Anion Exchange Protein in Southeast Asian Ovalocytes: Heterodimer Formation between Normal and Variant Subunits†

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ABSTRACT: Chemical cross-linking has been used to determine the composition of the erythrocyte band 3 protein dimer in Southeast Asian ovalocytes (SAO). Individuals with SAO are heterozygous for a mutation in which residues 400−408 of band 3 are deleted. Normal and variant protein are present in equal amounts, but the SAO protein does not transport anions or bind stilbenedisulfonates with high affinity. We find that the rate constant for $^{35}SO_4^{2-}$ efflux from SAO cells is about 50% that of normal cells, but the time course is a single exponential, indicating that there is no detectable heterogeneity in the distribution of SAO band 3 in the population of cells. Treatment of intact cells with the homobifunctional crosslinker BS^3 (bis[sulfosuccinimido]suberate) produces similar amounts of covalent dimer in both normal and SAO cells. In SAO cells, copies of normal band 3 can be distinguished from SAO band 3 by treating with H_2DIDS to form a crosslink between major chymotryptic fragments (60 kDa and 35 kDa) within one subunit. Successive treatment of cells with [^{3}H]-4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate ([^{3}H]H_2DIDS), BS^3, and chymotrypsin gives ^{3}H-labeled products that include homodimer of normal band 3 as well as products of crosslinking normal band 3 with the 60- and 35-kDa fragment of SAO band 3. The results are in semiquantitative agreement with a model in which covalent dimer forms between normal and SAO subunits with the same probability as between two normal subunits. These results indicate that the normal copy of band 3, complexed in a heterodimer with SAO band 3, reacts with H_2DIDS as in normal cells. The normal copy of band 3 in the heterodimer may also be able to carry out anion transport, although more transport data will be necessary to settle this issue.

The 95-kDa transmembrane protein of the human erythrocyte membrane is known as band 3 (Fairbanks et al., 1971) and consists of two domains (Kopito & Lodish, 1985; Tanner et al., 1988). The N-terminal ~400 residues constitute a water-soluble cytoplasmic domain, the function of which is to serve as an attachment site for the membrane skeleton (Low, 1986; Bennett, 1985). The cytoplasmic domain also binds hemoglobin (Walder et al., 1984) and cytoplasmic enzymes (Low et al., 1989). The C-terminal ~500 residues form a hydrophobic membrane domain, which functions as an anion transporter [see Passow (1986)]. The physiological mode of this anion transport is the exchange of Cl^- for HCO_3^- as part of the process of CO_2 elimination (Wieth & Brahm, 1985). Because of its abundance in a readily available membrane, band 3 has served as a model system for the study of structure−function relations in a transport protein (Knauf, 1979; Passow, 1986; Jennings, 1989; Salhany, 1990).

A variety of biochemical and biophysical data have shown that band 3 is oligomeric, very likely a tetramer consisting of a pair of dimers rather than a tetramer with 4-fold rotational symmetry (Casey & Reithmeier, 1991; Schubert et al., 1992; Jennings, 1984). A recent structural map derived from two-dimensional crystals of the membrane domain dimer indicates extensive close contact between subunits in the portion of the protein that is imbedded in the bilayer (Wang et al., 1994).

There is considerable evidence for allosteric interactions between band 3 subunits, especially with regard to inhibitor binding (Salhany, 1989). For example, the binding of stilbenedisulfonate to one subunit lowers the affinity for reversible binding to the other subunit (Macara & Cantley, 1981; Pimplikar & Reithmeier, 1988; Salhany et al., 1993). Moreover, the covalent binding of DIDS[1] alters the temperature of unfolding of the membrane domain, but the relationship between subunit occupancy and transition temperature shift is not linear (Van Dort et al., 1994), indicating an interaction between subunits. The kinetics of transport inhibition by water-soluble carbodiimides also indicates that, at least under some conditions, reaction with one subunit affects the rate of reaction with the other (Bjerrum et al., 1989).

Although there is abundant evidence for subunit interactions as detected by inhibitor binding, the role of the oligomeric structure in the transport function of the protein is unclear. Inhibition of transport by covalently bound DIDS or H_2DIDS is a strictly linear function of occupancy (Cabantchik & Rothstein, 1974; Passow, 1986; Wieth & Brahm, 1985), with a maxium stoichiometry of 1 H_2DIDS/subunit (Jennings & Passow, 1979). Therefore, complete inhibition of one subunit does not prevent transport through the adjacent subunit. However, there may be subtle interac-

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[1] Abbreviations: SAO, Southeast Asian ovalocyte; BS^3, bis(sulfosuccinimido)suberate; H_2DIDS, 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; MOPS, 3-(*N*-morpholino)propanesulfonate; PVDF, poly(vinylidene difluoride); TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

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tions between subunits that can be significant determinants of transport rates under some conditions (Salhany & Cordes, 1992).

The functional properties of monomeric band 3 are controversial. Band 3 in nonionic detergent solution was shown by gel-filtration experiments to be dimeric or tetrameric (Casey & Reithmeier, 1991). Dissociation of dimers into monomers could not be accomplished without denaturing the protein (Boodhoo & Reithmeier, 1984; Casey & Reithmeier, 1991). On the other hand, reconstitution experiments by Schubert and co-workers have provided evidence that monomeric band 3 has functional (transport) properties that are similar to those of dimeric band 3 (Schubert et al., 1992). The reasons for the divergent results on this issue are not clear.

