A Role for Band 4.2 in Human Erythrocyte Band 3 Mediated Anion Transport†

Saira Malik, Malkit Sami, and Anthony Watts*

Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

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ABSTRACT: Human erythrocyte band 3 was purified essentially free of peripheral proteins, in particular band 4.2, using affinity chromatography. Band 3 protein was then reconstituted into liposomes of lipid type and ratio approximating that of erythrocyte membranes. Stilbenedisulfonate inhibition of band 3 mediated efflux of radiolabeled sulfate from preloaded liposomes was used to test the functionality and correct orientation of the protein. When sulfate efflux, mediated by purified band 3, was compared with partially purified band 3, which contained detectable amounts of bands 4.1 and 4.2, a clear difference in efflux was measured. Sulfate efflux was ~30% faster from liposomes containing purified band 3 compared with those containing partially purified protein. In order to investigate further any specific effect of band 4.2 protein on band 3 mediated anion transport, band 4.2 was purified. Increasing amounts of band 4.2 were complexed with purified band 3 and then reconstituted into liposomes. Increasing amounts of band 4.2 complexed with band 3 caused a decrease in band 3 mediated anion transport. The effect of band 4.2 on band 3 mediated anion transport appears to be specific since increasing concentrations of band 4.2 added exogenously to band 3 in reconstituted vesicles (rather than complexed with band 3 before reconstitution) produced no significant changes in sulfate efflux. Further, when increasing amounts of band 4.2 were added to the functionally active transmembrane domain of band 3 and then reconstituted into vesicles, there was also no significant change in sulfate efflux. These results indicate that one of the roles for band 4.2 *in situ* is possibly as a modulator of band 3 anion transport through its specific interaction with the cytoplasmic domain of band 3.

The erythrocyte anion transporter, band 3, is a 95-kDa integral membrane glycoprotein and is responsible for anion exchange in the red cell (Jay & Cantley, 1986; Jennings, 1989; Salhany, 1990). Erythrocyte anion exchange has been shown to be sensitive to inhibition by stilbenedisulfonates (Cabantchik & Rothstein, 1974), and these inhibitors have been used to provide evidence for the functional integrity of reconstituted protein samples (Scheuring *et al.*, 1986). Band 3 accounts for ~50% of the intrinsic membrane protein of the erythrocyte (Steck, 1974) and has two distinct domains which can be obtained on mild trypsin treatment of erythrocyte membranes (Steck *et al.*, 1976). The 55-kDa transmembrane domain contains the anion-transporting site and can mediate anion exchange independently of the cytoplasmic domain (Grinstein *et al.*, 1978). The underlying erythrocyte cytoskeletal network is linked to the erythrocyte membrane via ankyrin binding to the 40-kDa cytoplasmic domain of band 3, which contains the binding sites for ankyrin, hemoglobin, and glycolytic enzymes (Low, 1986). The cytoplasmic domain also has a distinct binding site for band 4.2 (Korsgren & Cohen, 1988), a protein for which no specific function has been attributed. However, there has been a report that band 4.2 interacts with ankyrin and may stabilize ankyrin in the membrane (Rybicki *et al.*, 1988). The amino acid sequences of mouse (Kopito & Lodish, 1985), chicken (Cox & Lazirides, 1988), and human (Tanner *et al.*, 1988) erythrocyte band 3 proteins have been deduced by cDNA sequencing. The sequences of the transmembrane domain are highly conserved and it has been predicted that the transmembrane domain transverses the membrane up to 14 times using hydropathy data (Lux *et al.*, 1989).

Although initial studies on the anion transport capability of the transmembrane domain show that anion transport will occur in the absence of the cytoplasmic domain (Grinstein *et al.*, 1978), there have been reports in the literature indicating the possibility of heterotropic allosteric modulation of band 3 anion transport: phosphorylation of the cytoplasmic domain of band 3 increases anion transport (Bursaux *et al.*, 1984). In addition, light scattering measurements have shown that hemoglobin binds with greater affinity to the cytoplasmic domain of band 3 when DIDS[1] is bound to the transmembrane domain (Salhany *et al.*, 1980), and addition of hemoglobin to band 3 reconstituted vesicles tends to increase band 3 mediated phosphate transport (Ducis *et al.*, 1988). Ankyrin is another possible modulator of the transmembrane domain conformation of band 3, as it has been shown that the binding of H_2DIDS results in a tightly bound state of ankyrin to the cytoplasmic domain of band 3 (Hsu & Morrison, 1983).

To assess the effect of band 4.2 association on band 3 mediated anion transport, band 3 was purified essentially free of any associated proteins using affinity chromatography and reconstituted functionally. Inhibition of band 3 transport by selected inhibitors was used as an indicator of the integrity of the reconstituted protein system and to show the correct orientation of band 3 in reconstituted vesicles.

