-link between the two chymotryptic fragments (Jennings and Passow, 1979). A third reactive lysine, Lys c, is in a 8,000–10,000-dalton peptic fragment (Kempf et al., 1981). This residue is probably located near the intracellular surface of the membrane (Brock et al., 1983) and reacts with phenylisothiocyanate under conditions in which transport is inhibited (Sigrist et al., 1980).

Although it is very clear that these amino group modifications inhibit transport,

the functional roles of lysine residues in the anion exchange process are not understood. With one exception, the above chemical modifications introduce negative charge and/or bulky aromatic rings into the protein. The modifications could therefore block transport, even though the lysines themselves may have no essential role in the process. There is now good evidence from two laboratories that some of the positive charge at the extracellular substrate binding site is provided by one or more arginine residues (Wieth et al., 1982*a*, *b*; Bjerrum et al., 1983; Zaki, 1981). In fact, the extracellular pH dependence (pH > 7) of the chloride self-exchange flux can be interpreted as resulting from the titration of two arginine side chains (Wieth and Bjerrum, 1982). If this is the correct interpretation, then the state of protonation of outward-facing lysine residues has no effect on the anion flux.

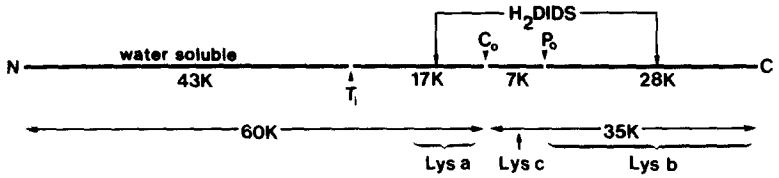

FIGURE 1. Locations in the band 3 primary structure of lysine residues of possible functional importance in anion transport. The sites of in situ proteolytic cleavage by intracellular trypsin (T_i) (Steck et al., 1976), extracellular chymotrypsin (C_o) (Cabantchik and Rothstein, 1974*b*), and extracellular papain (P_o) (Jennings et al., 1984) are indicated. There are two additional papain cleavage sites very close to C_o (Jennings et al., 1984). Lys a and Lys b are the two lysines that react covalently with the single bound H_2DIDS molecule (Jennings and Passow, 1979).

In order to understand more fully the role of the exofacial lysine residues in band 3–catalyzed anion exchange, we have employed chemical modification with the active ester cross-linker bis(sulfosuccinimidyl)suberate (BSSS), which was developed by Staros (1982) as an impermeant probe of membrane protein subunit interactions. Staros and Kakkad (1983) showed that BSSS forms both inter- and intramolecular cross-links at the outer aspect of band 3. In this paper, we show that the intermolecular cross-link has no detectable effect on anion transport and that the intramolecular cross-link is probably between Lys a and Lys b. Unlike H_2DIDS, BSSS does not introduce a bulky, negatively charged mass into the protein (Fig. 2). We have found that anion exchange catalyzed by the BSSS-modified band 3 is not completely inhibited, and thus the function of the modified protein can be characterized. The results indicate that neither of the BSSS-modified lysines (at the stilbene disulfonate site) participates directly in the ion translocation, but that the positive charge normally provided by one of the lysines does appear to be necessary for monovalent anion exchange. Moreover, one of the BSSS-reactive lysines at the stilbene disulfonate site is necessary for the outward-facing "modifier" site at which extracellular bromide or iodide inhibits the exchange of intracellular chloride for extracellular halide (Gunn and Fröhlich, 1979; Wieth and Bjerrum, 1982). This modifier site is largely responsible for the selectivity (Br $\gg$ I) of the maximum rate of Cl-halide heteroexchange.

MATERIALS AND METHODS

Materials

Human red blood cells (EDTA anticoagulant) were obtained from the Lipid Research Laboratory or the DeGowin Blood Donor Center of The University of Iowa. Cells were used after at most 4 d of storage as packed cells in plasma at 4°C. [^{3}H]H_2DIDS was prepared as described previously (Jennings et al., 1984). BSSS was synthesized as described by Staros (1982) or purchased from Pierce Chemical Co., Rockford, IL. DNDS (4,4′-dinitrostilbene-2,2′-disulfonate) was purchased from Aldrich Chemical Co., Milwaukee, WI. Papain was purchased from Boehringer Mannheim, Indianapolis, IN. Endo-β-galactosidase (*Escherichia freundii*) was from Miles Laboratories, Inc., Elkhart, IN. All other reagents, salts, and buffers were obtained from Sigma Chemical Co., St. Louis, MO, or Fisher Chemicals, Itasca, IL.

Effect of BSSS on Cl-Br or Cl-I Exchange

Cells were washed three times in 10 vol of 150 mM KCl, 10 mM Na-phosphate, pH 7.4[1] (PBK 7.4), and resuspended at 50% hematocrit in the same medium containing 0–5 mM

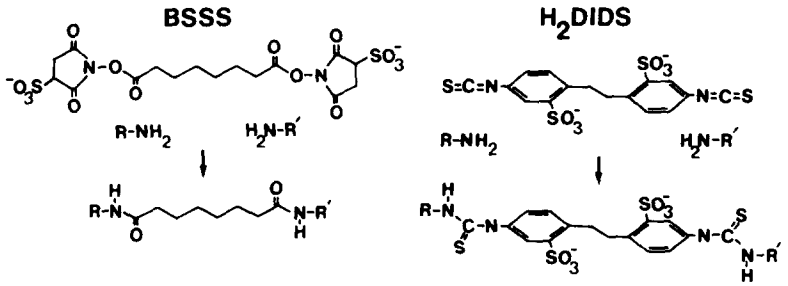

FIGURE 2. Comparison of BSSS (left) and H_2DIDS (right), each reacting with two primary amines to produce a covalently cross-linked product.