The purpose of this study was to investigate subunit interactions in the band 3 dimer in Southeast Asian ovalocytes (SAO). SAO band 3 has a deletion of amino acid residues 400−408, i.e., at the junction of the cytoplasmic and membrane domains (Jarolim et al., 1991; Mohandas et al., 1992; Schofield et al., 1992b). Red cells from individuals who are heterozygous for this deletion are more rigid than normal (Mohandas et al., 1984; Saul et al., 1984) and are also relatively resistant to invasion by malarial parasites (Kidson et al., 1981). Red cells from SAO heterozygotes (denoted as SAO red cells here) have roughly equal amounts of normal and SAO band 3 but about half the normal sulfate influx and half the normal number of high-affinity stilbenedisulfonate sites (Schofield et al., 1992a; Sarabia et al., 1993). These data strongly indicate that SAO band 3 does not transport anions or bind stilbenedisulfonates.

SAO band 3 has been expressed in *Xenopus* oocytes in the presence and absence of the expression of normal band 3; therefore, normal band 3 is not required for translocation of SAO band 3 to the oocyte plasma membrane (Groves et al., 1993). SAO band 3 has a slightly higher tendency than normal to form tetramers or higher oligomers when isolated in nonionic detergent (Sarabia et al., 1993). Crosslinking experiments with Cu^{2+}/*o*-phenanthroline showed that band 3 in SAO red cells has the same extent of cross-linking as in normal cells (Moriyama et al., 1992). However, it is not known whether normal and SAO band 3 subunits can associate in a heterodimer.

In this study we have used the homobifunctional active ester BS^3 (bis[sulfosuccinimido]suberate), which was originally shown by Staros (1982) to cross-link band 3 to a covalent dimer in intact cells. Normal band 3 was distinguished from SAO band 3 on the basis of labeling and intramolecular cross-linking of chymotryptic fragments with [^{3}H]H_2DIDS (Jennings & Passow, 1979). The results indicate that BS^3 forms both homodimers of two normal or two SAO subunits as well as heterodimers consisting of one normal and one SAO subunit.

EXPERIMENTAL PROCEDURES

Materials. Human blood from an individual who is heterozygous for the SAO mutation was generously provided by Professor P. S. Low, Purdue University. Band 3 from this donor has been characterized by Low and co-workers (Moriyama et al., 1992). The blood was drawn into citrate−phosphate−dextrose and stored at 4 °C until use. Human blood from each of the authors was drawn into EDTA and stored at 4 °C. Most of the studies were performed after the blood was stored less than 5 days, but no effect of length of storage was noted. Bis(sulfosuccinimido)suberate (BS^3) was purchased from Pierce, Rockford, IL. [^{3}H]H_2DIDS (4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate) was synthesized as described previously (Jennings et al., 1984). Enzymes (trypsin, chymotrypsin, endo-β-galactosidase) were purchased from Boehringer-Mannheim. Other buffers, salts, and electrophoresis reagents were obtained from Bio-Rad, Fisher, or Sigma Chemical Co.

Anion Transport Measurements. Cells were washed three times in at least 10 volumes of 100 mM K_2SO_4/10 mM MOPS, pH 7.0, with a 10-min incubation at 37 °C before each spin to allow Cl^- to exit the cells in exchange for SO_4^{2-}. Cells were then incubated 1 h, 37 °C, in the same medium containing 5 μCi/mL $Na^{35}SO_4$. Immediately prior to the efflux measurement, cells were washed three times in ice-cold K_2SO_4/MOPS and then resuspended at a 3% hematocrit in the same medium at 25 °C. At various times, aliquots were removed and centrifuged 30 s in a microfuge, and 200 μL of each supernatant was removed for determination of radioactivity. Results are plotted as $-\ln(1 - [\text{cpm}/\text{cpm}^\infty])$ vs time, where cpm^∞ was determined by lysing duplicate aliquots in 10% trichloroacetic acid.

Treatment with [^{3}H]H_2DIDS, Chymotrypsin, and BS^3. Cells were washed three times in 150 mM $KHCO_3$, pH 9, and then incubated at a 5% hematocrit in the same medium plus 10 μM [^{3}H]H_2DIDS (100 mCi/mmol) for 1 h, 37 °C. Cells were then washed three times in 150 mM KCl/10 mM HEPES, pH 7.4, and resuspended at a 50% hematocrit in KCl/HEPES plus 5 mM freshly dissolved BS^3. After an incubation of 1 h, 37 °C, remaining active ester was removed by adding either 50 mM glycine or 25 mM Tris and washing twice in 20 volumes of medium. Cells were treated with chymotrypsin (1 mg/mL) in 150 mM KCl/10 mM HEPES, for 1 h, 37 °C. Cells were then washed in 150 mM potassium acetate/20 mM sodium phosphate, pH 6, and incubated in the same medium 30−45 min at 37 °C, 75% hematocrit, with 0.01 unit endo-β-galactosidase/mL of cells to remove the polylactosyl carbohydrate from band 3; the resultant band on gels is considerably sharper (Mueller et al., 1979; Jennings et al., 1984).

Membrane Isolation. Cells were lysed in 40 volumes of 5 mM $NaHCO_3$, pH ~8, at 0 °C and centrifuged 20 min at 19 000 rpm. Two further washes in cold 5 mM $NaHCO_3$ produced nearly white membranes. In some experiments, unsealed membranes were treated with trypsin (50 μg/mL) in 150 mM KCl/10 mM HEPES, pH 7.4, for 30−45 min at 37 °C to remove the cytoplasmic domain of band 3. Trypsin was inhibited with TLCK (50 μg/mL), the membranes were centrifuged, and the loosely bound polypeptides were stripped by washing once in 0.1 N NaOH (Steck & Yu, 1973). Membranes were washed once more in 5 mM $NaHCO_3$, and protein was solubilized by heating 2 min at 100 °C in electrophoresis sample buffer. Electrophoresis was carried out in the Laemmli (1970) buffers, with acrylamide concentrations specified in the figure legends. Gels were stained and radioactivity was determined as described previously (Jennings et al., 1984). For N-terminal sequencing of the 20-kDa tryptic fragment, protein from a polyacrylamide gel was electroblotted to PVDF membrane (Millipore) in 20% methanol (Towbin et al., 1979). The band at 20 kDa was excised, and Edman degradation was performed by J. S.