Transport was measured in reconstituted membranes containing purified band 3 and compared with complexes of band 3–band 4.2 containing varying amounts of band 4.2. To demonstrate the specificity of the effect of band 4.2 on band

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* To whom correspondence should be addressed.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMSF, phenylmethanesulfonyl fluoride; PS, phosphatidylserine; SM, sphingomyelin; TMD, transmembrane domain of band 3; TLC, thin-layer chromatography; Tris tris(hydroxymethyl)methylamine; *p*-cmb, *p*-chloromercuribenzoic acid; Tris, tris(hydroxymethyl)aminomethane.

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3 mediated transport, sulfate efflux was also measured for band 3 reconstituted vesicles where band 4.2 was added exogenously after vesicle formation. Further, sulfate efflux mediated by the transmembrane domain of band 3 in the presence and absence of band 4.2 was also measured.

MATERIALS AND METHODS

Recently outdated human blood was obtained from the Oxfordshire Blood Transfusion Service, lipids were purchased from Lipid Products, Triton X-100 (reduced form) was obtained from Aldrich Chemical Co., Bio-Gel P6-DG desalting gel and the SM2 Bio-Beads were from Bio-Rad, [^{3}H]Triton X-100 was purchased from New England Nuclear Chemicals, Sepharose 4B was obtained from Pharmacia, and [^{3}H]octyl glucoside was obtained from Amersham International. The rest of the materials were of the highest analytical grade.

Preparation of Protein Samples. (*A*) *Band 3.* Erythrocyte membranes (20 mL; protein content, 3 mg/mL), prepared as described in Dodge *et al.* (1963), were extracted on ice for 1 h with 2 volumes of extraction buffer (10 mM Tris-HCl, pH 8, 10 mM NaCl, and 0.5 mM EDTA) containig 1% Brij-58. Brij-58 specifically removes glycophorin from the membrane. The extracted ghosts were then centrifuged (30000*g*, 20 min, 4 °C). The supernatant, enriched with glycophorin, was discarded and the pellet was resuspended in twice the original amount of erythrocyte membrane volume of buffer containing 0.5% Triton X-100. This suspension was then extracted on ice for 1 h. The extract was centrifuged (30000*g*, 20 min, 4 °C). The pellet, enriched with peripheral proteins, was discarded and the supernatant was applied to a preequilibrated DEAE-cellulose column. The column was first washed with twice the column volume of buffer containing 0.5% Triton X-100 and 100 mM NaCl, which removed the majority of contaminating proteins. The column was then eluted with twice the column volume of buffer containing 0.5% Triton X-100 and 200 mM NaCl. This eluant, containing band 3 and some peripheral proteins, was then applied directly to a preequilibrated *p*-cmb affinity gel column. The affinity gel was prepared according to Lukacovic *et al.* (1981). This column was washed with twice the column volume of buffer containing 0.5% Triton X-100 and 1 M KCl. The high-salt step removes proteins most strongly associated with band 3, mainly band 4.2. Band 3 was finally eluted with twice the column volume of buffer containing 0.5% Triton X-100 and 15 mM β-mercaptoethanol. Band 3 could be stored for up to 3 days in 25 mM β-mercaptoethanol before aggregation started to occur, as shown by analytical centrifugation.

(*B*) *Band 4.2.* Band 4.2 was prepared according to Korsgren and Cohen (1986).

(*C*) *TMD of Band 3.* The preparative method followed for the transmembrane domain was that of Maneri and Low (1988).

(*D*) *Preparation of Band 3–Band 4.2 Complexes.* A number of complexes of band 3–band 4.2 were prepared. Band 3 (10 mg) and varying amounts of band 4.2, to give mole ratios of band 4.2 associated with band 3 of 0, 0.2, 0.25, 0.33, and 0.5, were incubated (4 °C, 6 h). Uncomplexed band 4.2 was removed by gel filtration as follows. A Sepharose 4B column (1 cm × 90 cm) was initially calibrated with thyroglobulin (Stokes radius 8.6 nm) and aldolase (Stokes radius 4.6 nm) (LeMaire *et al.*, 1980). The column was then equilibrated with buffer (10 mM Tris-HCl, pH 8, 10 mM NaCl, and 0.5 mM EDTA) before addition of the complex. Aliquots (2 mL) were collected from the column and run on SDS gels. The fractions containing the complex were pooled.