freshly dissolved BSSS. After a 1-h incubation at 37°C, the reaction was terminated by adding 10 vol of 50 mM glycine in PBK 7.4. The cells were then washed three times in PBK 7.0 containing 0.2% bovine serum albumin (BSA). After each resuspension, the cells were incubated for 10 min at 22°C to allow the intracellular pH to equilibrate with the pH 7.0 medium. Then the cell pellet was resuspended in 0.5 cell volumes of PBK 7.0 containing 0.2% BSA and 2 μCi ^{36}Cl/ml. After an incubation of at least 5 min at 22°C, the cells were packed by a 1-min centrifugation in a microfuge, and the ^{36}Cl efflux was initiated by resuspending 0.1 ml of packed cells in 5 ml of the media specified in the figure legends. The flux media were ice-cold (<0.1°C), and the tracer efflux was measured in a cold room. Aliquots of suspension were centrifuged at 30-s intervals and ^{36}Cl radioactivity in 0.5 ml of supernatant was measured by liquid scintillation counting. This radioactivity was compared with that (cpm^{∞}) of 0.5 ml of a lysate of suspension in 10% trichloroacetic acid; the rate constant for the tracer efflux was calculated from a plot of $-\ln[1 - (cpm/cpm^{\infty})]$ vs. time.

In the experiments in which extracellular pH was varied, the intracellular pH remained constant at 7.0 ± 0.1. This was verified by lysing aliquots of packed cells at the end of flux experiments carried out at extracellular pH 4 and 10. No special precautions were

[1] All pH values represent the pH of the solution measured at 22°C.

taken to remove CO_2; the durations of the experiments at 0°C were not sufficient to allow significant intracellular pH shifts. Phenylglyoxal modification of the BSSS-treated cells was carried out by a procedure adapted from Bjerrum et al. (1983). Packed cells (in PBK 7.4) were resuspended in 5 vol of 100 mM Na-phosphate, pH 10, containing 10 mM phenylglyoxal. After a 1-min incubation at 25°C, a 10-fold excess of 150 mM KCl, 10 mM Na-phosphate, pH 6, was added to quench the reaction and return the extracellular pH to neutrality. The cells were then washed and equilibrated with PBK 7.0 plus 0.2% BSA and loaded with ^{36}Cl as described above.

Enzyme/H_2DIDS Treatment and Gel Electrophoresis

After pretreatment with BSSS (Fig. 5), intact cells were treated for 1 h at 37°C, pH 6, with 1 mg/ml papain that had been activated with 2 mM cysteine. After the papain treatment, cells were washed twice and treated with endo-β-galactosidase, and membranes were isolated, treated with trypsin, alkali-stripped, and analyzed by gel electrophoresis, all as described previously (Jennings et al., 1984). [^{3}H]H_2DIDS incubation after BSSS pretreatment was with 8 μM [^{3}H]H_2DIDS, in 150 mM $NaHCO_3$, pH 9, for 1 h, 37°C, followed by two washes in 150 mM $NaHCO_3$, 0.2% BSA. Membrane isolation, gel electrophoresis, and determination of radioactivity in gel slices were as described previously (Jennings, 1982).

RESULTS

BSSS Prevents Irreversible H_2DIDS Binding

Staros and Kakkad (1983) first showed that BSSS, acting on intact cells, forms both intermolecular and intramolecular cross-links in band 3 (Fig. 3). Subsequent work in our laboratory (Jennings and Nicknish, 1985) showed that the reversibly binding stilbene disulfonate DNDS (Barzilay et al., 1979; Fröhlich, 1982) can block the intramolecular, but not the intermolecular, BSSS cross-link. Fig. 4 shows that, conversely, BSSS pretreatment can completely prevent irreversible attachment of [^{3}H]H_2DIDS to band 3. Intact cells were incubated at pH 7.4 in the presence of no additive, BSSS alone, or BSSS and DNDS together. The cells were then washed and incubated with [^{3}H]H_2DIDS. Membranes were isolated and solubilized, and the protein was separated by gel electrophoresis. In control cells, as expected, the counts were localized mainly in band 3 (Cabantchik and Rothstein, 1974*a*). In the cells treated with BSSS alone, the irreversible H_2DIDS binding to band 3 was <2% of that in the control cells. In the cells treated with BSSS plus DNDS, the total H_2DIDS binding was somewhat reduced and was localized to the region of the gel that corresponds to the band 3 dimer. This indicates that it is the intramolecular BSSS cross-link, not the intermolecular cross-link, that prevents covalent H_2DIDS binding.

In the experiment in Fig. 4, aliquots of cells were removed before the [^{3}H]-H_2DIDS binding measurement for assay of band 3–mediated anion exchange (^{36}Cl efflux at 0°C into a phosphate-buffered isotonic KBr medium at pH 7). The same BSSS treatment that completely prevented irreversible H_2DIDS binding only partially (65%) inhibited anion exchange measured under these conditions. The residual flux in the BSSS-treated cells was not a nonspecific leak; the flux had characteristics that indicated that it was catalyzed by the modified band 3 (see below). Thus, although >95% of the copies of band 3 reacted with BSSS

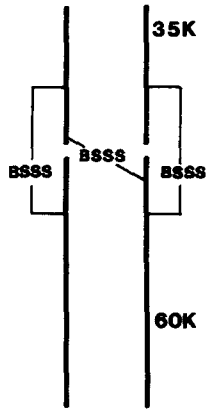

FIGURE 3. Intermolecular and intramolecular cross-links in band 3 formed by BSSS (Staros and Kakkad, 1983). The 60,000-dalton and 35,000-dalton chymotryptic fragments are indicated.

