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FLUORESCENCE LABELING OF THE HUMAN ERYTHROCYTE ANION TRANSPORT SYSTEM

SUBUNIT STRUCTURE STUDIED WITH ENERGY TRANSFER

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Summary

The anion transport system of human red cells was isolated in vesicles containing the original membrane lipids and the 95 000 dalton polypeptides (band 3) by the method of Wolosin et al. (J. Biol. Chem. (1977) 252, 2419—2427). The vesicles have a functional anion transport system since they display sulfate transport that is inhibited by the fluorescent probe 8-anilinonaphthalene 1-sulfonate (ANS) with similar potency as in red cells. The vesicles were labeled with the SH-specific probe fluorescein mercuric acetate (FMA). Labeling lowers FMA fluorescence, and is prevented or reversed by dithiothreitol, suggesting that the reaction is with a thiol group on the protein. Fluorescence titrations show a maximum labeling stoichiometry of 1.3 ± 0.4 mol FMA/mol 95 000 dalton polypeptide. The polarization of bound FMA fluorescence is high indicating that the probe is highly immobilized. Pretreatment with Cu^{2+} + *o*-phenanthroline under conditions that crosslink band 3 in ghosts decreases FMA labeling 50%. Differences in kinetics of FMA labeling in sealed and leaky vesicles suggest that the reactive SH group is located in the intravesicular portion of the protein (corresponding to the cytoplasmic surface of the red cell) and that FMA can cross the membrane. Inhibitors of anion transport have no effect on FMA labeling kinetics suggesting it is not transported via the anion

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Abbreviations: ANS, 8-anilinonaphthalene 1-sulfonate; DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonate; DNDS, 4,4′-dinitro-2,2′-stilbene disulfonate; FMA, fluorescein mercuric acetate; SDS, sodium dodecyl sulfate.

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transport system. Sulfate transport in the labeled vesicles remains fully functional.

We detected self-energy transfer between bound FMA molecules by fluorescence depolarization. With excitation at 450—500 nm P decreases from 0.4, when less than half of the proteins are labeled, to 0.1 at saturation. This depolarization is not observed with red edge excitation (510—530 nm). Addition of 0.1% sodium dodecyl sulfate (SDS) changes P to 0.32, regardless of the excitation wavelength or degree of saturation with FMA. These results indicate that the band 3 proteins are close enough to allow energy transfer between fluorophores (R_0 = 37.4 Å), which does not occur upon red edge excitation or when the proteins are separated by SDS. We conclude that the functional anion transport system exists as a dimer or higher oligomer of band 3 proteins in these membranes, confirming previous suggestions derived using other methods. Future applications are discussed.

Introduction

The anion transport system of human red cells mediates a rapid electroneutral exchange of inorganic anions. Its kinetic and chemical characteristics have been extensively studied (for recent reviews see refs. 1—4), and it has been suggested that a 95 000 dalton polypeptide known as band 3 [5] is involved in anion transport on the basis of chemical labeling [6—8] and reconstitution studies [9—11].

We have attempted to develop fluorescence techniques to study the structure and conformational dynamics of the anion transport system. Previous studies showed that the fluorescent probe 8-anilinonaphthalene 1-sulfonate (ANS) and its isomers are substrates and inhibitors of anion transport in red blood cells [12]. However, nanosecond fluorescence studies indicated that ANS interacts with both membrane proteins and lipids [13] so that the analysis of observations with ANS is complex.

In order to minimize nonspecific interactions, we have used membrane vesicles that contain band 3 as the predominant (>90%) membrane protein and retain the functional characteristics of anion transport in the intact red cell [14]. Because band 3 appears to have a single reactive SH group [7,11,15] we used the sulfhydryl reagent fluorescein mercuric acetate (FMA) as a probe. FMA offers the advantage that its spectroscopic parameters are sensitive to interaction with SH groups in proteins (refs. 16 and 17 and Jesaitis, A.J. and Fortes, P.A.G., unpublished) and to the properties of its immediate environment (Jesaitis, A.J. and Fortes, P.A.G., unpublished).

In the present work, we have studied the interaction of FMA with band 3 vesicles. FMA labels band 3 with a 1 : 1 stoichiometry at a SH group located on the cytoplasmic portion of the protein without perturbing sulfate transport in these vesicles. Fluorescence polarization measurements show significant self-energy transfer between bound FMA molecules indicating that band 3 exists as a dimer or higher oligomer in these membranes. A preliminary account of this work has been published in abstract from [18].