Preparation of Reconstituted Samples. A lipid mixture (30 mg) approximating the type and ratio found in the human erythrocyte membrane (egg PC 25%, egg PE 22%, bovine PS 10%, bovine SM 18%, cholesterol 25%) (Devaux & Seigneuret, 1985) in chloroform/ethanol was first dried under nitrogen and then under vacuum. The dried lipid film was hydrated with reconstitution buffer (10 mM Tris-HCl, pH 8, 10 mM NaCl, 0.5 mM EDTA, and 25 mM β-mercaptoethanol) and was solubilized by mixing with a minimum volume of a 10% (w/v) solution of octyl glucoside until the solution went clear. To this lipid mixture was then added protein (10 mg) to give the desired lipid/protein ratio, and the lipid/protein mixture was vortexed briefly and dialyzed overnight against reconstitution buffer (4 L) containing SM2 Bio-Beads (3 g). The opalescent solution was centrifuged (60000*g*, 60 min) to pellet the vesicles, which were resuspended in a minimum volume of buffer and then layered on top of a linear sucrose density gradient (12–50%) and centrifuged (50000*g*, 18 h). The largest band of vesicles, located near the middle of the tube, was removed and dialyzed against reconstitution buffer (5 L) containing SM2 Bio-Beads (3 g) for 72 h, with the buffer and Bio-Beads being changed every 12 hours. The reconstituted protein samples were finally collected by centrifugation (50000*g*, 60 min).

Protein-free vesicles were prepared as described above except that prior to dialysis the volume of the protein solution was replaced by an equivalent volume of buffer (10 mM Tris-HCl, pH 8, 10 mM NaCl, and 0.5 mM EDTA).

Transport Assay. The transport of radiolabeled sulfate under equilibrium exchange conditions was measured for protein-free and protein-containing vesicles at 37 °C as described in Scheuring *et al.* (1986).

Analytical Methods. (*A*) *Protein Content.* Protein content was quantified using the modified Lowry method and samples were analyzed by SDS–7% polyacrylamide slab gels (Laemmli, 1970), stained with Coomassie Blue, and scanned at 660 nm with a densitometer. In samples containing band 3 and band 4.2 proteins, protein content was calculated by comparing densitometer scans of the complexes with densitometer scans of known quantities of band 3 and band 4.2.

(*B*) *Lipid Content.* The presence of endogenous lipids in each protein sample measured in all the protein preparations. Each protein preparation was exhaustively extracted with a chloroform/methanol mixture (1:1), and TLC analysis was performed on the extract against lipid standards.

(*C*) *Vesicle Protein Content.* The protein content of the samples was routinely calculated using amino acid analysis. A vesicle preparation (10 μL) was hydrolyzed at 105 °C in 6 M HCl, and the amino acids were separated in a Waters chromatographic autoanalyzer using multimethod SM261190. In order to calculate the amino acid composition of the sample, standards of known concentration were used for calibration. The band 3 content was derived from the concentrations of four representative amino acids, arginine, glutamate, glycine, and leucine; the proportion in which they occur in band 3 is given in Lux *et al.* (1989).

(*D*) *Vesicle Lipid Content.* The phospholipid content of the vesicles was determined using a phosphorus assay according to Rouser *et al.* (1970).

(*E*) *Electron Microscopy.* Samples for freeze–fracture were prepared by freeze etching according to Sternberg *et al.* (1989). The samples were analyzed in a Siemens electron microscope.

(*F*) *Detergent Concentration.* The final detergent concentration of the reconstituted samples was monitored using [^{3}H]Triton X-100 and [^{3}H]octyl glycoside.

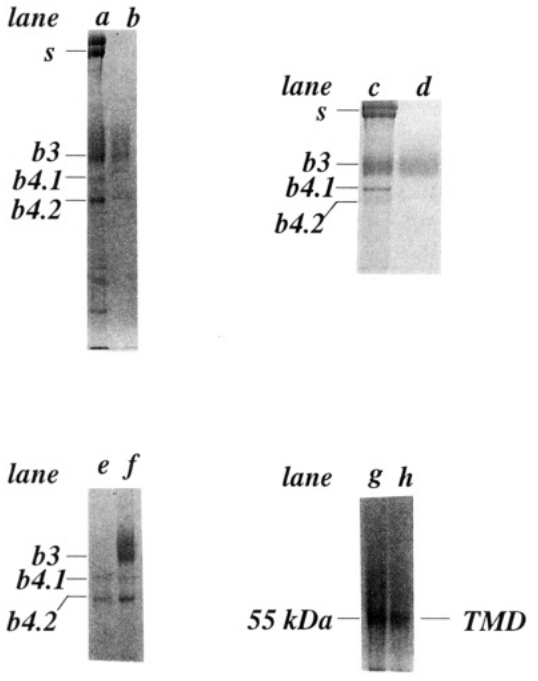

FIGURE 1: SDS gel electrophoresis of ghost membranes (lane a) and partially purified band 3 (ion-exchange chromatography only) (lane b); ghost membranes (lane c) and purified band 3 (affinity chromatography) (lane d); band 4.2 (lane e) and band 4.2–band 3 complex, mole ratio 0.5 (lane f); 55-kDa molecular mass marker (lane g) and the transmembrane domain of band 3 (lane h). Gels were silver stained and lanes a–f were loaded with 10 μg of protein. Lanes g and h were loaded with 20 μg of protein. (s) represents spectrin, (b3) represents band 3 protein, (b4.1) represents band 4.1 protein, (b4.2) represents band 4.2 protein, and (TMD) represents the transmembrane domain of band 3.