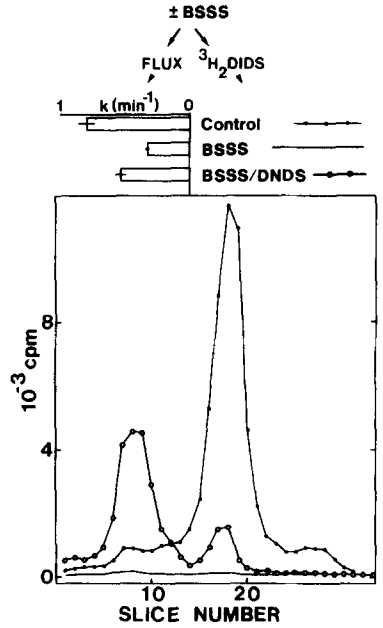

FIGURE 4. (Bottom) Effect of BSSS (5 mM, pH 7.4, 1 h, 37°C) on subsequent labeling of red cells with [^{3}H]H_2DIDS. Cells were incubated with or without BSSS, then washed in 150 mM $NaHCO_3$ and incubated at pH 9 in 8 μM [^{3}H]H_2DIDS for 1 h, 37°C. Membranes were then isolated, solubilized, and separated on a 5–12% gradient polyacrylamide gel (0.1% sodium dodecyl sulfate). The distribution of radioactivity over the top half of each lane is shown; the remainder of the gel contained only a small amount of radioactivity, which migrated with the tracking dye. The band 3 monomer is centered at slice 18, the dimer at slice 8. For the data designated BSSS/DNDS, the BSSS incubation was carried out in the presence of 1 mM DNDS. (Top) Effect of the same pretreatments on the rate constant (k) for ^{36}Cl efflux into a 140 mM KBr medium, 0°C, buffered at pH 7.0 with 10 mM phosphate.

at the stilbene disulfonate site, the Cl-Br exchange at pH 7 was not completely inhibited.

About half the inhibition of transport by BSSS was prevented by the presence of DNDS during the BSSS treatment, just as the DNDS partially protected the H_2DIDS binding site from covalent reaction with BSSS. Therefore, the inhibition of anion exchange appears to be related to BSSS modification of the stilbene disulfonate site. In contrast, the intermolecular BSSS cross-link is not affected by DNDS (Jennings and Nicknish, 1985), which suggests that the intermolecular cross-link has no effect on anion exchange.

Papain Fragments Cross-Linked by BSSS

We showed previously (Jennings and Nicknish, 1985) that the intermolecular BSSS cross-link involves a lysine residue that is within four residues of the extracellular chymotrypsin cleavage site. The covalent dimers formed by the BSSS can be cleaved back to monomers by extracellular papain, because papain cleaves the polypeptide on both sides of the cross-linked lysine residue (see Jennings and Nicknish, 1985). The BSSS cross-links that remain after extracellular papain digestion are therefore intramolecular. Fig. 5 shows that the intramolecular BSSS cross-link is between the same major papain fragments that are cross-linked by H_2DIDS. Cells were incubated at pH 7.4 in the presence or absence of 5 mM BSSS, then washed and incubated with papain, followed by endo-β-galactosidase to remove much of the band 3 carbohydrate (Mueller et al., 1979). Membranes were isolated, treated with trypsin to remove the cytoplasmic domain, alkali-stripped to remove peripheral proteins (Steck and Yu, 1973), and then analyzed by gel electrophoresis. The band 3 fragments of 28, 17, and 7 kD are indicated. The 28-kD and 7-kD fragments are weakly stained and tend to aggregate. BSSS cross-links the 28-kD and 17-kD fragments to produce a new major band at ~45 kD. The same two fragments are cross-linked to each other by H_2DIDS (Jennings et al., 1984). This finding, and the fact that BSSS completely prevents covalent H_2DIDS binding (Fig. 4), suggest that BSSS and H_2DIDS cross-link the same two lysine side chains.

Activation of Cl-Br Exchange by Extracellular H^+

Chloride self-exchange in red cells is inhibited by lowering the extracellular pH below 7 (Wieth et al., 1982*a*; Milanick and Gunn, 1982). Not surprisingly, Cl-Br exchange has a similar dependence on extracellular pH (Fig. 6). BSSS pretreatment at pH 7.4 dramatically changes the pH dependence of the transport. The Cl-Br exchange in the treated cells is ~95% inhibited when the flux is assayed at an extracellular pH above 8. Lowering the extracellular pH (at a constant intracellular pH of 7) activates the flux by a factor of 15 to a maximum between pH 5 and 6. At pH 6, the flux is equal to the control flux. As mentioned previously, the proton-activated Cl-Br exchange in the BSSS-treated cells is not a nonspecific leak: exposure of the BSSS-treated cells to 10 mM phenylglyoxal at extracellular pH 10 for 1 min, followed by extensive washing at pH 7, causes 80% inhibition of the acid-activated Cl-Br exchange flux (Fig. 6, triangle). Thus, although all copies of band 3 have an intramolecular BSSS cross-link at the

stilbene disulfonate site, phenylglyoxal is still able to modify an essential arginine residue (Wieth et al., 1982*a*) and inhibit the transport. We have also found that phloretin (40 μM) inhibits the Cl-Br exchange in the BSSS-treated cells by 90%. Phloretin similarly inhibits Cl-Cl exchange in native red cells (Wieth et al., 1973). Finally, lowering the extracellular pH from 5 to 4 strongly inhibits the anion exchange, which suggests that the extracellular H^+ titration (pK 5) on band 3 that inhibits monovalent anion exchange in native cells (Wieth et al., 1982*a*; Milanick and Gunn, 1982) is also effective in the BSSS-modified cells. These

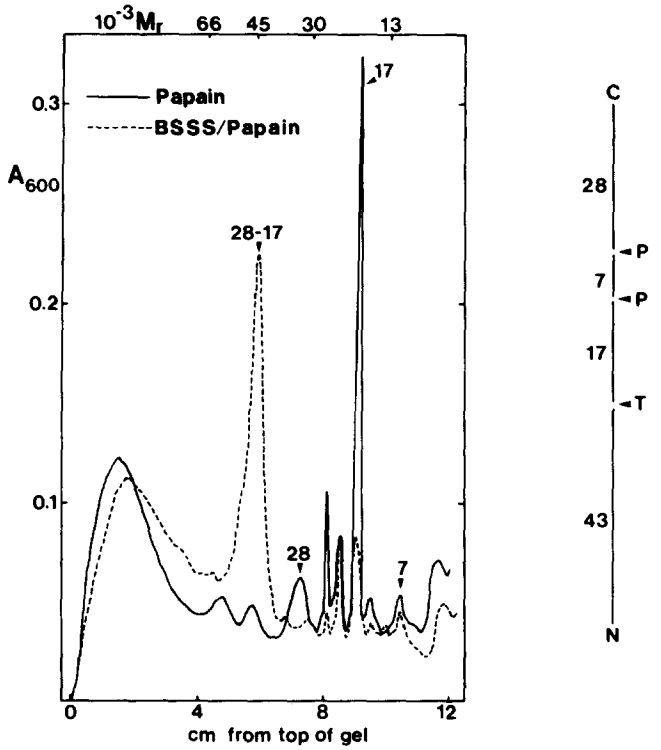

FIGURE 5. Intramolecular BSSS cross-link between the 28,000-dalton and 17,000-dalton papain fragments of band 3. See text.

findings all indicate that band 3 catalyzes the Cl-Br exchange flux that is activated by lowering the extracellular pH of the BSSS-treated cells.

