Evidence That Inhibitors of Anion Exchange Induce a Transmembrane Conformational Change in Band 3*

(Received for publication, April 12, 1982)

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The transport inhibitor, eosin 5-maleimide, reacts specifically at an external site on the membrane-bound domain of the anion exchange protein, Band 3, in the human erythrocyte membrane. The fluorescence of eosin-labeled resealed ghosts or intact cells was found to be resistant to quenching by CsCl, whereas the fluorescence of labeled inside-out vesicles was quenched by about 27% at saturating CsCl concentrations. Since both Cs⁺ and eosin maleimide were found to be impermeable to the red cell membrane and the vesicles were sealed, these results indicate that after binding of the eosin maleimide at the external transport site of Band 3, the inhibitor becomes exposed to ions on the cytoplasmic surface. The lifetime of the bound eosin maleimide was determined to be 3 ns both in the absence and presence of CsCl, suggesting that quenching is by a static rather than a dynamic (collisional) mechanism.

Intrinsic tryptophan fluorescence of erythrocyte membranes was also investigated using anion transport inhibitors which do not appreciably absorb light at 355 nm. Eosin maleimide caused a 25% quenching and 4,4'-dibenzamidostilbene-2,2'-disulfonate caused a 7% quenching of tryptophan fluorescence. Covalent labeling of red cells by either eosin maleimide or BIDS (4-benzamido-4'-isothiocyanostilbene-2,2'-disulfonate) caused an increase in the susceptibility of membrane tryptophan fluorescence to quenching by CsCl. The quenching constant was similar to that for the quenching of eosin fluorescence and was unperurbed by the presence of 0.5 mM KCl. Neither NaCl nor Na citrate produced a large change in the relative magnitude of the tryptophan emission. The tryptophan residues that can be quenched by CsCl appear to be different from those quenched by eosin or BIDS and are possibly located on the cytoplasmic domain of Band 3. The results suggest that a conformational change in the Band 3 protein accompanies the binding of certain anion transport inhibitors to the external transport site of Band 3 and that the inhibitors become exposed on the cytoplasmic side of the red cell membrane.

Band 3 is the major integral membrane protein of the erythrocyte (1, 2). It has a molecular weight of about 95,000 and is organized into two separate domains that perform distinct functions: a membrane-bound domain (55,000 daltons) catalyzes the rapid exchange of chloride and bicarbonate (or other anions) across the membrane (3) and a cytoplasmic domain (40,000 daltons) acts as an anchor for the cytoskeleton (4). Anion exchange is specifically inhibited by stilbene disulfonates and by eosin 5-maleimide, which bind exclusively at the external transport site of Band 3 when added to intact red cells (5, 6). This site is buried within the membrane in a hydrophobic environment (7), only 35-40 Å from sulfhydryl residues on the cytoplasmic domain. Temperature-jump kinetic studies of the reversible interaction with the fluorescent stilbene disulfonate DBDS have suggested that a rapid initial binding step is followed by a much slower conformational change that locks the inhibitor in place (8).

In the present study we have further investigated this conformational change, which is assumed to accompany the binding of transport inhibitors to Band 3. We have observed changes in intrinsic membrane tryptophan emission that occur on binding and changes in the ability of small hydrophilic molecules to quench both the intrinsic tryptophan fluorescence and the fluorescence of the covalently bound transport inhibitor, eosin maleimide. The eosin maleimide was found to be associated exclusively with the 55-kilodalton membrane-bound domain of Band 3. The results support the hypothesis that certain transport inhibitors induce a conformational change in Band 3 and further suggest that after binding to the external transport site, they become partially exposed to the cytoplasmic side of the membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—Eosin 5-maleimide was obtained from Molecular Probes, Inc., DIDS was from Pierce Chemical Co., DBDS and BIDS were prepared as described by Rao et al. (7), and HDBDS was prepared by exhaustive dehydrogenation using a palladium-charcoal catalyst (9). SDS, Bio-Gel P-10, and acrylamide were obtained from Bio-Rad, and N-ethylmaleimide, trypsin, and α-chymotrypsin were from Sigma. [U-¹⁴C]Sucrose and [³⁵S]CsCl were from New England Nuclear, and H₂O was from Amersham Radiochemicals.

**Membrane and Vesicle Preparations**—Human red blood cells were obtained from the American Red Cross and were washed three times before use in phosphate-buffered saline (5 mM NaCl, 150 mM NaCl, 1 mM EDTA, pH 7.5). Cells were labeled with eosin 5-maleimide as described by Nigg and Cherry (10) or with BIDS as described by Rao et al. (7). Stoichiometries of labeling were determined by

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§Recipient of Grant GM 26199 from the National Institutes of Health in support of this work. Established Investigator of the American Heart Association.