(*G*) *Intravesicular Volume.* The internal volume of the vesicles was calculated by measuring the content of $[^{35}S]SO_4^{2-}$ in the vesicles before and after gel filtration on a Bio-Gel P6-DG column.

(*H*) *Turnover Numbers.* Turnover numbers (TN) (defined as sulfate molecules transported per mole of band 3 per minute at a standard sulfate concentration) were calculated for all protein containing samples by (Scheuring *et al.*, 1986)

$$TN = kCV/a$$

where k (minutes^{-1}) is the rate coefficient of the fraction of sulfate efflux sensitive to inhibition by externally added H_2-DIDS, C (moles per liter) is the standard sulfate concentration in the vesicles, V (liters per mole of phospholipid) is the intravesicular volume of the vesicles, and a is the molar protein/lipid ratio. The standard sulfate concentration used for all experiments was 10 mM.

RESULTS AND DISCUSSION

Protein Purification

(*i*) *Band 3.* Band 3 protein purified using ion-exchange chromatography (partially purified band 3) was found to contain band 4.1 and band 4.2 proteins (Figure 1, lane b). These proteins were identified by comparison with erythrocyte ghost membranes (Figure 1, lane a). The majority of band 4.1 and band 4.2 proteins were removed using affinity chromatography as the final purification step. Band 3 protein purified using affinity chromatography was over 95% pure as determined by silver staining of SDS gels (Figure 1, lane d) when compared with erythrocyte ghost membranes (Figure 1, lane c). This final affinity chromatography step, however, resulted in a reduced protein yield (~17% of the initial band 3 content of erythrocyte membranes) when compared with protein prepared using only ion-exchange chromatography (~35% of the initial band 3 content of erythrocyte membranes). However, as the effects of band 4.2 protein were to be investigated, it was important to obtain band 3 protein with minimal contamination from band 4.2 and other peripheral proteins, and thus affinity-purified band 3 was subsequently used in all experiments.

(*ii*) *Band 4.2.* Band 4.2 protein used in these experiments was judged to be over 85% pure as shown by silver staining (Figure 1, lane e) with some contamination (<15%) from other proteins, as judged by densitometer scanning and integration.

(*iii*) *Band 3–Band 4.2 Complex.* Figure 1, lane f, shows the SDS gel electrophoresis of a band 3–band 4.2 complex obtained after removal of uncomplexed proteins by gel filtration. This gel shows that band 4.2 does bind to band 3 protein as shown by the two distinct silver-stained bands of the two proteins.

(*iv*) *The TMD of band 3* was prepared essentially pure from any contamination as shown by silver staining of SDS gel electrophoresis (Figure 1, lane h) when compared with a 55-kDa molecular mass marker.

(*v*) *Lipid Content.* The affinity-purified and TMD protein samples were found to have no detectable endogenous phospholipids as determined by TLC (not shown).

Vesicle Characterization

Detergent Concentration. The concentration of detergents in the reconstituted vesicles was monitored by including radiolabeled detergent in the sample prior to dialysis and counting the radioactivity remaining in the samples over the dialysis period. The removal of octyl glucoside was found to be faster than that of Triton X-100 in equivalent samples, as expected. Dialysis was carried out until detergent levels detected were less than 1 molecule of detergent for 100 lipid molecules. The concentration of octyl glucoside fell below this level after 48 h, while for Triton X-100, the time taken to reach this level was 72 h. The amount of each detergent in the final vesicle samples was found to be less than 1 molecule of detergent for 100 molecules of phospholipid. This concentration of detergents is 1 order of magnitude below the detergent concentration affecting band 3 mediated transport (Köhne *et al.*, 1983).

Electron Microscopy. Freeze–fracture electron microscopy was used to study the morphology of reconstituted vesicles of band 3 in the presence and absence of band 4.2. Figure 2a shows freeze–fracture electron micrograph samples of band 3 containing vesicles, while Figure 2b shows the band 3–band 4.2 complex containing vesicles. In both micrographs, the vesicle preparations appear to be unilamellar and homogeneously distributed with minimal vesicular clustering. The band 3 containing vesicles were of an average diameter of 400 nm, while the band 4.2 containing vesicles were much smaller (~150 nm). In both preparations, there appears to be little protein aggregation and a homogeneous distribution is observed throughout the protein vessels. The band 3 containing vesicles were found to be approximately twice the diameter of band 3 vesicles containing band 4.2 (see Figure 2b), and it therefore appears that binding of band 4.2 may cause band 3 molecules to pack in a way which reduces the number of lipids around band 3.