Materials and Methods

Preparation of vesicles. Ghosts were prepared from recently outdated red cells following the method of Dodge et al. [19]. After the final wash the ghosts were resuspended in 120 mM NaCl, 10 mM KCl, 0.2 mM dithiothreitol, 20 mM potassium phosphate, pH 7.0, and stored under nitrogen at 4°C overnight. Band 3 vesicles were prepared from the ghosts by the method of Wolosin et al. [14] and resuspended in 0.2 M sucrose, 0.2 mM dithiothreitol, 5 mM sodium phosphate, pH 7.0; the suspension (1—2 mg/ml) was divided in 1-ml aliquots, frozen in liquid nitrogen and stored at —75°C.

For either flux or fluorescence measurements appropriate amounts of vesicles were thawed, centrifuged at 48 000 × *g* for 20 min (Sorvall RC-5, SS-34 rotor) and washed three times, in the buffer in which experiments were to be done, to remove the dithiothreitol. For flux measurements the vesicles were resuspended in 0.1 M sorbitol, 70 mM Na_2SO_4, 2 mM $MnSO_4$, 20 mM Tris-H_2SO_4 pH 6.5, sonicated 5 min at 0—4°C in a bath sonicator and incubated 1 h at 37°C. To minimize oxidation of SH groups, all solutions were bubbled with nitrogen or argon. The titrations and polarization measurements presented were done in vesicles resuspended in 50 mM histidine-HCl, pH 7.3, without incubation at 37°C. Control experiments showed no significant spectroscopic differences (except rates of reaction) caused by the composition of the buffer, the presence of divalent cations, sonication, or incubation at 37°C, except that the extinction coefficient of FMA is 30% lower at pH 6.5 than 7.3. Details of the conditions used are given in the figure legends.

Protein content of the vesicles was determined by the method of Lowry et al. [20] using crystalline bovine serum albumin as a standard. It should be noted that with this method the erythrocyte membrane proteins are less reactive than albumin [20] and therefore the protein content is underestimated. Gel electrophoresis in 7% acrylamide gels containing 0.1% sodium dodecyl sulfate was done according to Fairbanks et al. [5]. A single band at approx. 95 000 daltons was observed after staining with Coomasie Blue.

$^{35}SO_4^{2-}$ Influx. Sulfate influx was measured exactly as described by Wolosin et al. [14], except that 2 mM ANS was included in the stopping solution to inhibit any efflux of $^{35}SO_4^{2-}$ during separation of the vesicles from the medium. In experiments with FMA and ANS the vesicles were preincubated 25 min in the presence of the indicated concentrations of the probes. Rate constants for SO_4^{2-} self-exchange were determined from the slope of a plot of the approach to equilibrium vs. time [21] according to Eqn. 1:

$$\ln \frac{X_\infty}{X_\infty - X_t} = kt \tag{1}$$

where X_t and X_∞ are the radioactivity in the vesicles at time t and equilibrium, respectively, and k is the rate constant for sulfate self-exchange.

Fluorescence measurements. All measurements were done in 1-cm path cuvettes in a Perkin-Elmer, MPF-4 spectrofluorimeter equipped with a differential corrected spectra unit, polarization attachment, and a thermostated cell holder. Kinetic measurements and titrations were recorded with band-widths of 2 and 10 nm for excitation (490 nm) and emission (530 nm), respectively. In

addition, a 350 nm ultraviolet cut-off excitation filter was used to eliminate second-order radiation. Solutions of known FMA concentration were titrated with 5-μl aliquots of band 3 vesicle suspensions (2—10 μg protein) and the fluorescence of the suspension at equilibrium (approx. 15 min) was determined. Since FMA was found to bind to Teflon-coated stirrers, mixing was accomplished by inverting the cuvette several times after each addition. To minimize photobleaching of FMA the suspension was exposed to the excitation light only during short periods for measurement.

Corrected emission spectra were recorded with 460 nm excitation. Corrected excitation spectra were recorded with emission detected at 560 nm. Bandwidths for all spectra were 10 nm.