1 The abbreviations used are: DBDS, 4,4'-dibenzamidostilbene-2,2'-disulfonate; SDS, sodium dodecyl sulfate; HDBDS, 4,4'-dibenzamidodihydrostilbene-2,2'-disulfonate; BIDS, 4-benzamido-4'-isothiocyanostilbene-2,2'-disulfonate; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate; IOV, inside-out vesicles; EDTA, ethylenediaminetetraacetic acid; TEMPOBS, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-betaine sulfonate; BSA, bovine serum albumin; RSG, right-side-out resealed ghosts.
mined from the absorbances of the fluorophores in SDS-solubilized samples of membranes (6). Membranes were prepared by lysis of cells in a large volume of 5 mM Na phosphate, pH 8.0 (°C), and were washed by repeated centrifugation (20,000 × g for 20 min) until free of hemoglobin. When necessary, membranes were quick-frozen in dry ice/acetone and stored at -80 °C. IOVs were prepared as described by Steck and Kant (11), sealed vesicles being separated from unsealed membranes on a cushion of 4% Dextran T-40 (12). Sedimentation was determined from the latency of acetylcholinesterase activity, or of neuraminic acid accessibility to neuraminidase, using Triton X-100 to permeabilize the membranes (11). IOV preparations of less than 80% purity were discarded. Resealed ghosts were prepared from intact cells elutriated in about 40 volumes of 5 mM Na phosphate, pH 8.0, followed by incubation at 37 °C for 40 min in 5 mM Na phosphate, 150 mM NaCl, 1 mM MgSO₄, pH 8.0, or by lysis in about 80 volumes of 5 mM Na phosphate containing 1 mM MgSO₄, pH 4.0 (11). The resealed ghosts were washed extensively before use in phosphate-buffered saline or in the phosphate-Mg lysis buffer.

Veinçle Permeability—CsCl permeability was measured by trapping 143Cs⁺ within IOVs and, after extensive washing, passing the vesicles through a Bio-Gel P-10 column (1 × 10 cm) in order to determine if the 143Cs⁺ remained associated with the vesicles. The volume excluded by 143Cs⁺ to 143CsCl also was compared with that to 143Cs⁺, which is membrane-impermeant, and H₂O, which very rapidly penetrates biological membranes. For the latter measurement, vesicles were suspended to a concentration of 4 mg of protein/ml in 5 mM Na phosphate, pH 8.0, containing 2 mM CsCl and 2 mM sucrose. Isotopes were added separately to duplicate 0.5-ml samples, which were then centrifuged at 80,000 × g for 30 min. Counts per unit volume in the supernatant were compared with those from the uncentrifuged suspension, and the fractional excluded volumes were calculated using the formula (S - T)/S, where S and T represent counts in the supernatant and total uncentrifuged suspension, respectively.

Fluorescence Measurements—Fluorescence lifetimes were determined on an SLM 4800 spectrofluorometer as described previously (7), from phase shifts and changes in modulation, with 505-nm excitation oscillating at 6, 18, and 39 MHz. Emission was monitored through a Corning 3-67 filter. Steady state emission was also determined as described previously, using the SLM 4800 spectrofluorometer, controlled by a PET microcomputer (6). Eosin was excited at 505 nm, BIDS at 360 nm and membrane tryptophan at 295 nm. Narrow (2 nm) excitation slits were used to avoid bleaching the probes, and microcuvettes (5 × 3 mm) were used to minimize scattering and inner filter effects. Screens were mixed with a Pasteur pipette. Membrane protein concentration was generally 0.1-0.2 mg/ml.

Titrations were performed at 25 °C. In experiments with intact red cells, front-face emission measurements were made using a triangular cell, at a red cell hematocrit of 20% in 29 mM Na citrate, 205 mM sucrose, pH 7.4. At this cell concentration, the absorbance due to hemoglobin is about 5 absorbance units, and incident light will only penetrate about 2 mm. The uncorrected emission peak of eosin maleimide could be detected in the 40-kilodalton cytoplasmic domain that contains 3 of the 5 reactive sulfhydryl groups in Band 3 and that the eosin maleimide is bound to the same 17-kilodalton membrane-bound fragment (Fig. 1C) as is labeled by anti-Band 3 antibodies (8). Membranes were prepared from cells treated with eosin 5-maleimide, which has previously been shown to block anion exchange and to react almost exclusively at the external transport site of Band 3 (6, 10). In our preparations, the eosin moiety was detected almost exclusively at the Band 3 position after sodium dodecyl sulfate gel electrophoresis (Fig. 1A). Furthermore, limited digestion with α-chymotrypsin demonstrated (Fig. 1B) that no eosin maleimide could be detected in the 40-kilodalton cytoplasmic domain that contains 3 of the 5 reactive sulfhydryl groups in Band 3 and that the eosin maleimide is bound to the same 17-kilodalton membrane-bound fragment (Fig. 1C) as is labeled by stilbene disulfonates (17). Eosin fluorescence (at 560 nm) was monitored during a titration with CsCl (23). Typical results are shown in Fig. 2A. Surprisingly, quenching saturated at only 25%. A similar proportion of the eosin emission was quenchable by acrylamide and by a switzenic anion, TEMPOBS (data not presented). However, no quenching by CsCl was observed using either free eosin (in the same buffer) or BSA labeled with eosin maleimide (Fig. 2A). Quenching of free eosin maleimide by acrylamide saturated at 27%. These results indicate that the inability to attain 100% quenching is an intrinsic property of the solution interactions of these molecules rather than a result of occlusion on the protein.