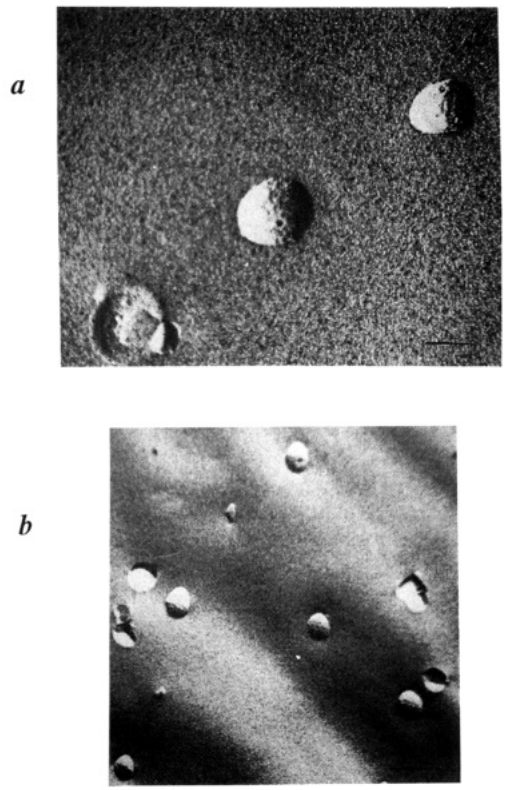

FIGURE 2: Freeze–fracture electron micrographs of affinity-purified band 3 reconstituted vesicles (a) and vesicles containing a band 4.2–band 3 complex, mole ratio 0.5 (b). Protein/lipid ratios are given in Table II. Bar indicates 400 nm.

Protein/Lipid Ratios. The final lipid and protein recoveries of vesicular samples used for sulfate transport measurements were used to calculate molar protein/lipid ratios (*a*) for use in calculating TN values. Lipid recoveries were in the range 68–73% while protein recoveries were generally lower, in the range 53–61%. Further attempts to increase the yield were not made since lipid and protein were readily available. Protein recoveries were measured by amino acid analysis, as the more common Lowry method was found to be unreliable in the presence of the amount of lipid used.

Transport Studies

Sulfate Efflux Measurements. When protein-free lipid vesicles, prepared in the same way as the protein-containing vesicles, were loaded with $[^{35}S]SO_4^{2-}$ and then transferred to label-free buffer, the vesicles released their intravesicular radioactivity very slowly (Figure 3, curve a) when compared with the protein-containing vesicles, which showed a rapid efflux, over minutes (Figure 3, curves c–e). In protein-containing samples, sulfate flux could be inhibited by the non-membrane-permeable inhibitor H_2DIDS at micromolar concentrations (Figure 3, curve b) (Jennings, 1989).

The data shown in Figure 3 shows the change in occluded vesicular radioactivity over a time course of 5 min, during which time band 3 mediated transport occurs to approximately 50% of the initial radiolabeled sulfate concentration. Band 3 mediated transport is defined here as that part of sulfate efflux which could be inhibited by 25 μM H_2DIDS. A representative curve for transport in the presence of H_2DIDS is shown for band 3 containing vesicles (Figure 3, curve b). All sulfate efflux measurements were routinely performed in the absence and presence of externally added H_2DIDS in parallel samples from one vesicle population so that the functionality of the reconstituted protein could be verified

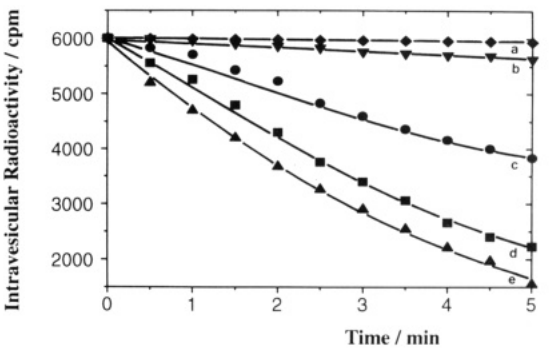

FIGURE 3: Sulfate efflux measured over time from preloaded vesicles for protein-free vesicles (a, diamond), vesicles containing partially purified band 3 (c, circles), vesicles containing band 3 protein purified using affinity chromatography (d, squares), vesicles containing the TMD of band 3 (e, triangles) and vesicles containing affinity-purified band 3 reconstituted vesicles in the presence of 25 μM H_2DIDS (b, inverted triangles). Protein/lipid ratios are given in Table I.