BSSS Eliminates the Extracellular "Modifier" Site

Since the Cl-Br exchange flux at extracellular pH 6 in the BSSS-treated cells is similar to the control flux, we chose this pH for an examination of the effects of BSSS on some of the kinetic characteristics of Cl-Br exchange. As in Fig. 6, the intracellular pH was 7 during the flux, and the BSSS pretreatment was at pH 7.4. Fig. 7 is a plot of the rate of Cl-Br exchange as a function of extracellular Br concentration (varied at the expense of gluconate). As we found previously

(Jennings, 1982), the $K_{1/2}$ for extracellular Br in control cells is ~8 mM in a gluconate medium; BSSS increases the $K_{1/2}$ to ~25 mM. Thus, the apparent affinity for extracellular substrate is reduced by a factor of ~3.

Gunn and Fröhlich (1979) showed that extracellular Br at concentrations above 30 mM inhibits Cl-Br exchange; that is, the extracellular bromide inhibits its own influx. We found similar inhibition by extracellular Br, although at

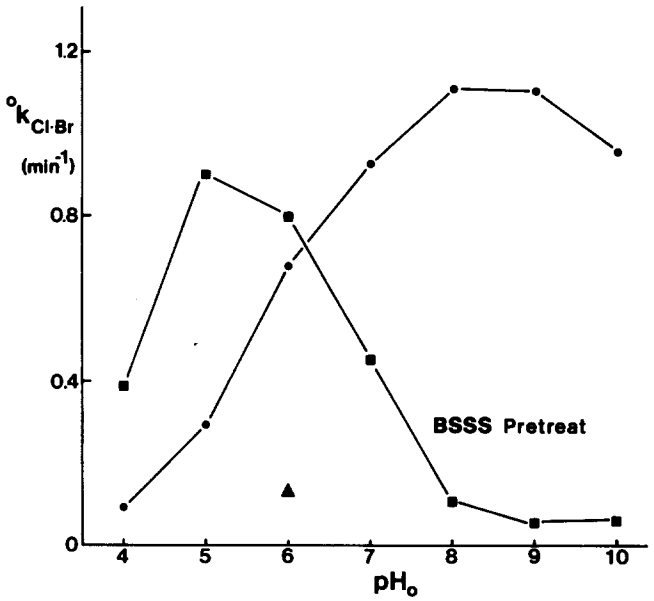

FIGURE 6. Effect of extracellular pH on the rate constant ($°k_{Cl\text{-}Br}$) for ^{36}Cl efflux into 140 mM KBr, buffered with 10 mM gluconate, phosphate, and borate at pH 4–10. Control cells (solid circles) were equilibrated with 150 mM KCl (extracellular pH 7) and loaded with tracer immediately before the efflux measurement. BSSS-pretreated cells (squares) were incubated 1 h, 37°C, pH 7.4, in 150 mM KCl medium containing 5 mM BSSS, and then washed and equilibrated with pH 7 medium and loaded with tracer. The triangle refers to cells treated with 10 mM phenylglyoxal, pH 10, after the BSSS, with the flux subsequently measured at extracellular pH 6 as for the other cells. Each data point represents the mean of two efflux measurements (four time points each) performed on each of at least two preparations of cells. For each data point, the standard error of the mean is not more than 16%.

somewhat higher concentrations than those used by Gunn and Fröhlich (1979). With BSSS-treated cells, however, there was no evidence that high Br concentrations are inhibitory (Fig. 8). This effect of BSSS on self-inhibition was even more dramatic for the exchange of intracellular Cl for extracellular iodide (Fig. 9). In control cells, rather low concentrations of extracellular iodide inhibited Cl-I exchange. This finding is in qualitative agreement with the work of Dalmark (1976), who found that iodide has a higher affinity than the other halides for the modifier site. In the BSSS-treated cells, there was no self-inhibition over the

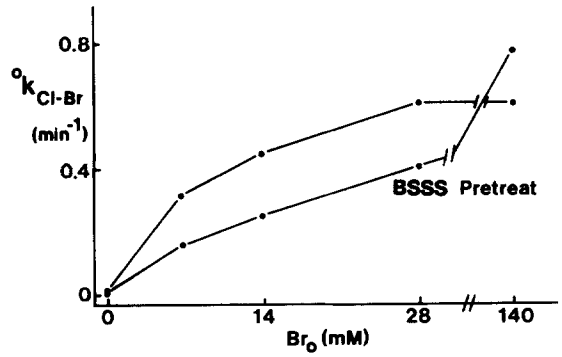

FIGURE 7. Effect of BSSS on the activation of ^{36}Cl efflux by extracellular Br. Cells were pretreated with or without BSSS and loaded with ^{36}Cl exactly as in Fig. 6, and the tracer efflux was measured at 0°C into 150 mM K gluconate media in which varying amounts of KBr were substituted for the gluconate. All media were buffered at pH 6 with 10 mM phosphate. Each data point represents the mean of two measurements on one (control) or two (BSSS-pretreated) preparations of cells. SEMs are 5–13%.

concentration range examined. Because of the lack of self-inhibition, the Cl-I exchange flux was much higher in the BSSS-treated cells than in control cells. Also, the $K_{1/2}$ for extracellular iodide was much larger in the BSSS-treated cells. This could be the result of a true change in affinity of the transport site for iodide, but much of the effect could be the result of removal of self-inhibition. The same consideration applies to the effect of BSSS on the $K_{1/2}$ for extracellular bromide.