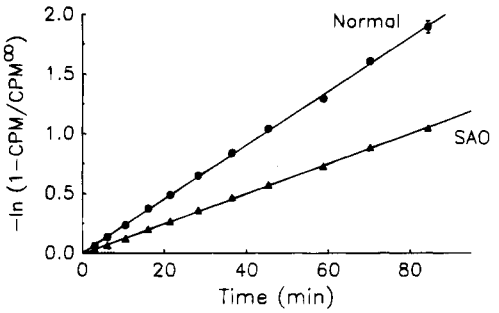

FIGURE 1: Sulfate transport in normal and SAO red cells. Cells were washed in 100 mM K_2SO_4/10 mM MOPS, pH 7.0, with incubation between washes to allow efflux of Cl^- and influx of SO_4^{2-}. The cells were then loaded with $^{35}SO_4^{2-}$, and the efflux was measured at 25 °C under conditions of constant SO_4^{2-} concentration and pH in the same K_2SO_4/MOPS medium. Duplicate suspensions were prepared for both normal and SAO cells, with a data point taken for each suspension at the indicated times. The mean of the two data points is plotted. Except for the last time point for normal cells, the range of the data (error bars) was smaller than the symbol size.

Smith, using an Applied Biosystems Model 475A sequencer.

RESULTS

Apparent Homogeneous Distribution of Normal and Variant Band 3. Schofield et al. (1992a) showed that erythrocytes from an individual heterozygous for the SAO variant have a $[^{35}S]SO_4^{2-}$ influx equal to about half (40% ± 6%) that of normal cells. The SO_4^{2-} influx in the study by Schofield et al. (1992a) was measured under conditions of initial flux, which gives a quantitative measure of the total number of ions crossing a unit area of membrane per minute. Initial influx, however, does not provide information about the distribution of transporters among different cells. To determine whether there is a major heterogeneity in the distribution of normal and variant band 3 in red cells from an SAO donor, the efflux of $[^{35}S]SO_4^{2-}$ was measured under equilibrium exchange conditions. If there were a subset of cells that contained either more or less of the variant band 3, then the efflux of tracer should not follow a single exponential time course.

Figure 1 shows that, in both normal and SAO red cells, the time course of $[^{35}S]SO_4^{2-}$ efflux at Donnan equilibrium in an all-SO_4^{2-} medium is not distinguishable from a single exponential. The rate constant for efflux in SAO cells is 0.55 times that in normal cells. We did not measure the intracellular SO_4^{2-} and water contents of these cells, but it is known that the density of Malayan ovalocytes is the same as that of normal cells (Mohandas et al., 1984). It is reasonable to expect, then, that the Donnan ratio and water content of SAO cells are normal. Therefore, the equilibrium anion exchange flux (ions per cell per minute) in SAO cells is about half normal. Moreover, the single-exponential time course is consistent with the idea that normal and SAO band 3 are distributed uniformly in the population of red cells. That is, the time course gives no evidence for a subpopulation of red cells that is enriched in either normal or SAO band 3. For example, if there were a distinct subpopulation of cells that has a rate constant for SO_4^{2-} efflux that is only half that of the remaining SAO cells, the size of this subpopulation is at most about 3% of the total (estimated by fitting the data to the sum of two exponentials differing in rate constant by a factor of 2). The experiments in the remainder of this paper are analyzed with the assumption that each red cell has an equal amount of normal and SAO band 3.

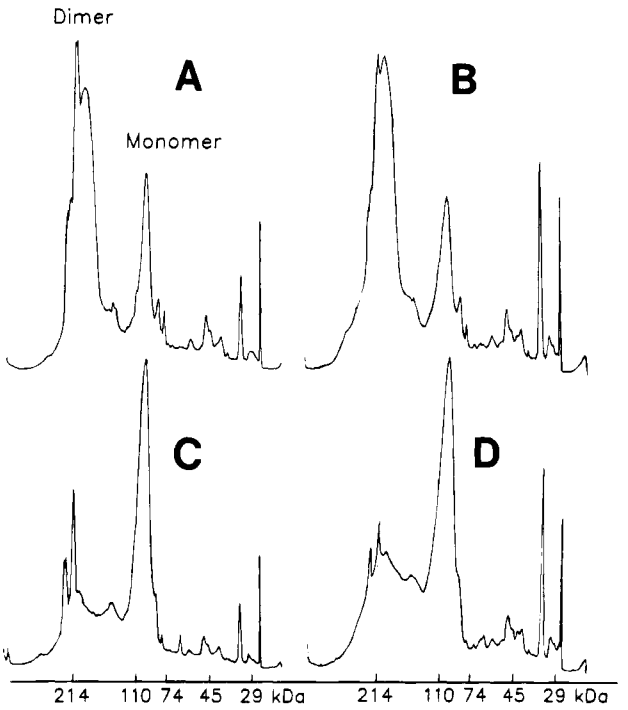

FIGURE 2: Cross-linking of normal and SAO band 3 to dimer with BS^3. Normal or SAO cells were incubated with or without 5 mM BS^3 for 1 h, 37 °C, in 150 mM KCl/10 mM HEPES, pH 7.4. The reaction was quenched with 25 mM Tris, and the cells were then treated with endo-β-galactosidase to remove most of the band 3 carbohydrate (Mueller et al., 1979). Membranes were isolated and stripped of peripheral protein with 0.1 N NaOH, and the protein was separated on a 5−12% polyacrylamide gel in SDS and stained with Coomassie blue. The positions of the band 3 dimer and monomer are indicated. The dimer comigrates with residual spectrin (the sharp bands superimposed on the broader dimer peak). (A) Normal cells, 5 mM BS^3. (B) SAO cells, 5 mM BS^3. (C) Normal cells, no BS^3. (D) SAO cells, no BS^3.