(data not shown). Also, for non-protein-mediated leakage, a control of protein-free lipid vesicles prepared in the same way as those containing protein was used. No significant efflux was observed from protein-free lipid vesicles over the period of the assay (a representative curve is shown in Figure 3, curve a), which illustrates that the vesicles were well sealed with minimal leakage. However, when inhibited band 3 mediated efflux for protein-containing vesicles was compared with efflux from protein-free lipid vesicles, some nonmediated band 3 transport was found to occur in all protein-containing samples.

Orientation of Band 3 Protein in Reconstituted Vesicles. The H_2DIDS binding site on band 3 is accessible to the inhibitor on the extramembranous side; when H_2DIDS is introduced on the cytoplasmic side the inhibitor has no effect (Cabantchik *et al.*, 1978). The effect of externally added H_2DIDS therefore gives information regarding the orientation of the reconstituted protein molecules. Under the conditions of the efflux measurements, addition of externally added 25 μM H_2DIDS reproducibly led to >90% inhibition for band 3 mediated sulfate efflux for all protein-containing samples. This indicates that in the majority of reconstituted band 3 molecules the binding site for H_2DIDS is intact and accessible to the inhibitor. H_2-DIDS does not penetrate the membrane on the time scale for sulfate transport (minutes) (Cabantchik *et al.*, 1978), and these results therefore indicate that nearly all the functional band 3 molecules are incorporated into the vesicles with the inhibitor binding site oriented to the extramembranous surface, implying a "right-side-out" orientation of band 3 when compared with erythrocytes, in all protein-containing samples. Further, the levels of inhibition (>90%) for reconstituted vesicles containing band 4.2–band 3 as a complex were similar to band 3 containing vesicles (>90%). This would imply that binding of band 4.2 protein to band 3 does not significantly affect the orientation of band 3 into vesicles during reconstitution. It is possible that the non-protein-mediated transport for protein-containing vesicles, which accounted for <10% of the total sulfate efflux, may be due to the leakage at the protein–lipid interface (Watts, 1987). Introduction of protein molecules may disrupt the packing of the bilayer lipid at the protein–lipid interface, providing a pathway for nonspecific ion permeation.

Reconstituted Band 3 Vesicles. A comparison of reconstituted band 3 samples (Table I) shows that there is a difference between the transport numbers (TN) for reconstituted band 3 and partially purified band 3 (no affinity chromatography), where partially purified band 3 shows TN values of ~30%

Table I: Comparison of Sulfate Efflux for Reconstituted Vesicles Containing Partially Purified Band 3, Purified Band 3, and the Transmembrane Domain of Band 3

protein reconstituted into vesicles	intravesicular volume, v [L (mol of phospholipid)$^{-1}$]	rate constant, k (min^{-1})	molar protein/lipid ratio, a ($\times 10^{-3}$)	transport number, TN[a] (min^{-1})
partially purified band 3	4.3	100	5.1	790 ± 60
band 3	3.5	150	4.8	1100 ± 50
TMD	2.4	300	5.7	1300 ± 70

[a] Transport numbers were calculated as described in the text. Standard deviation is given for five determinations.

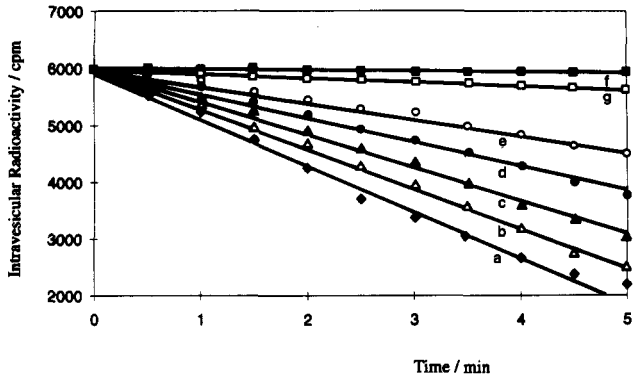

FIGURE 4: Sulfate efflux measured over time mediated by band 3 in reconstituted vesicles complexed with increasing amounts of band 4.2 protein. Radiolabeled sulfate efflux from preloaded vesicles containing band 4.2–band 3 at a mole ratio of 0 (curve a, diamonds), 0.2 (curve b, open triangles), 0.3 (curve c, filled triangles), 0.4 (curve d, filled circles), and 0.5 (curve e, open circles). Radiolabeled sulfate efflux was also measured for protein-free vesicles (curve f, filled squares). Sulfate efflux for all protein-containing samples was also measured in the presence of 25 μM H_2DIDS, and a representative curve for a band 4.2–band 3 complex-containing sample (mole ratio 0.5) is shown (curve g, open squares).

less than those for affinity-purified band 3 reconstituted vesicles. Partially purified samples of band 3 contained significant quantities of band 4.1 and band 4.2 proteins (Figure 1, lane b), which would indicate that the presence of these peripheral proteins was affecting the TN values for band 3 anion transport. Reconstituted TMD of band 3 gave similar TN values as reconstituted band 3 vesicles (1300 and 1100 min^{-1}), indicating that removal of the cytoplasmic domain of band 3 does not have a significant effect on the anion transport capability of the TMD.