To try and determine the cause of these effects, the quantum yields and fluorescent lifetimes were measured of free eosin maleimide and of BSA and red cell membranes labeled with eosin maleimide, in the presence or absence of 100 mM CsCl. The results are provided in Table I. They show firstly that the lifetimes of the eosin in each environment can be well...
described by a single exponential decay ($\tau$), although the phase modulation technique used to measure the lifetimes would not detect a component of small amplitude with an exceptionally long lifetime (>30 ns). They show secondly that the lifetime of eosin when bound to red cell membranes is considerably longer than when free in solution or bound to BSA, but is not decreased by the presence of 100 mM CsCl. These data suggest that eosin is located in a hydrophobic environment in the red cell membrane. They also indicate that the most likely mechanism for CsCl quenching of the eosin emission is a static mechanism in which Cs$^+$ binds to sites very close to the fluorophore, allowing virtually instantaneous quenching. The quenching by Cs$^+$ is apparently not due to a general ionic strength effect since it still occurs in the presence of 0.5 mM KCl. Quenching also occurred in the presence of 0.1% BSA, indicating that the observed changes are not artifacts caused by adhesion of the membrane fragments to the cuvettes or to the pipettes used for mixing. When the external transport site of Band 3 was blocked by preincubation of cells with DIDS, labeling with eosin maleimide decreased by 85%, and the maximal quenching of the residual eosin emission by CsCl was only 8% (Fig. 2A). These results indicate that the eosin molecules which are being quenched by CsCl are specifically bound to Band 3.

To determine the sidedness of the Cs$^+$-accessible eosin on the red cell membranes, titrations were performed on IOVs and on RSGs prepared from eosin maleimide-treated red cells. Results are shown in Fig. 2B. Remarkably, when labeled IOVs were used in place of unsealed membrane fragments, the extent to which Cs$^+$ quenched the eosin emission appeared virtually identical. The eosin emission of resealed ghosts, on the other hand, was significantly less susceptible to quenching by Cs$^+$ (Fig. 2B). The resistance to quenching by Cs$^+$ was independent of the method of resealing the ghosts (37 °C incubation or 4 °C incubation in the presence of Mg$^2+$. Moreover, no significant quenching of eosin emission was detectable by front-face fluorescence measurements of intact red cells upon addition of CsCl (Fig. 2B).

These results implied that the eosin becomes exposed to the cytoplasmic side of the membrane upon binding to Band 3. However, it was important to test rigorously two alternative possibilities: 1) that the red cell membranes become permeable to Cs$^+$ during preparation of IOVs and 2) that the eosin maleimide was penetrating the red cells and reacting with sulphydryl residues in the cytoplasmic domain of Band 3.

Evidence that Cs$^+$ is impermeable was obtained in two ways. Firstly, $^{134}$Cs was trapped within IOVs by incubation with $^{134}$CsCl during vesiculation; the amount of $^{134}$Cs remaining associated with the IOVs was then determined after extensive washing and passage through a Bio-Gel P-10 column. Secondly, the volume excluded from $^{134}$CsCl by IOVs was compared with that excluded to $^{14}$C-sucrose and $^3$H$_2$O. The results are presented in Table II. They demonstrate that IOVs are impermeable to $^{134}$Cs at 25 °C within periods similar to those required for fluorescence titrations (~30 min). About 80% of the $^{134}$Cs associated with the vesicles after washing remained associated during passage through the column (Table II). Virtually no $^{134}$Cs added after the vesiculation step remained associated. The exclusion volume for $^{134}$Cs was identical (within experimental error) with that for the impermeant $^{14}$C-sucrose (Table II). As further corroboration, the zwitterionic nitroxide radical TEMPOBS was also shown to be effective as a collisional quencher of eosin fluorescence in labeled IOVs (data not presented). Since this reagent has two permanent charges it is very unlikely to be permeant to red cell membranes.

The second possibility, that eosin maleimide penetrates the red cells during labeling, was excluded in several ways (see Table III). 1) No time-dependent decrease in eosin maleimide was detected in the supernatant from red cells incubated (at 25% hematocrit) with 125 μM of the reagent over 1 h at 25 °C. (This result contrasts with that obtained using free eosin which is taken up by red cells with a half-time of ~4 min at 25 °C (6).) 2) Preincubation of red cells with 25 mM N-ethylmaleimide, a permeant sulphydryl reagent which has been shown previously to react with the cytoplasmic sulphydryl groups of Band 3 (7), did not significantly block labeling of the cells with eosin maleimide. 3) Pretreatment with DIDS blocked labeling by eosin maleimide by 82%, indicating that less than 18% is nonspecifically bound (Table III). 4) Limited proteolysis of alkali-stripped membranes was employed to remove the cytoplasmic domain of Band 3 (18). The transport