Covalent Intermolecular Cross-Linking of Band 3 from SAO Blood. The homobifunctional active ester BS^3 (bis-[sulfosuccinimido]suberate) cross-links band 3 in intact cells into a covalent dimer (Staros, 1982). The purpose of the current study was to determine whether, in SAO blood, the covalent cross-link forms preferentially between two copies of normal band 3 or if the cross-link can form as readily between normal and SAO band 3. Figure 2 compares the extent of covalent dimer formation following BS^3 treatment of normal and SAO red cells. In both cases BS^3 cross-links about 80% of the copies of band 3 into a covalent dimer. There is very little higher oligomer. (Salhany et al. [1990] showed that BS^3 can induce noncovalent tetramers [dimer of dimers] in band 3, but these dissociate under the high-temperature solubilization conditions used here.) We conclude that both normal and SAO band 3 can be cross-linked by BS^3 into covalent dimers, with negligible formation of covalent higher oligomers.

Intramolecular Cross-Linking of Normal Band 3 with H_2-DIDS. The dimer peak for SAO blood in Figure 2 is some combination of homodimers (normal/normal and SAO/SAO) and heterodimers of normal and SAO. To determine whether covalent heterodimers form between normal and SAO band 3, the copies of normal band 3 in SAO cells were reacted with H_2DIDS under conditions in which a covalent *intra*molecular cross-link is formed between the two major chymo-

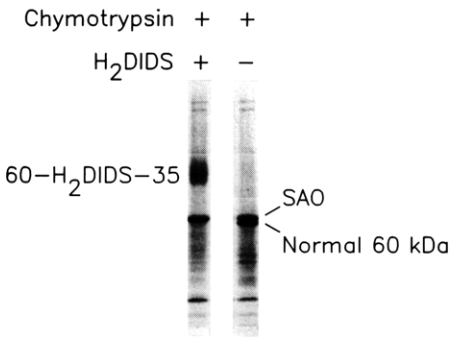

FIGURE 3: Intramolecular cross-linking of normal but not SAO band 3 fragments in SAO red cells. SAO cells were treated with chymotrypsin at pH 7.4, followed by incubation at pH 9 with or without 10 μM H_2DIDS. Membranes were isolated and alkali-stripped, and protein was separated on a 5−12% polyacrylamide gel. The positions of the 60-kDa fragment of normal band 3, its counterpart in SAO band 3, and the position of normal 60- and 35-kDa fragments cross-linked with H_2DIDS are indicated.

tryptic fragments (60 kDa and 35 kDa). Treatment of intact cells with chymotrypsin cleaves normal band 3 at Tyr 553 to produce membrane-bound fragments of about 60 and 35 kDa [see Steck et al. (1978)]. The SAO variant (Jones et al., 1990; Liu et al., 1990), in addition to the deletion of residues 400−408, also contains the Memphis mutation, which is a fairly common band 3 variant originally discovered by Mueller and Morrison (1977). The Memphis mutation is a point substitution of Glu for Lys at position 56 (Yannoukakos et al., 1991), which causes the N-terminal chymotryptic fragments in SAO red cells to migrate on SDS−polyacrylamide gels as a doublet of 60 kDa (normal) and 63 kDa (SAO). The nine-residue SAO deletion on the background of the Memphis mutation may shift the 63-kDa fragment slightly, but it is still easily resolved from the normal 60-kDa chymotryptic fragment.

It is known that, at high pH, H_2DIDS forms a covalent intramolecular cross-link between the 60- and 35-kDa fragments of normal band 3 in intact cells (Jennings & Passow, 1979; Okubo et al., 1994). When SAO red cells are treated with chymotrypsin at pH 7.4 followed by 10 μM H_2DIDS at pH 9, only the normal copies of the protein are cross-linked (Figure 3). The variant 63-kDa fragment and the complementary glycosylated 35-kDa fragment are neither labeled nor cross-linked by H_2DIDS, in agreement with the literature on stilbenedisulfonate binding by SAO band 3 (Schofield et al., 1992a; Sarabia et al., 1993).

Combined Cross-Linking with H_2DIDS and BS^3. The selective *intra*molecular cross-linking of normal band 3 with H_2DIDS provides a way to distinguish normal from SAO band 3 in an *inter*molecular cross-linking experiment with BS^3. The idea of the experiment is shown schematically in Figure 4. The cross-link formed by BS^3 is between the 60-kDa chymotryptic fragment of one subunit and the 35-kDa fragment of the other (Staros & Kakkad, 1982). The H_2-DIDS cross-link is between the 60- and 35-kDa fragments of the same subunit [see Jennings and Nicknish (1985) and Okubo et al. (1994)]. Therefore, a combination of H_2DIDS, BS^3, and chymotrypsin treatment of intact cells should give a product of 190 kDa for the normal homodimer (with two molecules of H_2DIDS per dimer). Heterodimers should migrate on gels more rapidly than homodimers, since only one subunit is internally cross-linked with H_2DIDS. The heterodimer should consist of a 95-kDa normal band 3 cross-linked to either the 60- or 35-kDa fragment of SAO band 3 (Figure 4).