Effect of Increasing Band 4.2 Protein Content on Band 3 Mediated Sulfate Transport in Reconstituted Band 4.2–Band 3 Complexes. The effect of increased band 4.2 concentration on the TN values for band 3 mediated anion transport can be seen in Figure 4. Increasing the mole ratio of band 4.2 in the reconstituted band 4.2–band 3 complexes results in decreasing sulfate efflux from the protein-reconstituted vesicles. The sulfate efflux curves plotted against increasing band 4.2 protein content in band 3 containing vesicles show that there is an inverse correlation between sulfate efflux and band 4.2 protein content, with increasing band 4.2 protein content resulting in decreasing sulfate efflux. This inverse relationship is reflected in the TN values shown in Table II. Increasing band 4.2 protein content in band 3 containing vesicles results in decreasing TN values (TN value of 890 min^{-1} when band 4.2 is present at a mole ratio of 0.2 compared with a TN value of 500 min^{-1} when band 4.2 is present at a mole ratio of 0.5). This would indicate that band 4.2 has a

Table II: Effect of Increasing Band 4.2 Concentration on Band 3 Mediated Sulfate Transport in Band 4.2–Band 3 Complexes

mole ratio associated with band 3	intravesicular volume, v [L (mol of phospholipid)$^{-1}$]	rate constant, k (min^{-1})	molar protein/lipid ratio, a ($\times 10^{-3}$)	transport number, TN[a] (min^{-1})
	Band 4.2			
0	4.1	120	4.7	1040 ± 50
0.2	1.6	330	5.7	890 ± 40
0.3	1.6	250	5.2	760 ± 60
0.4	1.9	140	4.3	640 ± 40
0.5	1.8	100	3.9	500 ± 50
	Bovine Serum Albumin			
0	3.6	160	5.3	1090 ± 70
0.2	4.1	150	6.2	980 ± 80
0.3	5.1	100	4.9	1070 ± 50
0.4	4.3	130	5.1	1080 ± 60
0.5	3.9	120	3.9	1100 ± 60

[a] Transport numbers were calculated as described in the text. Standard deviation is given for three determinations.

Table III: Effect of Exogenously Added Band 4.2 on Band 3 Sulfate Transport Efflux from Band 3 Containing Vesicles

mole ratio of exogenous band 4.2:band 3	intravesicular volume, v [L (mol of phospholipid)$^{-1}$]	rate constant, k (min^{-1})	molar protein/lipid ratio, a ($\times 10^{-3}$)	transport number, TN[a] (min^{-1})
0	5.2	100	5.0	1000 ± 60
0.2	4.9	88	4.8	890 ± 60
0.3	3.9	130	4.5	1100 ± 70
0.4	4.6	120	5.3	1000 ± 80
0.5	4.9	100	5.4	990 ± 50

[a] Transport numbers were calculated as described in the text. Standard deviation is given for three determinations.

specific effect on band 3 mediated anion transport. Increasing band 4.2 concentration appears to result in decreasing band 3 transport capability. The effect of band 4.2 on band 3 transport appears to be specific as shown by the TN values measured for equivalent vesicles containing BSA (Table II), which was used as a control. For the samples containing BSA, no correlation between the concentration of BSA and the TN values for band 3 transport could be observed; that is, similar TN values were obtained for all concentration of BSA. These results show that the inhibition of transport by band 4.2 is a specific effect on band 3 mediated anion transport.

Effect of Exogenous Band 4.2 on Band 3 Mediated Sulfate Transport. The addition of increasing concentrations of band 4.2 to band 3 vesicles after reconstitution has little effect on band 3 transport (see Table III; the TN value for vesicles containing exogenous band 4.2 at mole ratios of 0.2 and 0.5 were similar, 890 and 990 min^{-1}, respectively). This is in contrast to the effect of increasing concentrations of band 4.2 reconstituted with band 3 into vesicles (see above). Band 4.2 has a specific binding site on the cytoplasmic domain of band 3 (Korsgren & Cohen, 1988). These results therefore confirm that band 3 is reconstituted in the right orientation since band 4.2 added exogenously does not appear to bind to band 3, indicating that the cytoplasmic domain is intramembranous. Further, these results confirm the finding that the specific interaction of band 4.2 with band 3 causes reduction of the anion transport capacity of band 3 rather than unspecific effects caused by the presence of unbound band 4.2 protein.