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**Fig. 1.** SDS-polyacrylamide gel profiles of red cell membranes labeled with eosin maleimide. Intact red cells were labeled with eosin maleimide as described under "Experimental Procedures" to a stoichiometry of 1.53 nmol/mg of protein. A portion of the cells (C) was then treated with 1 mg/ml of α-chymotrypsin (37 °C, 1 h), converted to ghosts, stripped of peripheral proteins with 10 mM NaOH, 0.1 mM EDTA (0 °C), and treated with 100 μg/ml of trypsin (0 °C, 1 h). The remainder of the cells was converted to ghosts and a portion (B) was treated with 2 μg/ml of α-chymotrypsin (+0.2 μg/ml of soybean trypsin inhibitor) for 1 h at 0 °C. Proteolysis was stopped by addition of 200 μg/ml of phenylmethylsulfonyl fluoride. Aliquots (300 μg of protein) were solubilized in SDS and electrophoresed on 10% (A, B) or 15% (C) polyacrylamide tube gels (0.5 X 10 cm) using the procedure of Laemmli (16). The gels were then removed and scanned at 280 nm for protein, 520 nm for eosin, and 400 nm as a control to detect flaws in the gels. Myosin, phosphorylase b, bovine albumin, ovalbumin, lactate dehydrogenase, carbonic anhydrase, ribonuclease, cytochrome c, and insulin were used as molecular weight markers. Gel A is a control (unproteolized), gel B shows membranes clipped with chymotrypsin to remove the 40-kilodalton cytoplasmic domain of Band 3, and gel C shows stripped membranes cut both with external chymotrypsin and with trypsin.

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Effects of reaction of eosin maleimide with BSA and erythrocyte membranes on the eosin fluorescence lifetime

Fluorescence lifetimes were measured on an SLM 4800 phase shift spectrophotometer, using 505 nm excitation modulated at 6, 18, and 32 MHz. Emission was monitored through a Corning 3-67 filter. Both phase shifts and changes in modulation were used to determine lifetimes, as described previously (7). Samples were in 10 mM Na citrate, pH 7.4, at concentrations of 1-3 μM eosin maleimide (EMA). Where present, the CsCl concentration was 100 mM. The data were well fit by a single exponential decay (7). % T represents the relative amplitudes of the two exponentials.

Table I

| Sample                  | τ1    | % T  | τ2    | % T  | Absolute τ (μs) |
|-------------------------|-------|------|-------|------|-----------------|
| Eosin maleimide         | 0.91  | 97   | 3.26  | 3    | 0.3 ns          |
| EMA-BSA                 | 1.45  | 98   | 4.66  | 2    | 0.3 ns          |
| EMA-ghosts              | 2.96  | 100  |       |      |                 |
| EMA-ghosts + CsCl       | 2.96  | 100  |       |      |                 |

Effects of reaction of eosin maleimide with BSA and erythrocyte membranes.

Table II

| Isotopic marker          | Volume inaccessible to isotope (μl) |
|--------------------------|------------------------------------|
| H2O                      | 3 μl ± 8 μl                         |
| 14C-sucrose               | 32 μl ± 13 μl                       |
| 133 CsCl                  | 35 μl ± 13 μl                       |

Impermeability of inside-out vesicles from red cell membranes to Cs

A. Isotopic marker

B. % Counts trapped in vesicles

Fig. 2. Effect of Cs+ on eosin maleimide fluorescence. A, quenching of eosin maleimide emission by Cs+.

BSA was labeled as described under “Experimental Procedures” and titrated against CsCl (7). Similar titrations were performed using unsealed hemoglobin-free membranes prepared from intact red cells labeled either directly with eosin maleimide (C) or after prior reaction with a saturating concentration (200 μM) of DIDS (A). Excitation was at 505 nm and emission at 500 nm (2 nm excitation, 8-nm emission slit). Both the BSA and membranes were suspended in 28 mM Na citrate, pH 7.0, to a concentration of 0.1 mg/ml and titrated by addition of 0.2 or 2 mM CsCl in the same buffer. Relative fluorescence changes were corrected for dilution, which never exceeded 10%. B, quenching of eosin maleimide-labeled vesicles by Cs+. Sealed inside-out (○) vesicles and right side-out ghosts (□) were prepared by the methods of Steck and Kant (11) from intact red cells labeled with 1.9 nmol of eosin maleimide/mg of membrane protein. The inside-out vesicles were 98% pure of contamination by unsealed or right-side-out vesicles, as determined by neuraminic acid assay (11). Titrations of the eosin emission were performed as described above. Titrations of intact eosin-labeled red cells by Cs+ was also performed by front-face fluorescence measurements, using a triangular cell, as described under “Experimental Procedures” (6).

Impermeability of inside-out vesicles from red cell membranes to Cs

A. Isotopic marker

B. % Counts trapped in vesicles

1IOVs were prepared and characterized by the methods of Steck and Kant (11) and were 85% pure of unsealed or right-side-out vesicles. Isotopes were added to 2 mg of membrane protein in 500 μl of total volume and centrifuged at 40,000 × g for 30 min. Counts remaining in the supernatant were compared to those from the uncentrifuged suspension. Excluded volumes were calculated using the formula \( V(S - T)/S \) where \( V = 500 \mu l, T = \text{total counts/μl} \) in the suspension, and \( S = \text{counts/μl} \) given ± 1 S.D. (n = 4).