Absorption spectra were recorded with a Perkin-Elmer 356 spectrophotometer with sample and reference cuvettes in the secondary compartment, far away from the photomultiplier, to avoid detection of fluorescent light.

Quantum yields of bound and free FMA were determined from the areas of corrected emission spectra using fluorescein in 0.1 M NaOH as a standard of quantum yield = 0.92 [22], according to:

$$\Phi_x = \frac{F_x}{F_s} \cdot \frac{A_s}{A_x} \cdot \Phi_s \tag{2}$$

where Φ_s and Φ_x are the quantum yields, A_s and A_x are the absorbances at the exciting wavelength (460 nm), and F_s and F_x are the areas of the corrected emission spectra of the standard and FMA, respectively.

The value of the overlap integral, J [23], between FMA absorption and corrected emission spectra was estimated from absorption and fluorescence values at 5-nm intervals using the equation:

$$J = \frac{\sum_{\lambda} F(\lambda)\epsilon(\lambda)\lambda^4\Delta\lambda}{\sum_{\lambda} F(\lambda)\Delta\lambda} \tag{3}$$

where $F(\lambda)$ and $\epsilon(\lambda)$ are, respectively, the fluorescence and the extinction coefficient (in $M^{-1} \cdot cm^{-1}$) at wavelength λ. J has units of cm^6/mmol.

Fluorescence polarization, p, was determined with vertically polarized excitation (λ_{ex} = 500 nm, 2 nm band-pass) and emission intensity (λ_{em} = 550 nm, 10 nm band-pass) detected with the analyzer parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the excitation, according to Eqn. 4:

$$p = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} \tag{4}$$

correction for intrinsic polarization of the instrument was made by measuring the ratio of parallel and perpendicular intensities with horizontal excitation.

Since the polarization is constant across the emission band, excitation polarization spectra were determined by scanning the excitation with the different polarizer settings and measuring emission through a Corning CS 2-62 filter instead of the emission grating, to increase the signal to noise ratio. P was calculated from Eqn. 4 across the excitation spectrum at 5-nm intervals.

Corrections for light scattering were unncessary since 'blank' spectra with unlabeled vesicles at identical concentrations showed negligible intensity.

Materials. FMA and $MgANS_2$ were obtained from Eastman Organic Chemicals. $^{35}SO_4^{2-}$ was obtained from New England Nuclear. DNDS was a gift from Dr. Z.I. Cabantchik. Water was deionized and distilled in glass. Recently outdated human red cells were obtained from the San Diego Blood Bank. All other chemicals used were reagent grade.

FMA was dissolved in 0.1 M NaOH, diluted into 50 mM histidine-HCl, pH 7.5, and the concentration was determined by absorbance at 497 nm using a molar extinction coefficient $\epsilon_{497} = 7.4 \cdot 10^4\ M^{-1} \cdot cm^{-1}$. Solutions were prepared immediately before use.