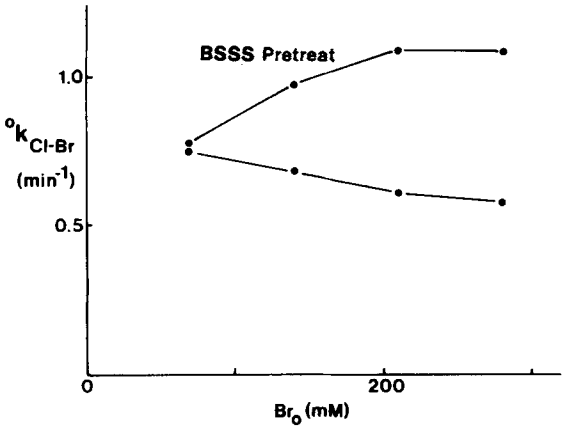

FIGURE 8. Effect of BSSS on self-inhibition of Cl-Br exchange by extracellular Br. Cells were pretreated and loaded with tracer as in Figs. 6 and 7, and the tracer flux was measured in a hypertonic medium containing 70–280 mM KBr, varied at the expense of gluconate. All media were buffered at pH 6 with 10 mM phosphate. The shrinkage of the cells in the hypertonic medium has no effect on monovalent anion exchange (Funder and Wieth, 1976). Each data point represents the mean of three to six measurements; SEMs are 4–14%.

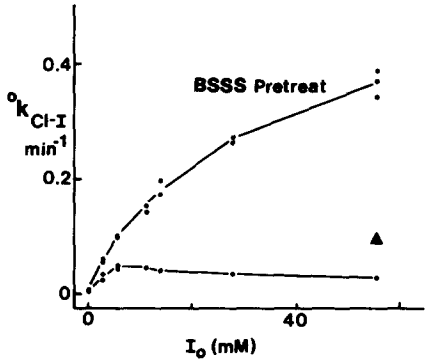

FIGURE 9. Effect of BSSS on the activation of ^{36}Cl efflux by extracellular iodide. Cells were pretreated and loaded with tracer as in Figs. 6–8, and the tracer efflux was measured at extracellular pH 6 in a 140 mM K gluconate medium, with 0–56 mM iodide substituted for gluconate. The triangle refers to cells pretreated with BSSS and then with phenylglyoxal as in Fig. 6.

As is true for the inhibition of Cl-Br exchange assayed at alkaline or neutral pH, the acceleration by BSSS of Cl-I exchange (flux measured at pH 6) is a consequence of BSSS modification of the stilbene disulfonate site. This was demonstrated (Fig. 10) by incubating cells at pH 7.4 in 0.5 mM BSSS in the presence or absence of 1 mM DNDS. The DNDS prevents much of the acceleration of the Cl-I exchange (flux measured at extracellular pH 6) and also prevents the inhibition of the Cl-Br exchange (flux measured at pH 8).

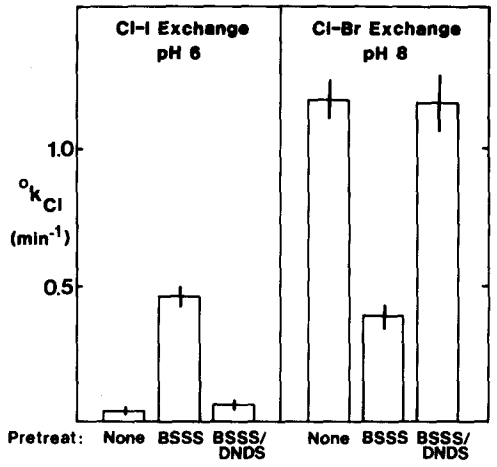

FIGURE 10. Protection by DNDS of BSSS inhibition of Cl-Br exchange (measured at extracellular pH 8) and acceleration of Cl-I exchange (measured at extracellular pH 6). Cells were pretreated as above, except that the BSSS concentration was 0.5 rather than 5 mM. At the lower BSSS concentration, the protective effect of 1 mM DNDS is larger than in Fig. 6. The data represent the means (± SEM) of two measurements on two separate preparations of cells.

DISCUSSION

In these studies, we have shown that although BSSS irreversibly modifies at least two amino groups at the stilbene disulfonate site of human red cell band 3 protein, it does not cause total irreversible inhibition of band 3–mediated anion transport.[2] This has allowed us to study the characteristics of the transport in the modified cells and gain insight into the possible functions of lysine residues at the outer surface of band 3. BSSS forms a covalent intramolecular cross-link between the same two papain fragments that are cross-linked by H_2DIDS. The length of the BSSS cross-link is several angstroms shorter than that of H_2DIDS (Fig. 2), but, given the length and flexibility of lysine side chains, it is possible (and we believe likely) that BSSS and H_2DIDS cross-link the same two residues. Our major conclusions are as follows.

(*a*) The two lysines at the stilbene disulfonate site that are cross-linked by BSSS are not absolutely necessary for transport. This is demonstrated by activation of the Cl-Br exchange flux to control levels at low extracellular pH (Fig. 6), even though virtually all copies of band 3 have been irreversibly modified by BSSS. Although the lysine residues themselves are not absolutely required for Cl-Br exchange, BSSS does cause >90% transport inhibition when the flux is assayed at pH above 8. This inhibition is probably not caused by steric blockage of access to the transport pathway, because lowering the extracellular pH overrides the inhibitory effects of the irreversibly bound BSSS. The most reasonable explanation for the inhibition is that the positive charge normally provided by one of the lysines at the stilbene disulfonate site has a necessary role in the transport. This positive charge can apparently be replaced by lowering the extracellular pH.