133 CsCl trapped within IOVs was measured by passing vesicles through a P-10 Bio-Gel column equilibrated with 5 mM Na phosphate, pH 8.0, and measuring the 133 Cs eluting at V5 with the vesicles and at V0 (free Cs+).
The results indicate that a change in the environment of certain membrane tryptophan residues accompanies the binding of anion exchange inhibitors to the external transport site of Band 3. The observed quenching is consistent with increased exposure of these tryptophan residues to a more polar environment. However, the possibility that certain tryptophan residues in the membrane are very close to the H₂DBDS-binding site (<14 Å) cannot be excluded.

Quenching of Membrane Tryptophan Emission—To test whether any change in exposure of membrane tryptophan to the medium occurs upon binding of anion exchange inhibitors, quenching by CsCl was again employed. Cs⁺ is a relatively efficient collisional quenching agent for the fluorescence of free tryptophan in aqueous solution (Kq = 2.1 M⁻¹; efficiency, γ = 0.2) and of exposed residues in proteins (13). We observed that 10–15% of the total membrane protein tryptophan emission was quenched by saturating CsCl concentrations, using either unsealed membrane fragments or IOVs (86% inside-out). However, less than 4% of the tryptophan emission of RSGs was susceptible to quenching by CsCl (Fig. 5). Since we already have shown the IOVs to be impermeant to Cs⁺, these results suggest that a conformational change in Band 3 accompanies the binding of anion exchange inhibitors at the external transport site of the protein. This possibility was investigated by titration of the tryptophan emission of red cell membranes against eosin maleimide and a novel nonfluorescent stilbenedisulfonate derivative, H₂DBDS. These reagents were chosen because their absorption bands overlap only to a small extent with the emission spectrum of tryptophan (see Fig. 3). Quenching of the emission via singlet-singlet resonance energy transfer is thereby considerably reduced, as compared to that between tryptophan and fluorescent stilbene disulfonates such as DBDS (6, 7). The titration of membrane tryptophan emission against eosin maleimide is shown in Fig. 4A.

A saturable decrease in fluorescence was observed that extrapolated to 25% of the initial fluorescence at a saturating concentration of eosin maleimide. Measurements were taken 2 min following the addition of aliquots of the maleimide, after which time little further change in fluorescence was detectable (inset to Fig. 4A). The data were corrected for a small inner filter effect by performing a parallel titration on free tryptophan. When similar measurements were made on membranes from cells previously reacted with excess DIDS, no decrease in tryptophan fluorescence was detectable. This result demonstrates that the observed quenching was not a consequence of nonspecific binding or of reaction with sulfhydryl residues on the cytoplasmic domain of Band 3.

To determine whether the quenching is a result of reaction at the transport site or of reversible binding, titrations were also performed using the stilbene disulfonate analog, H₂DBDS. This compound was prepared by hydrogenation of DBDS, a fluorescent stilbene disulfonate which is a highly specific and potent competitive inhibitor of anion exchange (7, 8). The H₂DBDS has no detectable absorption peak at 355 nm (Fig. 3) and the critical distance, R₀, at which this molecule will quench tryptophan fluorescence by resonance energy transfer is less than 14 Å (assuming an orientation factor of 2/3 and a quantum yield of 0.1 for tryptophan). H₂DBDS was shown to compete with DBDS for binding to red cell membranes with a Kᵢ of ~2 μM (data not presented). The effect of H₂DBDS on membrane tryptophan emission, after correction for inner filter effects, is shown in Fig. 4B. Quenching reached a maximum value of 7% at saturating concentrations of H₂DBDS. The dissociation constant is 1.5 μM. Blocking of the external transport sites of Band 3 with DIDS significantly reduced the observed quenching by H₂DBDS. Limited proteolysis of membranes with trypsin, under conditions which clip the cytoplasmic domain off Band 3 but which do not affect transport properties of the protein (3), also reduced quenching by H₂DBDS to about the same level as did pretreatment with DIDS (Fig. 4B).

The data were corrected for a small inner filter effect by performing a parallel titration on free tryptophan. When similar measurements were made on membranes from cells previously reacted with excess DIDS, no decrease in tryptophan fluorescence was detectable. This result demonstrates that the observed quenching was not a consequence of nonspecific binding or of reaction with sulfhydryl residues on the cytoplasmic domain of Band 3.