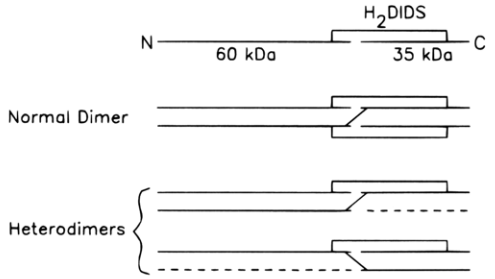

FIGURE 4: Rationale of combined intramolecular and intermolecular cross-linking experiment. (Top) The band 3 sequence is shown schematically, with the N-terminal 60- and 35-kDa chymotryptic fragments of the same subunit cross-linked covalently with H_2DIDS (Jennings & Passow, 1979). (Middle) The normal band 3 dimer is depicted, with the chymotryptic fragments of each subunit cross-linked with H_2DIDS, and an intermolecular cross-link formed by BS^3 (diagonal line). The cross-link is between unlike chymotryptic fragments on adjacent subunits (Staros & Kakkad, 1983). (Bottom) The two possible covalent heterodimers formed between normal and SAO band 3. Normal band 3 is internally cross-linked with H_2DIDS. BS^3 forms a cross-link between normal band 3 and one of the chymotryptic fragments of SAO band 3. The dashed lines represent SAO fragments that are not covalently cross-linked. The heterodimer therefore migrates on gels as the covalent complex consisting of two 60-kDa fragments and one 35-kDa fragment or one 60-kDa fragment and two 35-kDa fragments.

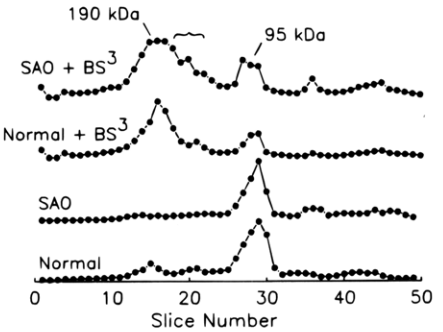

FIGURE 5: Combined [^{3}H]H_2DIDS labeling and cross-linking with BS^3. Normal or SAO cells were treated successively with chymotrypsin at pH 7.4 and [^{3}H]H_2DIDS at pH 9 and then incubated with or without 5 mM BS^3 for 1 h at 37 °C. Membranes were isolated and alkali-stripped, and the protein was separated electrophoretically as in Figure 2. The profiles of radioactivity (cpm/slice) as a function of distance from the top of the resolving gel (slice 1) have been normalized for the purpose of comparison such that the peak cpm is the same height for each lane, to make up for different amounts of protein in each lane. The peak cpm/slice were 920 (normal), 590 (normal + BS^3), 1250 (ovalocyte), and 750 (ovalocyte + BS^3). The approximate position of counts attributable to heterodimer (between homodimer and monomer) is indicated by the bracket.

Figure 5shows the result of the combined cross-linking of band 3 in chymotrypsin-treated normal and SAO red cells with [^{3}H]H_2DIDS (intramolecular) and BS^3 (intermolecular). To a reasonable first approximation, the only species labeled with ^{3}H are the copies of normal band 3. In normal cells without BS^3, label is mainly in band 3; BS^3 cross-links about 80% of the label into the position of the dimer, consistent with the gel scans of unlabeled protein in Figure 2. This result confirms that although stilbenedisulfonates alter the dimer−tetramer associations of band 3 (Salhany et al., 1990, 1991), the presence of H_2DIDS does not interfere with covalent intermolecular cross-linking by BS^3 (Jennings & Nicknish, 1985).

In SAO cells the same combined cross-linking protocol gives a different labeling pattern. Without BS^3, the [^{3}H]H_2-DIDS is mainly in band 3, as expected; there is less labeling per unit protein, because only half the copies of band 3 are labeled. BS^3 causes much of the label to move to the position of the dimer, but a significant amount of label migrates between the monomer and dimer, consistent with the idea that normal band 3 has been cross-linked with either the 60- or 35-kDa fragment of SAO band 3, i.e., as a heterodimer.

Combined H_2DIDS and BS^3 Cross-Linking Followed by Internal Trypsin. Although the data in Figure 5 (and two other similar experiments) provide evidence for heterodimer formation between normal and SAO band 3, the relative amounts of homodimer and heterodimer cannot be estimated quantitatively because the bands are not well resolved. To try to get a semiquantitative estimate of the amount of heterodimer, the cytoplasmic domain of band 3 (residues 1−360) was removed by treatment of unsealed membranes with trypsin, which removes the cytoplasmic domain and thereby converts the 60-kDa chymotryptic fragment to a 17-kDa membrane-bound fragment (Steck et al., 1976, 1978; Mawby & Findlay, 1982). The rationale for using trypsin was to increase the resolution of the cross-linked homodimer relative to the heterodimer.

The conditions of trypsin digestion of unsealed membranes used in these experiments should cause cleavage only at Lys 360 (Mawby & Findlay, 1982) and not elsewhere in the membrane domain (Lepke & Passow, 1976). At much lower ionic strength, trypsin also cleaves at Lys 743, but this cleavage is prevented by covalently bound H_2DIDS (Jennings et al., 1986). We found, however, that SAO band 3 is cleaved by trypsin in 150 mM KCl to produce a 20-kDa C-terminal fragment, which does not stain well with Coomassie blue but is easily detected with a monoclonal antibody (data not shown). Edman degradation showed that the fragment has the same N-terminal sequence as the fragment produced at low ionic strength from normal band 3 (Jennings et al., 1986) and therefore results from cleavage at Lys 743.