(*b*) The modification of the lysines by BSSS lowers the apparent affinity of Br or I for transport sites and removes the self-inhibition at high extracellular Br or I concentration. Wieth and co-workers (Wieth et al., 1982*a*; Wieth and Bjerrum, 1982) have shown that self-inhibition of Cl-Cl exchange is reduced by raising the extracellular pH above 10. They interpreted these results as evidence that an arginine residue is responsible for the self-inhibition. Here we have used an amino group–specific modification that eliminates self-inhibition of Cl-halide exchange by extracellular Br and I. This is direct evidence for the participation of at least one of the BSSS-modified lysine residues in the self-inhibition. We believe, therefore, that the extracellular titratable group ($pK > 9$) associated with anion exchange self-inhibition (Wieth and Bjerrum, 1982) is a lysine rather than an arginine residue.

(*c*) There are at least two extracellular H^+-titratable groups ($pK_a < 8$) of functional significance in the BSSS-modified band 3. Previous evidence was consistent with a single titration in this pH range at the outer surface (Milanick and Gunn, 1982, 1984), but in the BSSS-treated cells, there are both stimulatory and inhibitory H^+ binding sites. In native band 3, protonation of the stimulatory site either does not occur in the pH range 4–7 or has no effect on the flux because the native positive charges are sufficient to provide an anion binding

[2] In contrast, H_2DIDS causes >98% irreversible inhibition of Cl-Br exchange (extracellular pH 4–7), measured exactly as in the experiments in Fig. 6.

site. The inhibitory H^+ binding site in the native and treated cells is probably a carboxyl group (Wieth et al., 1982*a*). The identity of the stimulatory H^+ binding site in the BSSS-treated cells is unknown. The apparent pK_a for protonation of this site is suggestive of a histidine residue, but we have no direct evidence for the participation of histidines in the transport. The BSSS-modified residues themselves are not titratable in this pH range, unless there is very severe torsional strain on the amide group (Dunker, 1982). It is unlikely that such strain could be caused by the flexible aliphatic chains on each side of the amide.

(*d*) Covalent intermolecular cross-linking of band 3 to a dimer has no detectable effect on anion transport. The evidence for this (Figs. 4 and 10) is that DNDS protects band 3 against two effects of BSSS: the inhibition of Cl-Br exchange (assayed at pH 7–8) and the acceleration of Cl-I exchange (assayed at pH 6). DNDS does not prevent the intermolecular BSSS cross-link (Fig. 4; see also Jennings and Nicknish, 1985). Thus, the intermolecular cross-link is not responsible for the effects on transport. Because the intermolecular cross-link is formed much more slowly than the intramolecular cross-link, it is difficult to find conditions under which only intermolecular cross-links are present. Thus, we cannot rule out subtle effects of the intermolecular cross-link on anion transport. The major effects, however, are attributable to the intramolecular cross-link at the stilbene disulfonate site. This is consistent with the concept that although band 3 is a dimer (or tetramer) in the membrane, subunit rearrangements are not important steps in the catalysis of anion exchange (see Jennings, 1984).

Which Lysine Residues Are Modified?

Given the reactivity of Lys a (see Passow et al., 1980), it would be surprising if this residue were not modified by BSSS under the conditions of our experiments. Our data suggest that Lys b is the other residue at the stilbene disulfonate site modified by BSSS, but this is not yet certain. The intramolecular BSSS cross-link definitely involves an extracellular lysine in the COOH-terminal 28,000-dalton papain fragment, and the BSSS intramolecular cross-link completely prevents covalent H_2DIDS binding (Figs. 4 and 5). Further evidence that BSSS reacts with Lys b is that BSSS pretreatment completely protects band 3 from the inhibitory effects of reductive methylation (two experiments not shown). We previously showed that reductive methylation of Lys b inhibits Cl-Br exchange by ~75% (Jennings, 1982). This inhibition persists even if the transport is assayed at extracellular pH 6. Thus, when the transport is assayed under these conditions, methylation of Lys b inhibits transport more than does modification with BSSS, which neutralizes the charge on the lysine. One possible explanation for this finding is that the methylated lysine may have a subtle steric effect on the conformational change associated with anion translocation, whereas the same lysine, if it is cross-linked by BSSS to Lys a, may be restrained in such a way as to prevent any steric interference with transport.

Relation to Previous Work

As stated above, we have interpreted the pH dependence of the anion exchange in BSSS-modified cells as evidence that one of the modified lysines normally

provides the positive charge that is necessary for anion exchange. If this is correct, why is the Cl-Cl exchange flux (at 165 mM Cl) nearly independent of extracellular pH, up to pH 11.5 (Wieth and Bjerrum, 1982)? One possibility is simply that the pK_a of the lysine side chain at 0°C in a chloride medium is >11. Kampmann et al. (1982) have estimated that the pK_a of Lys b at 37°C is ~10.0. The enthalpy of ionization of lysine side chains in proteins is usually 10–12 kcal/mol (Cohn and Edsall, 1965). Thus, a pK_a of 10 at 37°C corresponds to a pK_a of 11 at 0°C. Also, the pK_a estimated by Kampmann et al. (1982) applies to the protein in the presence of H_2DIDS bound covalently to Lys a. The extracellular pH dependence of the Cl-Cl exchange flux (Wieth and Bjerrum, 1982) reflects the titration of the protein with a chloride ion bound at the outward-facing transport site.[3] In both cases, the bound anion should raise the pK_a, but the quantitative effects may not be comparable. Thus, the existing data on the pH dependence of monovalent anion exchange are not inconsistent with our suggestion that a lysine residue provides an essential positive charge near the outward-facing transport site. It should be emphasized that the effects of pH on the anion flux may not result solely from direct electrostatic interactions; allosteric effects on the conformation of the protein may also be important.

Our evidence for functionally important lysine residues is not at variance with the chemical modification studies (Wieth et al., 1982*a*, *b*; Zaki, 1981) that have indicated that one or more arginine residues are necessary for anion transport by band 3. We do disagree with the hypothesis that the modifier site necessarily involves an arginine (see above), but we believe it is quite likely that an arginine is necessary for anion binding at the extracellular transport site. In fact, our data (Figs. 6 and 9) strengthen the evidence for an essential arginine: phenylglyoxal inhibits the transport (measured at pH 6) irreversibly in BSSS-pretreated cells. Therefore, under conditions in which extracellular amino groups have been modified, the phenylglyoxal still inhibits transport, which implies that the inhibition is unlikely to be the result of a side reaction between phenylglyoxal and a lysine residue.