Effect of Increasing Band 4.2 Concentration on Band 3 Mediated Transport for the TMD of Band 3. The effect of increasing band 4.2 concentration on the anion transport

Table IV: Effect of Band 4.2 on Sulfate Transport Mediated by the TMD of Band 3 Reconstituted Into Phospholipid Vesicles

mole ratio of band 4.2:band 3	intravesicular volume, υ [L (mol of phospholipid)$^{-1}$]	rate constant, k (min^{-1})	molar protein/lipid ratio, a ($\times 10^{-3}$)	transport number, TN[a] (min^{-1})
0	4.4	100	4.2	1000 ± 60
0.2	5.1	120	4.7	1200 ± 50
0.3	5.2	130	5.1	1000 ± 50
0.4	4.2	120	3.9	1000 ± 60
0.5	3.9	140	4.5	1100 ± 50

[a] Transport numbers were calculated as described in the text. Standard deviation is given for three determinations.

capacity of reconstituted TMD is shown in Table IV. There appears to be little effect on increasing band 4.2 concentration on the TN values for band 3 mediated transport of the TMD; band 4.2 present at a mole ratio of 0.2 in the protein complex gave a TN value of 1200 min^{-1} compared with a TN value of 1100 min^{-1} when band 4.2 is present at a mole ratio of 0.5 in protein complexes. This result is not unexpected as the only specific binding site for band 4.2 on band 3 has been reported to be on the cytoplasmic domain of band 3 (Korsgren & Cohen, 1988). These results appear to confirm further that the reduction in correctly oriented band 3 anion transport in the presence of band 4.2 is due to the specific interaction of band 4.2 with the cytoplasmic domain of band 3.

The results presented above appear to indicate that the specific interaction of band 4.2 with the cytoplasmic domain of band 3 reduces the anion-carrying capacity of band 3 protein. Transport of anions through band 3 is believed to occur through a gated channel formed by a specific arrangement of membrane helices 4, 5, 6, and 7 of band 3 where a salt bond is formed between the anion to be transported and an amino acid residue (believed to be arginine). This bond formation is believed to be necessary to trigger a conformational change in the protein backbone that causes the channel to "open" and allows anion transport (Jennings, 1989). It is possible, therefore, that band 4.2, through its interaction with the cytoplasmic domain, is keeping the TMD in a particular conformation that is not the most favorable for maximal anion transport in the cell.

It has been shown by differential scanning calorimetry measurements that there is a considerable destabilization of the TMD when the cytoplasmic domain and peripheral proteins, including band 4.2, are proteolytically removed from ghost membranes (Sami *et al.*, 1992). This lowering in thermal stability of the TMD is attributed to changes in the tertiary structure of the TMD on removal of the cytoplasmic domain of band 3 and peripheral proteins. This reduction in thermal stability of the TMD could be due to changes in the conformation of the TMD on removal of the cytoplasmic domain and peripheral proteins and could give rise to an increased rate of conformational reorientation of the transport site, leading to increased transport.

Changes in the conformation of the cytoplasmic domain leading to increased transport of anions on binding of hemoglobin have been reported (Ducis *et al.*, 1988). Hemoglobin binds to the first 23 amino acids of the cytoplasmic domain (Prasanna-Murthy *et al.*, 1984). Any changes that occur on binding would have to be structurally transmitted to the TMD. As the cytoplasmic domain is a dimer in the membrane (Low, 1986), these changes could occur through a change in conformation of the dimeric structure. The site of conformational change of the dimeric cytoplasmic domain due to hemoglobin binding is believed to be the cluster of sulfhydryl groups that lie ~100 Å from the N-terminus as shown by measuring sulfhydryl reactivity on hemoglobin binding (Salhany & Cassoly, 1989). Further, it has been reported that chemical modification of the sulfhydryl groups in the cytoplasmic domain of band 3 inhibits phosphate transport across the human eryhtrocyte membrane (Yamaguchi & Kimoto, 1992).

This model for conformational changes in the cytoplasmic domain transmitted to the TMD causing changes in the transport activity of the TMD gives support to the view that band 4.2 could act as a modulator of anion transport by band 3 by acting through the cytoplasmic domain.

The role of band 4.2 as a possible heterotropic allosteric modulator of band 3 anion transport adds to the considerable body of evidence obtained from model and native membranes (Salhany, 1990) indicating that band 3 could be a center for metabolic control for the erythrocyte.

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