The fact that SAO band 3, but not normal band 3, is cleaved by trypsin at Lys 743 in unsealed membranes provides an independent way to test for the presence of BS^3 heterodimer, without using chymotrypsin cleavage of intact cells. Cells were labeled with [^{3}H]H_2DIDS and then treated with BS^3 to produce covalent dimers. Membranes were isolated and treated with trypsin to remove the cytoplasmic domain and also to cleave SAO band 3 at Lys 743. As expected, the [^{3}H]H_2DIDS label migrates mainly in the position of the covalent dimer of membrane domains (104 kDa) in normal cells. In protein from SAO cells, however, there is a stained and labeled peak at about 85 kDa (Figure 6). In the same membranes, immunoblots show a large amount of 20-kDa C-terminal fragment (data not shown), which of course is not labeled by [^{3}H]H_2DIDS because it is produced only from the copies of SAO protein. The peak at 85 kDa represents a heterodimer of normal membrane domain cross-linked to SAO band 3, which has been cleaved by trypsin to remove about 20 kDa of mass, resulting in a product of about 85 kDa.

To try to increase further the difference in mobility between homodimer and heterodimer, an experiment was performed with chymotrypsin treatment of intact cells as well as trypsin treatment of unsealed membranes. Again, the rationale for the double proteolysis is to reduce the size of

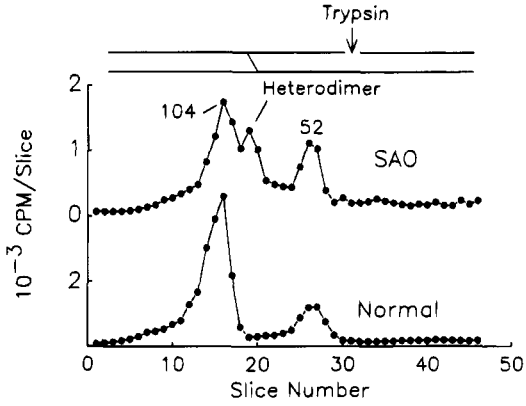

FIGURE 6: Combined [^{3}H]H_2DIDS labeling and cross-linking with BS^3, followed by trypsin treatment of unsealed membranes. Normal or SAO cells were treated with [^{3}H]H_2DIDS at pH 9 followed by 0 or 5 mM BS^3 at pH 7.4. Membranes were isolated and treated with 50 μg/mL trypsin in 150 mM KCl/10 mM HEPES, pH 7.4. Trypsin treatment under these conditions cleaves the 20-kDa C-terminal fragment from SAO but not from normal band 3. Distribution of radioactivity on a 6−18% gel is shown for normal (lower) and SAO (upper) cells; each lane was loaded with 75 μg of protein. The positions of the membrane domain monomer (52 kDa), homodimer (104 kDa), and heterodimer (about 85 kDa) are indicated.

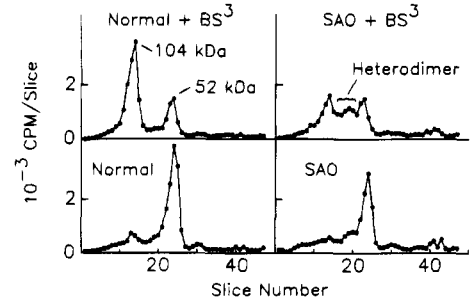

FIGURE 7: Combined [^{3}H]H_2DIDS labeling at pH 9 and cross-linking with 0 or 5 mM BS^3 at pH 7.4, followed by chymotrypsin treatment of intact cells and trypsin treatment of unsealed membranes. Distribution of radioactivity on a 6−18% gel (75 ± 15 μg of protein/lane) is shown for normal, normal + BS^3, SAO, and SAO + BS^3. The positions of the membrane domain monomer (52 kDa) and homodimer (104 kDa) are indicated. The heterodimer zone (bracket, upper right) is broader than in Figure 6, probably because it represents 52-kDa normal membrane domain cross-linked to either of two different SAO fragments (see text).

any fragment of SAO band 3 that is cross-linked to [^{3}H]H_2-DIDS-labeled normal band 3. Figure 7 shows an experiment in which band 3 in normal or SAO cells was labeled with [^{3}H]H_2DIDS at pH 9, then treated with or without BS^3, and then incubated with chymotrypsin. Unsealed membranes were isolated and treated with 50 μg/mL trypsin as in Figure 6.

In normal cells the result of these treatments is that most of the counts are in the position of the 104-kDa dimer of membrane domains. Each copy of the normal dimer has been cleaved with chymotrypsin into 17- and 35-kDa fragments, but the two fragments are cross-linked with H_2-DIDS, so the mobility of the normal dimer is unaffected by chymotrypsin. In SAO cells, however, there is substantial radioactivity migrating between the homodimer (104 kDa) and monomer (52 kDa). Since only the normal copies of band 3 have been labeled, this material must consist of normal band 3 (17−H_2DIDS−35) cross-linked to a fragment of SAO band 3. The zone attributed to heterodimer in Figure

7 is broader than that in Figure 6, probably because the cross-link can form with either of two different fragments of SAO band 3: the 17-kDa segment (Gly 361−Tyr 551) or the glycosylated fragment bounded by the external chymotrypsin site and the internal trypsin site at Lys 743. The total radioactivity in the heterodimer zone is roughly 75% that in the homodimer (see below).