For helpful and stimulating discussions during the early stages of this work, we are grateful to Prof. Jens Otto Wieth, in whose memory this paper is dedicated. We thank James V. Staros for informing us that *N*-alkyl amides can be protonated under some circumstances.
This work was supported by grant GM 26861 from the National Institutes of Health. M.L.J. is recipient of National Institutes of Health Research Career Development Award AM 01137.

Original version received 19 April 1985 and accepted version received 21 July 1985.

REFERENCES

Barzilay, M., and Z. I. Cabantchik. 1979. Anion transport in red blood cells. II. Kinetics of reversible inhibition by nitroaromatic sulfonic acids. *Membr. Biochem.* 2:255–281.

Bjerrum, P. J., J. O. Wieth, and C. L. Borders, Jr. 1983. Selective phenylglyoxalation of functionally essential arginine residues in the erythrocyte anion transport protein. *J. Gen. Physiol.* 81:453–484.

[3] Extracellular pH could also affect Cl-Cl exchange by titration of outward-facing sites on conformations of the protein in which the chloride binding site is facing inward.

Brock, C. J., M. J. A. Tanner, and C. Kempf. 1983. The human erythrocyte anion transport protein. Partial amino acid sequence, conformation, and a possible molecular mechanism for anion exchange. *Biochem. J.* 213:577–586.

Cabantchik, Z. I., M. Balshin, W. Breuer, and A. Rothstein. 1975. Pyridoxal phosphate. An anionic probe for amino groups exposed on the outer and inner surfaces of intact human red blood cells. *J. Biol. Chem.* 250:5130–5136.

Cabantchik, Z. I., and A. Rothstein. 1974*a*. Membrane proteins related to anion permeability of human red blood cells. I. Localization of disulfonic stilbene binding sites in proteins involved in permeation. *J. Membr. Biol.* 15:207–226.

Cabantchik, Z. I., and A. Rothstein. 1974*b*. Membrane proteins related to anion permeability of human red blood cells. II. Effect of proteolytic enzymes on disulfonic stilbene sites of surface proteins. *J. Membr. Biol.* 15:227–248.

Cacciola, S. O., H. Sigrist, M. Reist, Z. I. Cabantchik, and P. Zahler. 1984. Functional evidence for distinct interaction of hydrophobic arylisothiocyanates with the erythrocyte anion transport protein. *J. Membr. Biol.* 81:139–147.

Cohn, E. J., and J. T. Edsall. 1965. Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions. Hafner Publishing Co., New York. 445.

Dalmark, M. 1976. Effects of halides and bicarbonate on chloride transport in human red blood cells. *J. Gen. Physiol.* 67:223–234.

Drickamer, L. K. 1976. Fragmentation of the 95,000 dalton transmembrane polypeptide in human erythrocyte membranes. Arrangement of the fragments in the lipid bilayer. *J. Biol. Chem.* 251:5115–5123.

Dunker, A. K. 1982. A proton motive force transducer and its role in proton pumps, proton engines, tobacco mosaic virus assembly, and hemoglobin allosterism. *J. Theor. Biol.* 97:95–127.

Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.

Fröhlich, O. 1982. The external anion binding site of the human erythrocyte anion transporter: DNDS binding and competition with chloride. *J. Membr. Biol.* 65:111–123.

Funder, J., and J. O. Wieth. 1976. Chloride transport in human erythrocytes and ghosts. A quantitative comparison. *J. Physiol. (Lond.).* 262:679–698.

Grinstein, S., S. Ship, and A. Rothstein. 1978. Anion transport in relation to proteolytic digestion of band 3 protein. *Biochim. Biophys. Acta.* 507:294–304.

Gunn, R. B., and O. Fröhlich. 1979. Asymmetry in the mechanism for anion exchange in human red blood cell membranes. *J. Gen. Physiol.* 74:351–374.

Ho, M. K., and G. Guidotti. 1975. A membrane protein from human erythrocytes involved in anion exchange. *J. Biol. Chem.* 250:675–683.

Jennings, M. L. 1982. Reductive methylation of the two 4,4′-diisothiocyano-dihydrostilbene-2,2′-disulfonate-binding lysine residues of band 3, the human erythrocyte anion transport protein. *J. Biol. Chem.* 257:7554–7559.

Jennings, M. L. 1984. Oligomeric structure and the anion transport function of human erythrocyte band 3 protein. *J. Membr. Biol.* 80:105–117.

Jennings, M. L., M. Adams-Lackey, and G. H. Denny. 1984. Peptides of human erythrocyte band 3 protein produced by extracellular papain cleavage. *J. Biol. Chem.* 259:4652–4660.

Jennings, M. L., and J. S. Nicknish. 1984. Erythrocyte band 3 protein: evidence for multiple, membrane-crossing segments in the 17,000 dalton chymotryptic fragment. *Biochemistry.* 23:6432–6436.

Jennings, M. L., and J. S. Nicknish. 1985. Localization of a site of intermolecular cross-linking in human erythrocyte band 3 protein. *J. Biol. Chem.* 260:5472–5479.

Jennings, M. L., and H. Passow. 1979. Anion transport across the erythrocyte membrane, *in situ* proteolysis of band 3 protein, and cross-linking of the proteolytic fragments by 4,4′-diisothiocyano-dihydrostilbene-2,2′-disulfonate. *Biochim. Biophys. Acta.* 554:498–519.

Kampmann, L., S. Lepke, H. Fasold, G. Fritzsch, and H. Passow. 1982. The kinetics of intramolecular cross-linking of the band 3 protein in the red blood cell membrane by 4,4′-diisothiocyano-dihydrostilbene-2,2′-disulfonic acid (H_2DIDS). *J. Membr. Biol.* 70:199–216.

Kempf, C., C. Brock, H. Sigrist, M. J. A. Tanner, and P. Zahler. 1981. Interaction of phenylisothiocyanate with human erythrocyte band 3 protein. II. Topology of phenylisothiocyanate binding sites and influence of *p*-sulfophenylisothiocyanate on phenylisothiocyanate modification. *Biochim. Biophys. Acta.* 641:88–98.