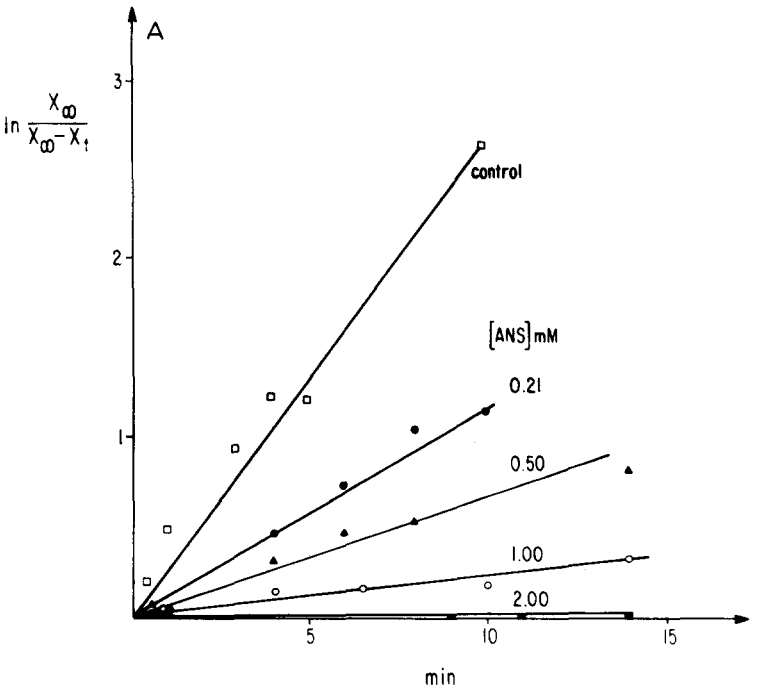
Results

Characterization of vesicles

Fig. 1 shows $^{35}SO_4^{2-}$ uptake into band 3 vesicles as a function of ANS concentration. In the absence of ANS the uptake follows first-order kinetics with a halftime of 3.5 min, in agreement with the findings of Wolosin et al. [14]. ANS, which inhibits Cl^- and SO_4^{2-} exchange in intact red cells [12], also inhibits SO_4^{2-} uptake in these vesicles with similar potency: 50% inhibition at 0.2 mM ANS and more than 99% inhibition at 2 mM ANS. Thus, in order to prevent any efflux of radioisotope during separation in the column, 2 mM ANS was included in the stopping medium in all subsequent experiments. If no divalent cations are present in the medium during sonication the vesicles are not sealed to SO_4^{2-} since no $^{35}SO_4^{2-}$ influx is observed (Fig. 2).

Interaction of FMA with band 3 vesicles

When FMA reacts with SH groups in proteins the fluorescence is quenched (refs. 16 and 17, and Jesaitis, A.J. and Fortes, P.A.G., unpublished). We used this effect to estimate the number of FMA molecules that bind to band 3 protein. Fluorescence titrations were carried out by measuring the fluorescence at equilibrium of a given concentration of FMA as a function of the protein concentration. A typical titration is shown in Fig. 3. FMA fluorescence decreases linearly with protein concentration up to a maximum, after which the fluorescence is independent of the protein concentration. The intersection of the lines drawn through the points above and below saturation gives the concentration of protein that saturates the given FMA concentration. The inset in Fig. 3 shows that the plot of the intercepts vs. protein concentration is linear. The results of nine similar titrations in three different preparations give a stoichiometry of 1.3 ± 0.4 mol FMA/mol band 3 assuming a molecular weight of 95 000 and 100% purity. This value is an upper limit, since the protein content of these vesicles is underestimated because of lower reactivity with the Lowry reagents than the albumin standard [11,20]. The labeling stoichiometry is the same in 'leaky' and 'sealed' vesicles (cf. Fig. 2), but the rate of the reaction, as measured by the kinetics of fluorescence quenching, is faster in the 'leaky' vesicles. This suggests that the slow rate observed in 'sealed' vesicles is due to inaccessibility of the SH group if it is exposed at the inner surface, since the rate-limiting step would be translocation of FMA across the membrane. FMA is



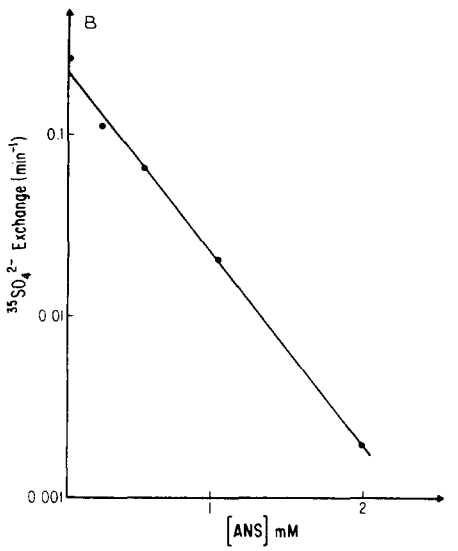


Fig. 1. Effect of ANS on sulfate influx in band 3 vesicles. (A) Band 3 vesicles (1.2 mg protein/ml) were sealed and equilibrated as described in Materials and Methods, suspended in 70 mM Na_2SO_4, 0.1 M sorbitol, 20 mM Tris-H_2SO_4, 2 mM $MnSO_4$, pH 6.5 containing the indicated ANS concentrations and $^{35}SO_4^{2-}$ (10^8 cpm/ml). The data are plotted as the logarithm of the approach to equilibrium vs. time (see Materials and Methods). (B) Rate constants of sulfate exchange versus ANS concentration.