DISCUSSION

Covalent Heterodimers of Normal and SAO Band 3. The main new finding presented here is that BS^3 treatment of SAO red cells produces cross-linked heterodimers consisting of normal and SAO band 3. In order to interpret these findings as evidence for heterodimers, we make the following two assumptions.

(1) H_2DIDS labels only normal and not SAO band 3. Schofield et al. (1992a) showed that, at pH 7, 5 μM [^{3}H]H_2-DIDS labels only the normal copies of band 3. The conditions used here (pH 9, 10 μM) could potentially lead to labeling of sites on SAO band 3 that are not labeled at pH 7. However, in SAO cells that have been treated with chymotrypsin, the 63-kDa SAO fragment persists as a major stained band on the gel, but this band is only very slightly labeled (<15% of the labeling of the normal copies of band 3, as found in Figure 5 and three other experiments).

(2) All the normal copies of band 3 have been labeled and internally cross-linked between the 60- and 35-kDa fragments. This assumption is verified by the almost complete (>90%) disappearance of the 60-kDa (normal) band in SAO cells treated with chymotrypsin followed by H_2DIDS (Figure 3).

Since essentially all the normal band 3, and very little of the SAO band 3, has been labeled and internally cross-linked with H_2DIDS, there is only one likely explanation for the presence of labeled, cross-linked products larger than the monomer but smaller than the homodimer: labeled copies of normal band 3 have been cross-linked to SAO band 3. The final product on the gel is smaller than the homodimer because SAO band 3 was subsequently cleaved at the outer surface of intact cells with chymotrypsin and/or at the cytoplasmic surface of unsealed membranes by trypsin. The products that we attribute to heterodimer do not arise from the cross-linking of some other protein with normal band 3 in SAO cells, because simple treatment of SAO cells with BS^3 (without proteolysis) causes formation of a dimer of the same electrophoretic mobility as in normal cells (Figure 2).

Is the Covalent Dimer Made of the Same Two Subunits as the Noncovalent Dimer? Our working assumption is that the covalent dimer formed by BS^3 is between the same two subunits that are tightly associated in a native dimer. However, consideration must be given to the possibility that the covalent cross-link is between two band 3 subunits that are not tightly associated in a dimer in the membrane. It is possible in principle that, in the native dimer, there is no pair of appropriately spaced external lysine residues to allow a cross-link with BS^3. Instead, BS^3 could cross-link two subunits of different dimers that either are associated in the tetramer or diffuse laterally and rotationally to the appropriate position and orientation to be cross-linked.

Although lateral and rotational diffusion no doubt take place during the cross-linking reaction, we believe that the BS^3 cross-link involves a preexisting stable structure, on the basis of the fact that the cross-link forms only between two unlike (60 kDa and 35 kDa) fragments of different subunits (Staros & Kakkad, 1983; Jennings & Nicknish, 1985). That is, there is no cross-link of two 60-kDa or two 35-kDa fragments. If the cross-link formed simply during lateral and rotational diffusion because the residues are on the periphery (in the plane of the membrane) of the protein, then we would expect there to be more possible combinations of cross-linked fragments (and other cross-linked products, e.g., between band 3 and glycoprotein or band 3 and lipid, thus lowering the yield of dimer). Instead, the predominant product is the dimer formed by cross-linking the 60-kDa fragment of one subunit with the 35-kDa fragment of the other, with essentially no covalent tetramer and no products of cross-linking with other proteins.

Another important fact in this regard is the finding of Casey and Reithmeier (1991) that covalent cross-linking of normal band 3 in intact cells with 3,3′-dithiobis(sulfosuccinimido) suberate does not change the distribution of dimer and higher oligomer observed during subsequent gel filtration of purified band 3. Therefore, the covalently cross-linked dimer appears to be between the same two subunits that tightly associate during band 3 purification. It is also worth mentioning that the combination of external cross-linking with BS^3 and cross-linking of cytoplasmic domains with Cu^{2+}/*o*-phenanthroline (Steck, 1972) produces both dimers and tetramers (Jennings & Nicknish, 1985). Cu^{2+}/*o*-phenanthroline by itself also produces both dimers and tetramers, with yields that are similar to those produced by combined external BS^3 and cytoplasmic Cu^{2+}/*o*-phenanthroline. These findings are evidence that the external and internal cross-links are between the same pair of subunits.

SAO band 3 has a higher tendency than normal to form oligomers larger than dimer (Sarabia et al., 1993). However, the extent of covalent dimer formation by BS^3 is very similar in normal and SAO red cells (Figure 2). If the BS^3 dimer were a consequence of a cross-link between subunits of two different dimers in the tetramer, there should be more covalent dimer in SAO than in normal cells. The fact that the extent of covalent cross-linking is so similar in SAO and normal cells is further indication that the covalent dimer involves the same two subunits that are associated in the native dimer.

Estimates of the Proportion of Heterodimers. If normal and SAO subunits associate into dimers without any preference for either form, then the population of dimers should be 50% heterodimers and 25% of each kind of homodimer (normal/normal or SAO/SAO). The proportions of covalent normal/SAO heterodimer and normal/normal homodimer can be estimated from the relative amounts of [^{3}H]H_2DIDS in the positions of homodimer and heterodimer in the gel in Figures 6 and 7. If there were twice as many heterodimers as normal/normal homodimers, there should be equal labeling of both peaks, because both subunits of the homodimer are labeled, whereas only one subunit of the heterodimer is labeled. In Figures 6 and 7, the heterodimer peak contains about 75% as much radioactivity as the homodimer peak, although there is considerable uncertainty in this estimate because the peaks are not completely resolved, and the choice of baseline is somewhat arbitrary. From the raw data, though, it appears that homodimer formation is more likely than that of heterodimer.