Knauf, P. A. 1979. Erythrocyte anion exchange and the band 3 protein: transport kinetics and molecular structure. *Curr. Top. Membr. Transp.* 12:249–363.

Knauf, P. A., and A. Rothstein. 1971. Chemical modification of membranes. I. Effects of sulfhydryl and amino reactive reagents on anion and cation permeability of the human red blood cell. *J. Gen. Physiol.* 58:190–210.

Lepke, S., and H. Passow. 1976. Effects of incorporated trypsin on anion exchange and membrane proteins in human red blood cell ghosts. *Biochim. Biophys. Acta.* 455:353–370.

Mawby, W. J., and J. B. C. Findlay. 1982. Characterization and partial sequence of diiodosulphophenyl isothiocyanate-binding peptide from human erythrocyte anion transport protein. *Biochem. J.* 205:465–475.

Milanick, M. A., and R. B. Gunn. 1982. Proton-sulfate cotransport. Mechanism of H^+ and sulfate addition of the chloride transporter of human red blood cells. *J. Gen. Physiol.* 79:87–113.

Milanick, M. A., and R. B. Gunn. 1984. Proton-sulfate cotransport: external proton activation of sulfate influx into human red blood cells. *Am. J. Physiol.* 247:C247–C259.

Mueller, T. J., Y.-T. Li, and M. Morrison. 1979. Effect of endo-β-galactosidase on intact human erythrocytes. *J. Biol. Chem.* 254:8103–8106.

Nanri, H., N. Hamasaki, and S. Minakami. 1983. Affinity labeling of erythrocyte band 3 protein with pyridoxal 5-phosphate. *J. Biol. Chem.* 258:5985–5989.

Obaid, A. L., A. F. Rega, and P. J. Garrahan. 1972. The effects of maleic anhydride on the ionic permeability of red cells. *J. Membr. Biol.* 9:385–401.

Passow, H. 1969. Passive ion permeability of the erythrocyte membrane. An assessment of the scope and limitations of the fixed charge hypothesis. *Prog. Biophys. Mol. Biol.* 19:425–467.

Passow, H., H. Fasold, L. Zaki, B. Schumann, and S. Lepke. 1975. Membrane proteins and anion exchange in human erythrocytes. *In* Biomembranes: Structure and Function. FEBS Symposium Series. G. Gardos and I. Szasz, editors. Elsevier/North-Holland, Amsterdam. 35:197–214.

Passow, H., L. Kampmann, E. M. Gartner, B. Legrum, W. Ruffing, and L. Zaki. 1980. Anion transport across the red blood cell membrane and the conformation of band 3. *Ann. NY Acad. Sci.* 341:361–383.

Ramjeesingh, M., A. Gaarn, and A. Rothstein. 1984. Pepsin cleavage of band 3 produces its membrane-crossing domains. *Biochim. Biophys. Acta.* 769:381–389.

Rudloff, V., S. Lepke, and H. Passow. 1983. Inhibition of anion transport across the red cell membrane by dinitrophenylation of a specific lysine residue at the H_2DIDS binding site of the band 3 protein. *FEBS Lett.* 163:14–21.

Sigrist, H., C. Kempf, and P. Zahler. 1980. Interaction of phenylisothiocyanate with human erythrocyte band 3 protein. I. Covalent modification and inhibition of phosphate transport. *Biochim. Biophys. Acta.* 597:137–144.

Staros, J. V. 1982. *N*-Hydroxysulfosuccinimide active esters: bis(*N*-hydroxysulfosuccinimide) esters of two dicarboxylic acids are hydrophilic, membrane-impermeant, protein cross-linkers. *Biochemistry.* 21:3950–3955.

Staros, J. V., and B. P. Kakkad. 1983. Cross-linking and chymotryptic digestion of the extracytoplasmic domain of the anion exchange channel in intact human erythrocytes. *J. Membr. Biol.* 74:247–254.

Steck, T. L., J. Koziarz, M. K. Singh, G. Reddy, and H. Kohler. 1978. Preparation and analysis of seven major, topographically defined fragments of band 3, the predominant transmembrane polypeptide of human erythrocyte membranes. *Biochemistry.* 17:1216–1222.

Steck, T. L., B. Ramos, and E. Strapazon. 1976. Proteolytic dissection of band 3, the predominant polypeptide of the human erythrocyte membrane. *Biochemistry.* 15:1154–1161.

Steck, T. L., and J. Yu. 1973. Selective solubilization of proteins from red blood cell membranes by protein perturbants. *J. Supramol. Struct.* 1:220–232.

Wieth, J. O., O. S. Andersen, J. Brahm, P. J. Bjerrum, and C. L. Borders, Jr. 1982*a*. Chloride-bicarbonate exchange in red blood cells: physiology of transport and chemical modification of binding sites. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 299:383–399.

Wieth, J. O., P. J. Bjerrum, and C. L. Borders, Jr. 1982*b*. Irreversible inactivation of red cell chloride exchange with phenylglyoxal, an arginine-specific reagent. *J. Gen. Physiol.* 79:283–312.

Wieth, J. O., and P. J. Bjerrum. 1982. Titration of transport and modifier sites in the red cell anion transport system. *J. Gen. Physiol.* 79:253–282.

Wieth, J. O., M. Dalmark, R. B. Gunn, and D. C. Tosteson. 1973. The transfer of monovalent inorganic anions through the red cell membrane. *In* Erythrocytes, Thrombocytes, Leukocytes. E. Gerlach, K. Moser, E. Deutsch, and W. Wilmanns, editors. Thieme, Stuttgart. 71–76.

Zaki, L. 1981. Inhibition of anion transport across red blood cells with 1,2-cyclohexanedione. *Biochem. Biophys. Res. Commun.* 99:243–251.

Zaki, L., C. Gitler, and H. Passow. 1971. The effect of trinitrobenzene sulfonate on passive ion permeability of the human red blood cell. *Proc. Int. Union Physiol. Sci. XXV Int. Congress.* 9:616.