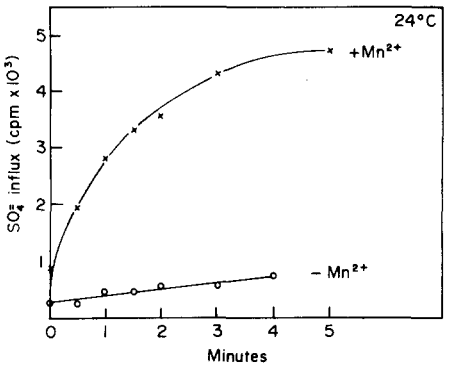


Fig. 2. Effect of Mn^{2+} and incubation at 37° C on $^{35}SO_4^{2-}$ influx in band 3 vesicles. X, vesicles (1.5 mg/ml) were prepared as in Fig. 1; ○, no Mn^{2+} during sonication or influx. Preequilibrated 1 h at 0° C instead of 37° C.

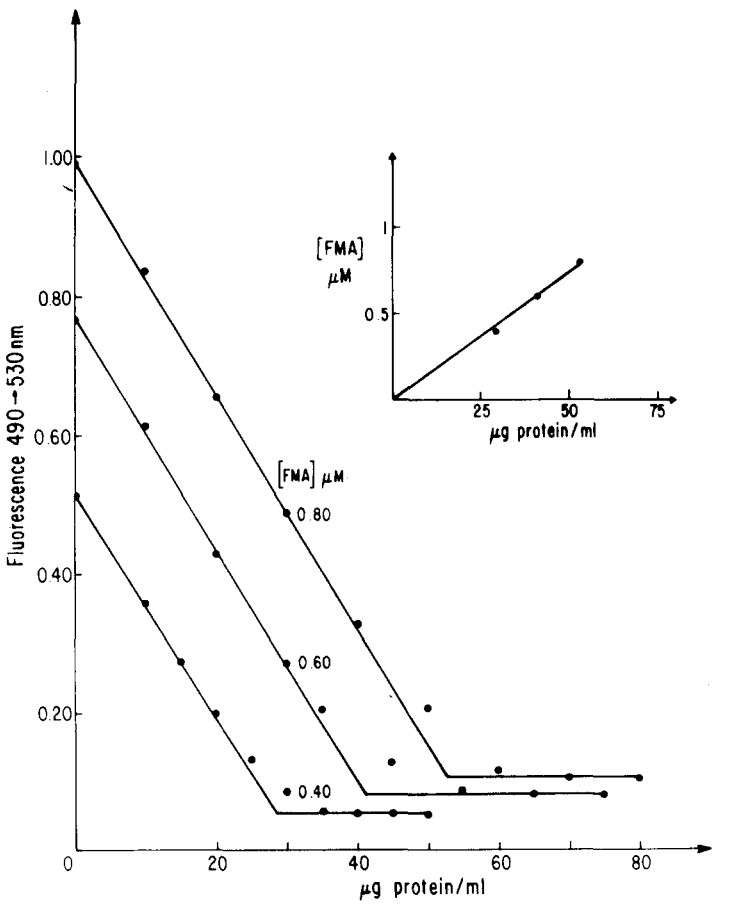


Fig. 3. Decrease in FMA fluorescence upon reaction with band 3 protein. Three FMA concentrations 0.8, 0.6 and 0.4 μM in 2 ml of 50 mM histidine, pH 7.3, 24° C were titrated by adding 5-μl (2 μg) aliquots of band 3 vesicles to cuvettes and the fluorescence at equilibrium (~15 min) was measured. The breaks in the curves show the amount of protein that saturates the given FMA concentration. The inset shows the saturation values as a function of the FMA concentration. The slope of the line gives the stoichiometry: 14.3 nmol FMA/mg protein.

also permeable in intact red cells, since it can label hemoglobin when added to the medium [24]. However, FMA does not appear to be transported via the anion transport system since neither the rate nor extent of FMA reaction with sealed or leaky vesicles are affected by ANS or the specific inhibitor DNDS [25] at concentrations that inhibit sulfate exchange more than 99%. These results indicate that FMA interacts with one SH group per mol of band 3, and suggest that this group is located in the intravesicular surface of the protein.