**TABLE III**

Characterization of eosin maleimide labeling of red blood cells

Membranes were prepared from cells labeled with eosin 5-maleimide, as previously described (15). Peripheral proteins were removed by alkali stripping in 10 mM NaOH, 0.5 mM EDTA, 10 °C, for 15 min (3). Where indicated, DIDS was incubated with cells for 1 h before addition of eosin maleimide, to a final concentration of 100 μM. N-ethylmaleimide was added to N₂-gassed cells to a final concentration of 25 mM or 1 h (in Tris-buffered saline, pH 7.4, 37 °C). Chymotrypsin (20 μg/ml) was incubated with alkali-stripped membranes for 1 h at 0 °C, then centrifuged for 30 min at 40,000 × g. Fluorescence quenching was measured as described under "Experimental Procedures." The fraction of total quenching in intact membranes which is attributable to eosin maleimide bound to chymotrypsin-releasable protein fragments was calculated using the formula:

\[
\text{(fraction released)} \times (\% \text{ quenching by supernatant})
\]

\[
\text{(\% quenching by native membranes)}
\]

The calculation assumes that no large changes in the accessibility of the label to Ca²⁺ occur when labeled fragments are released from the membrane by chymotrypsin.

| Treatment                          | Eosin % Protection | Eosin % Quenching by CsCl |
|------------------------------------|--------------------|---------------------------|
| A. Alkali-stripped                 | 2.89               | 24                        |
| + DIDS (before eosin maleimide)    | 0.51               | 82.3                      |
| + N-ethylmaleimide (before eosin maleimide) | 2.63               | 9                         |
| B. Chymotrypsin membranes          | 25.4               | 7.76                      |
| Supernatant                        | 0.26               | 0.28                      |
| Fraction released                  | 0.09               | 0.035                     |
| Fraction of total quenching        | 0.21               |                           |
| attributable to quenching of       |                    |                           |
| chymotrypsin-releasable label      |                    |                           |

**Fig. 3.** Absorption spectra of H₂DBDS and eosin 5-maleimide (EMA). The H₂DBDS and eosin maleimide absorption spectra were taken in 28 mM NaCl, pH 7.0. The uncorrected fluorescence spectrum of erythrocyte membrane tryptophan is shown for comparison. The membranes at a concentration of 0.2 mg of protein/ml were excited with 295 nm light (4-nm slits).
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for 1 h at 0 °C (A). Protein concentration in each titration was 0.1 labeled with 2.5 nmol of BIDS/mg of membrane protein, as described

EDTA

295

brane tryptophan emission by

8.0, 25 °C. Total dilution by CsCl was less than

under "Experimental Procedures." IOVs

of IOVs was stripped of peripheral proteins in 10 nM NaOH/0.5

under "Experimental Procedures." IOV purity was 86%. One fraction

in the absence of Cs' was normalized to 100%. Red cells were also

for in calculating per cent quenching. The fluorescence of all samples

were prepared by the methods of Steck and Kant (11) as described

FIG. 5. Effect of BIDS on quenching of erythrocyte membrane tryptophan emission by CsCl. Tryptophan was excited at 295 nm, and emission was measured at 335 nm. IOVs (■) and RSGs (□) were prepared by the methods of Steck and Kant (11) as described under "Experimental Procedures." IOV purity was 86%. One fraction of IOVs was stripped of peripheral proteins in 10 nM NaOH/0.5 mM EDTA (△), and an aliquot was then treated with trypsin at 100 μg/ml for 1 h at 0 °C (○). Protein concentration in each titration was 0.1 mg/ml, and vesicles were suspended in phosphate-buffered saline, pH 8.0, 25 °C. Total dilution by CsCl was less than 15% and was accounted for in calculating percent quenching. The fluorescence of all samples in the absence of Cs' was normalized to 100%. Red cells were also labeled with 2.5 nmol of BIDS/mg of membrane protein, as described under "Experimental Procedures." IOVs (○) and RSGs (△) were prepared in the same way as the unlabeled vesicles, described above. The relative fluorescence of the BIDS-labeled membranes in the absence of Ca' reflects a 30% quenching due to BIDS labeling.

results demonstrate that the accessible tryptophan residues are located almost exclusively on the cytoplasmic face of the membrane. Removal of peripheral proteins from the red cell IOVs by washing with 10 mM NaOH, 0.5 mM EDTA (0 °C) (15) had little effect upon the proportion of tryptophan fluorescence quenchable by Cs' (Fig. 5). Band 3 constitutes about two-thirds of the total protein remaining in membranes that have been stripped in this way, and the majority of tryptophan residues on the red cell membrane are located in this protein. It is, therefore, likely that the tryptophan residues being observed by collisional quenching are on Band 3. Mild protelysis of the stripped membranes (conditions which release the 40,000-dalton domain of Band 3 from the membrane) almost completely abolished tryptophan fluorescence quenching (Fig. 5).

Both IOVs and resealed ghosts were also prepared from cells labeled with BIDS, an anion exchange inhibitor that reacts specifically at the external transport site of Band 3 (7). The absorbance of this molecule overlaps the emission of Trp and the intrinsic membrane fluorescence is reduced by about 30% when the BIDS binding site is saturated (2.8 nmol/mg). CsCl was still capable of quenching the remaining Trp fluorescence of the IOVs but no significant quenching was observed using resealed ghosts prepared from BIDS-labeled cells (Fig. 5).

These results demonstrate that different sets of tryptophan residues are quenched by the BIDS and by CsCl and they suggest that reaction of the BIDS with Band 3 perhaps enhances the accessibility of certain tryptophan residues on the protein to Cs'. In order to further investigate this possibility, similar titrations were performed using membranes prepared in the same way as the unlabeled vesicles, described above. The relative fluorescence of the BIDS-labeled membranes in the absence of Ca' reflects a 30% quenching due to BIDS labeling.