As mentioned above, there is small but detectable labeling of SAO band 3 by [^{3}H]H_2DIDS. This could cause an overestimate of the number of heterodimers, but any such overestimate is very minor. For example, in Figure 7, there are detectable counts in the position of the 17-kDa fragment of SAO band 3 (slices 40−41, lower right). Some of these counts disappear after BS^3 cross-linking (Figure 7, upper right) and presumably contribute to the apparent heterodimer. However, the quantitative contribution of these counts is estimated to be only 6% of the total attributed to the heterodimer.

A potentially larger error in the estimate of heterodimer is in the other direction: the amount of homodimer could be overestimated because of an intramolecular BS^3 crosslink in SAO band 3. In normal band 3, BS^3 forms an intramolecular cross-link between the 60- and 35-kDa chymotryptic fragments (Staros & Kakkad, 1983). The exact locations of the cross-linked lysine residues are not known, but stilbenedisulfonates block the intramolecular BS^3 cross-link (Jennings & Nicknish, 1985). In SAO band 3, BS^3 does not appear to cause extensive intramolecular cross-linking of the two chymotryptic fragments; if there were intramolecular cross-linking by BS^3, then even the heterodimer would migrate in the position of the homodimer, and we would see no radioactivity other than in the homodimer and monomer position. However, there could be some intramolecular cross-linking of SAO band 3 by the high BS^3 concentrations used here. Accordingly, the amount of homodimer may be overestimated, because the homodimer peak on the gel could include heterodimer in which the 60-kDa (17-kDa) and 35-kDa SAO fragments have been cross-linked to each other with BS^3.

In summary, it is very difficult to make a quantitative estimate of the amounts of labeled homodimer and heterodimer, not only because the various species are not completely resolved on gels but also because there could be a significant overestimate of the amount of homodimer. Therefore, although there appear to be more counts in the homodimer than heterodimer peak in Figures 5−7, we cannot conclude that the homodimer forms preferentially. The data are also consistent with equal probability of homodimer (normal/normal) and heterodimer (normal/SAO) formation. The main conclusion that can be drawn is that significant covalent heterodimer is present, amounting to at least a third and possibly as much as half the normal copies of band 3.

Anion Transport Rates in SAO Cells. The $^{35}SO_4^{2-}/SO_4^{2-}$ exchange data in Figure 1 were carried out with symmetric, saturating concentration of substrate (Schnell & Besl, 1984). Therefore, minor abnormalities in the substrate affinity in SAO cells, if present, should have no effect on the results. Our data indicate that the SO_4^{2-} flux at Donnan equilibrium in SAO cells is about 55% of normal. This result is in approximate agreement with Schofield et al. (1992a), who found that the initial SO_4^{2-} influx into SAO red cells is 40% ± 6% of normal. Moriyama et al. (1992) found that in SAO red cells the influx of phosphoenolpyruvate, a band 3 substrate (Hamasaki & Kawano, 1987), is 42% of normal. In our view all of these data are consistent with an anion transport rate in SAO cells equal to 50% that in normal cells. It is of course possible that, under the equilibrium exchange conditions used here, the flux really is slightly larger than 50% of normal, and under net influx conditions the flux is slightly less than 50%. To establish the significance of these small differences would require much further study.

It will be important for future work to characterize more precisely the anion transport rate in SAO cells under a variety of conditions. If the actual transport rate is only 40% of normal, and if 33% of the normal band 3 is in heterodimers (lower limit of our estimates), then the anion flux mediated by heterodimers could be small, because the observed number of normal homodimers would be enough to account for most of the flux. On the other hand, if the transport rate is indeed 50% of normal, then heterodimers must make a major contribution to the flux.

In this context, it should be mentioned that Schofield et al. (1992) showed that, in SAO cells, there is a strictly linear decrease in SO_4^{2-} flux with increasing levels of covalently bound DIDS. Therefore, all DIDS binding sites (on normal band 3) appear to contribute equally to the anion flux. The data presented here indicate that a sizable portion (33−50%) of the H_2DIDS binding sites are in normal/SAO heterodimers. Our data, combined with the transport data of Schofield et al. (1992), are consistent with the idea that normal band 3 in a heterodimer can transport anions at essentially normal rates. However, this issue will not be settled without a much more thorough characterization of transport in SAO cells. Even if the heterodimer is eventually shown to transport anions, it would still not be possible to conclude that the subunits act independently; the heterodimer could still function by a concerted mechanism but at half the normal rate.

It should be emphasized that all the data related to heterodimer formation in this paper were obtained in cells treated with H_2DIDS. Salhany et al. (1990) showed that covalently bound DIDS alters the quaternary state of band 3. Treatment of cells with BS^3 in the presence of DNDS or pyridoxal phosphate at the stilbenedisulfonate site induces the formation of a tetramer consisting of a noncovalently associated pair of covalent dimers (Salhany et al., 1990). If DIDS or BS^3 is present at the stilbenedisulfonate site, very little tetramer is formed. Therefore, occupancy of the stilbenedisulfonate site influences the state of association of covalent dimers into tetramers, and it is possible that bound H_2DIDS in our experiments altered the associations between dimers. However, the focus of these studies is the nature of the dimer itself, and stilbenedisulfonates do not cause destabilization of the dimer (Casey & Reithmeier, 1991). It is therefore unlikely that the use of H_2DIDS affects the distribution of homodimers and heterodimers.

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