Since these vesicles appear to be 'right side out' because the DIDS inhibitory site is external as in the red cell [14], it is likely that the labeled SH group is the same one that can be crosslinked by Cu^{2+}-phenanthroline in ghosts to form covalent band 3 dimers [26,37]. To investigate this possibility, we tested the effect of pretreatment with Cu^{2+}-phenanthroline on the reactivity of FMA with band 3 vesicles. Fig. 4 shows that incubation of band 3 vesicles with Cu^{2+}-

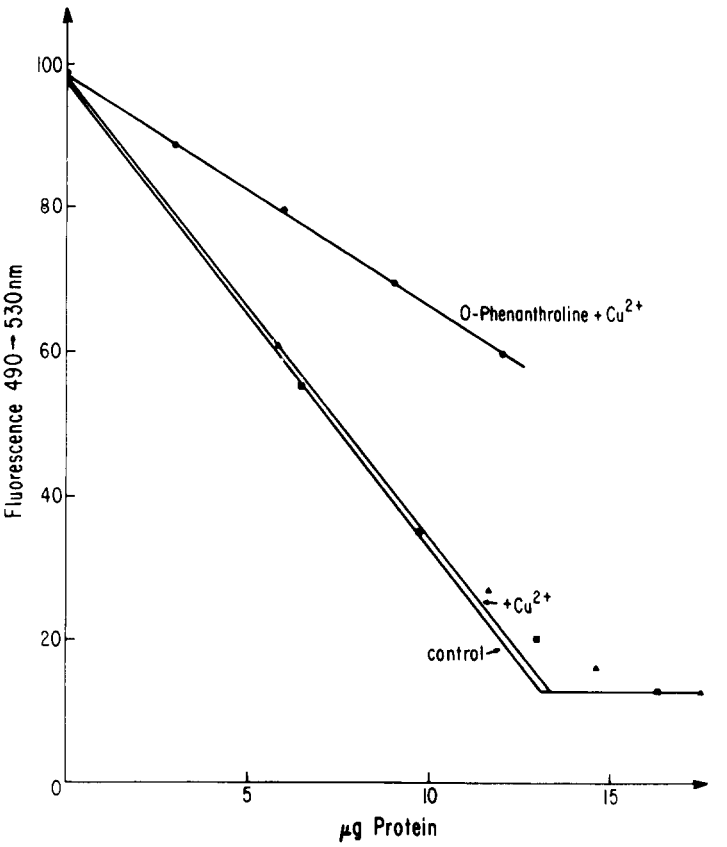


Fig. 4. Effect of Cu^{2+} and *o*-phenanthroline on FMA reaction with band 3 vesicles. Vesicles (1.3 mg/ml) were incubated 80 min at 24°C in 50 mM histidine, pH 7.3. Where indicated 20 µM Cu^{2+} and 100 µM *o*-phenanthroline were also present. After incubation the suspension containing *o*-phenanthroline was centrifuged 60 min at 48 000 × *g*, the supernatant was removed and the pellet was resuspended in 50 mM histidine, pH 7.3. Cuvettes containing 2 ml 0.8 µM FMA in 50 mM histidine, pH 7.3, were titrated with 5-µl aliquots of the suspensions and the fluorescence at equilibrium was measured. The saturation values were 9.7 nmol FMA/mg protein for the control ± Cu^{2+} and 5.4 nmol FMA/mg protein for the vesicles treated with Cu^{2+} + phenanthroline.

phenanthroline, under identical conditions as those used in ghosts [26,37], results in a 50% decrease in FMA sites per mg of protein. Cu^{2+} by itself has no effect. Thus, it appears that FMA reacts with the same SH group that has been characterized in ghosts, which is located on the cytoplasmic portion of the band 3 protein [26].

Although FMA is a dimercurial and can crosslink the $(Na^+ + K^+)$-ATPase (Jesaitis, A.J. and Fortes, P.A.G., unpublished) no crosslinking of band 3 proteins was observed as indicated by a single band at 95 000 daltons in SDS-gel electrophoresis of vesicles saturated with FMA.

The labeled vesicles retain a fully functional anion transport system. Fig. 5 shows that no differences in sulfate influx are observed in vesicles prereacted with saturating concentrations of FMA. This finding is consistent with reports that other SH reagents have no effect on anion transport in red cells [28,29].