FIG. 4. Quenching of membrane tryptophan fluorescence by anion transport inhibitors. A, effect of eosin 5-maleimide on erythrocyte membrane tryptophan fluorescence. Membranes were suspended to 0.2 mg/ml in 5 mM Na phosphate, pH 8.0, at 25 °C. Excitation was at 295 nm and emission at 340 nm. Freshly prepared eosin 5-maleimide was added to the membranes and emission was measured after 2 min (■). A similar titration was performed on membranes from cells pretreated with 100 μM DIDS (○). Inset shows the time course for tryptophan emission quenching after addition of 3.3 μM eosin maleimide. A membrane protein concentration of 0.2 mg/ml is equivalent to about 0.5 μM Band 3. B, effect of H2DBDS on erythrocyte membrane tryptophan fluorescence. Membranes were suspended to 0.2 mg/ml in 25 mM Na citrate, pH 7.0, at 25 °C. Excitation was at 295 nm and emission at 340 nm. Fluorescence was titrated against H2DBDS to 50 μM. Quenching was corrected for inner filter effects and dilution by performing a parallel titration using free tryptophan (0.1 mM) instead of membranes. Membranes were either from untreated cells (■) or cells labeled to saturation with DIDS (○). A portion of the membranes from untreated cells was incubated with trypsin (100 μg/ml, 1 h at 0 °C) and then washed in 5 mM Na phosphate, pH 8.0, before being titrated (△).
labeled with eosin maleimide (Fig. 5). In the absence of eosin maleimide, saturating concentrations of CsCl decreased the membrane tryptophan emission from a value of 100 (in arbitrary units) to ~89 (a decrease of 11 units). The presence of eosin maleimide, covalently attached to the membranes, reduced the initial fluorescence to a value of 75, as expected from the data described above (Fig. 4A). Titration of the eosin-labeled membranes with CsCl resulted in a further decrease, however, to a value of about 55, a difference of ~20 units. Thus, eosin maleimide brought about almost a 2-fold change (11 to 20) in the proportion of Cs\(^+\)-sensitive membrane tryptophan emission. Moreover, the quenching constant, \(K_0\), was decreased from about 500 m\(^{-1}\) to about 90 m\(^{-1}\) (inset to Fig. 6). Both results indicate an increased accessibility of tryptophan residues on the eosin-labeled membranes. The quenching data were well described by assuming a single class of accessible tryptophan residues. The effects are not unique to Cs\(^+\), since \(I\) detects similar changes in exposure (an absolute increase in quenching by \(I\) from 24 units in control membranes to 28 in eosin-maleimide-labeled membranes and a decrease in \(K_1\) from 400 to 170 m\(^{-1}\)). Nor are they only attributable to increases in ionic strength since titration with similar concentrations of NaCl or Na citrate produced virtually no change in fluorescence (data not shown).

The mechanisms for quenching of tryptophan emission by Cs\(^+\) are likely similar to involve collisions between the fluorophore and the free ions in solution, because the apparent quenching constants are at least an order of magnitude greater than those common to such quenching mechanisms in other (soluble) proteins. The apparent quenching constants are of similar value to those for quenching of the eosin emission, and quenching was found to be similarly unaffected by the presence of 0.5 M KCl. The most likely mechanism is, therefore, a static one, in which the local concentrations of quenching ions near the membrane are considerably higher than those in the bulk solution. All of the above results were reproducible using red blood cells from different donors and with different preparations of vesicles. However, the magnitude of the changes in the Cs\(^+\)-sensitive tryptophan emission diminished upon prolonged storage of the vesicles (>48 h) at 4 °C, possibly as a result of limited proteolysis by membrane-bound proteases. The quenching of eosin maleimide fluorescence was more stable during storage of vesicles although the magnitude of the quenching varied with different batches of eosin maleimide.

The above results all support the hypothesis that a conformational change in Band 3 accompanies binding of anion exchange inhibitors to the external transport site of the protein, causing an increase in the exposure of certain tryptophan residues on the cytoplasmic side of the membrane.