Spectroscopic properties of FMA in band 3 vesicles

The fluorescence intensity of FMA after reaction with band 3 vesicles is 11% that of FMA in buffer. The lower intensity is due to both a decrease in the extinction coefficient (53%) and a decrease in quantum yield (76%). In addi-

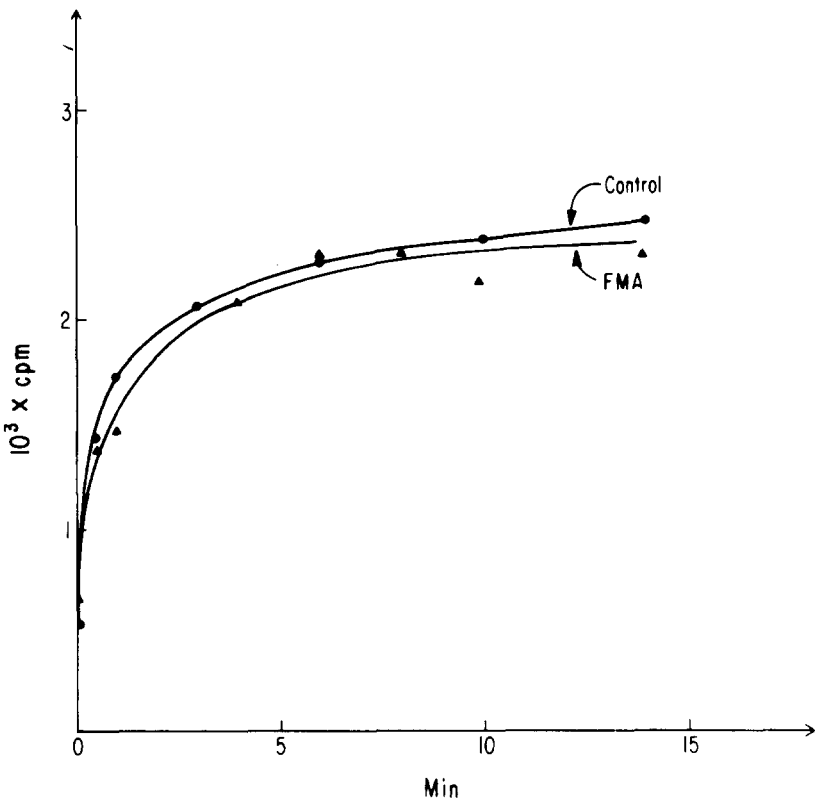


Fig. 5. Effect of FMA on sulfate influx in band 3 vesicles. Vesicles (1.5 mg/ml) were prepared as in Fig. 1 and preincubated 25 min at 24°C in the presence or absence of 20 μM FMA, $^{35}SO_4^{2-}$ was added and influx measured as described in Materials and Methods.

tion, the excitation and emission maxima shift to the red. The absorption and corrected emission spectra of FMA in band 3 vesicles are shown in Fig. 6. The decrease in absorbance and quantum yield is partly due to the formation of the mercaptide, since quenching is also observed upon reaction with dithiothreitol, mercaptoethanol and glutathione. In addition, the binding site appears to be somewhat shielded from water, since decreases in absorbance and quantum yield as well as spectral shifts to the red are observed with FMA dissolved in alcohols (Jesaitis, A.J. and Fortes, P.A.G., unpublished). No significant spectral differences between 'leaky' and 'sealed' vesicles or in the presence of DNDS or ANS were observed.

Fig. 6 also shows a substantial overlap between absorption and emission spectra indicating that self-energy transfer between FMA molecules can occur if they are close enough and properly oriented. The theory developed by Förster [23] states that the rate of nonradiative energy transfer is directly proportional to the degree of overlap between donor emission and acceptor absorption spectra, and inversely proportional to the sixth power of the distance between donor and acceptor, according to the equation:

$$R_0 = (8.71 \cdot 10^{23} \cdot J \cdot \kappa^2 \cdot \Phi \cdot n^{-4})^{1/6} \quad (5)$$

where R_0 is the distance in Å at which the transfer efficiency is 50% (the rate of transfer is equal to the rate of fluorescence emission); J is the overlap