**DISCUSSION**

We have examined the susceptibility of membrane tryptophan and eosin 5-maleimide fluorescence to quenching by CsCl after reaction of the eosin maleimide with intact human red blood cells. Eosin maleimide reacts specifically at the external anion transport site on the 17-kilodalton membrane-bound fragment and irreversibly inhibits anion exchange (10). We discovered that the eosin fluorescence was sensitive to CsCl present only on the cytoplasmic side of the red cell membrane. Little or no quenching was observed using eosin-labeled resealed right-side-out ghosts, or labeled intact cells, whereas the fluorescence of labeled inside-out vesicles was quenched by about 27% by CsCl. Since we have demonstrated that Cs\(^+\) cannot penetrate the vesicle membrane during the experimental period and that the membrane is also impermeable to eosin maleimide, the results imply that upon reaction with Band 3, the inhibitor becomes accessible to the aqueous environment on the cytoplasmic side of the membrane. Pasew and co-workers (19) have provided evidence for a local conformational change on binding of inhibitors to Band 3, by measuring changes in the rate of dimethylphenylation of the protein, and a slow conformational change in Band 3 has been inferred from kinetic studies of the binding of a stilbene disulfonate, DBDS, to Band 3 (8). The kinetic model proposed that a rapid initial binding of relatively low affinity (~0.5 \(\mu\)M) is followed by a slower step (\(t_{1/2} \approx 2 s\) at 25 °C) that locks the inhibitor in place, increasing the affinity about 10-fold. We suggest that this slow step may represent a partial translocation of the inhibitor and that it occurs when the protein attempts to transport the inhibitor as it would a substrate and switches from an outward- to an inward-facing state. Whether internal exposure of the eosin maleimide also results in occlusion on the external side of the membrane is not clear, despite the absence of quenching by external CsCl. The uncertainty arises from the dependence of quenching on the environment of the fluorophore. We found, for instance, that neither quenching of free eosin nor that of eosin maleimide-labeled BSA was susceptible to quenching by Cs\(^+\), at the concentration employed in titrating labeled erythrocyte membranes.

More direct evidence for a conformational change accom-
panying the binding of anion exchange inhibitors at the external site on Band 3 was obtained by observation of the intrinsic membrane tryptophan fluorescence. The reversible binding of stilbene disulfonates to Band 3 in red cell membranes has previously been shown to quench membrane tryptophan fluorescence (7, 15) but was assumed to do so largely through a singlet-singlet resonance energy transfer mechanism, allowed by the overlap between the tryptophan emission band centered at 335 nm and the absorption band of the stilbene disulfonates centered at about 360 nm. Such resonance energy transfer is in fact detectable as an enhancement of the emission of fluorescent stilbene disulfonates bound to Band 3 upon excitation of tyrosine and tryptophan residues at 280 nm (7). It is not possible, however, to decide from such observations what proportion of the total tryptophan fluorescence is quenched by the energy transfer mechanism because of the large number of tryptophan residues in the red cell membrane. In the present study we have determined the effect of eosin 5-maleimide and H2DBDS upon membrane tryptophan emission and observed substantial decreases at saturating concentrations of the two probes. Since the absorption bands of eosin and H2DBDS show very little overlap with the tryptophan emission spectrum the decrease in emission is probably, in part at least, the result of a conformational change in the protein that lowers the quantum yield of certain tryptophan residues. We cannot exclude the possibility that there are tryptophan residues in very close proximity to the bound inhibitors, oriented in positions favorable for resonance energy transfer. However, the observation that cleavage of the cytoplasmic domain of Band 3 with trypsin substantially reduces tryptophan quenching by H2DBDS strengthens the hypothesis that the quenching is the result of a conformational change. The limited proteolysis by trypsin does not affect anion transport by Band 3 (3) and does not alter binding of other stilbene disulfonates.

The possibility that a conformational change takes place on binding of the inhibitors was confirmed by the detection of an increase in the proportion of the tryptophan emission quenched by CsCl or KI. The residues accessible to Cs appear to occur almost exclusively on the cytoplasmic surface of the red cell membrane and are not the same tryptophans that are quenched by eosin maleimide or BIDS. Since they are removed by limited trypsin proteolysis, it is possible that they are present in the cytoplasmic domain of Band 3. These conclusions are supported by the results of Kleinfeld and co-workers (29, 21) who have used fluorescence resonance energy transfer between the tryptophans of Band 3 and anthroyloxy fatty acids in lipid bilayers to map the location of the tryptophan residues in the protein. Their data were best fit by assuming a bimodal distribution, with several tryptophans being located about 10 Å away from the bilayer, on the cytoplasmic side, and a larger group located close to the outer surface of the bilayer. The former set is presumably in the cytoplasmic domain of Band 3. A comparison of energy transfer in the presence and absence of the nonfluorescent stilbene disulfonate derivative, H2DBDS, suggested that the inhibitor induced a conformational change in which the tryptophan distribution of Band 3 is shifted toward the cytoplasmic side of the membrane (21).

The apparent quenching constants determined for the quenching of the membrane tryptophan residues by Cs+ and I- are considerably higher than those reported for free tryptophan (2.1 M^-1; Ref. 12) or for most other proteins (13, 22, 23). The reasons for this difference are not clear, but it implies either that the residues being quenched have an exceptionally long fluorescence lifetime or that quenching is occurring by a static mechanism that does not involve free diffusion (12, 13, 24). The former possibility is unlikely since 90% of Band 3 tryptophan fluorescence decays with a single lifetime of 5 ns (21). A third possibility, that Cs+ induces a conformational change in Band 3 which reduces the quantum yield of the tryptophan, is also unlikely since both NaCl and KCl have little effect on tryptophan emission. We suggest, therefore, that binding of certain inhibitors, such as eosin maleimide, causes a conformational change in Band 3, which results in exposure of the inhibitor to the opposite (cytoplasmic) side of the membrane, and a change in the environment of certain tryptophan residues also on the cytoplasmic side of the membrane.

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