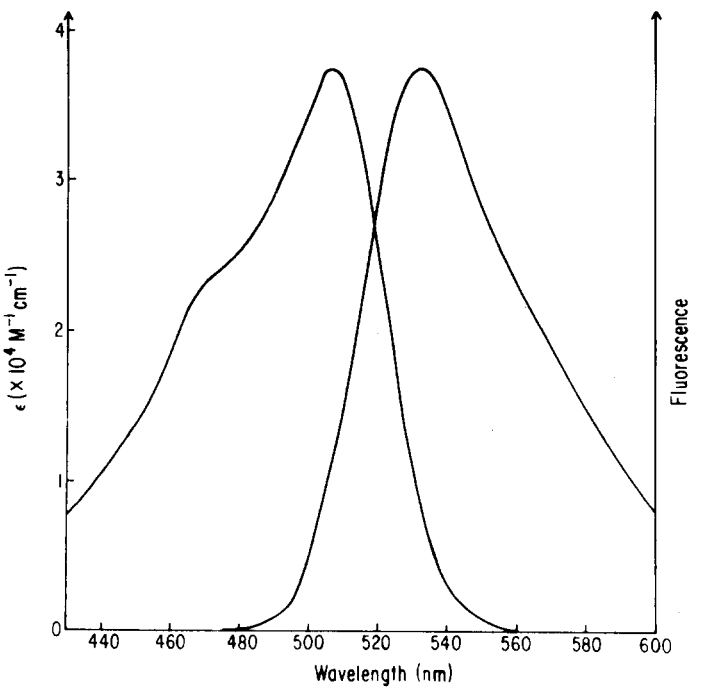


Fig. 6. 3.8 μM band 3 vesicles were reacted 30 min with 3 μM FMA in 50 mM histidine buffer, pH 7.3, 24°C, and the absorption spectrum was measured using unreacted vesicles at identical concentration as a reference. The suspension was diluted 10 times in the same buffer and the emission spectrum was measured exciting at 460 nm.

integral between donor emission and acceptor absorption spectra (see Materials and Methods); κ^2 depends on the relative orientation of donor and acceptor and can vary from 0 to 4 with an average value of 2/3 when donor and acceptor rotate isotropically; Φ is the fluorescence quantum yield of the donor in the absence of acceptor; and n is the refractive index of the medium (= 1.4 for proteins).

Owing to the inverse sixth power dependence of energy transfer efficiency on donor-acceptor separation, Stryer and Haugland [30] and Stryer [31] suggested the use of energy transfer as a spectroscopic ruler to estimate distances between appropriate pairs of chromophores in macromolecules (for a recent review see ref. 31) since:

$$E = \frac{R_0^6}{r^6 + R_0^6} \tag{6}$$

where E is the transfer efficiency, R_0 is the Förster critical distance defined above and r is the actual distance between donor and acceptor.

For FMA in vesicles, $J = 9.023 \cdot 10^{-14}$ cm^6/mmol and $\Phi = 0.2$. Substituting these values in Eqn. 5, $R_0 = 37.4$ Å, assuming a random orientation of FMA molecules (see below). Thus, self-energy transfer between FMA molecules in band 3 vesicles should occur if the distance between groups is less than 50 Å (=10% transfer). Energy transfer causes fluorescence depolarization. The degree of depolarization is proportional to the average angle, θ, between emission vectors of the donor and acceptor molecules and the average number of transfers, $\bar{n}$, that occur before emission, as shown by Weber [32]:

$$\frac{1}{p_{\bar{n}}} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)(1 + \tfrac{3}{2}\sin^2\theta \cdot \bar{n}) \tag{7}$$

where p_n is the polarization of the fluorescence emitted after $\bar{n}$ transfers and p_0 is the polarization in the absence of transfer. $\bar{n}$ is a measure of the probability of transfer and equals 1 when the transfer efficiency is 50% (i.e. $r = R_0$).

We studied self-energy transfer between FMA molecules by measuring the polarization of the fluorescence as a function of the extent of labeling with FMA. Fig. 7A shows two titrations done at different protein concentrations. Regardless of the concentration of protein, the polarization of FMA fluorescence is high at low FMA : protein ratios, decreases progressively as the protein is saturated, and the decrease in polarization becomes more pronounced after half of the proteins have been titrated. The high polarization at low FMA : protein ratios indicates that the mobility of the bound FMA is either slow or restricted. The observed depolarization suggests that it is due to self-energy transfer, and that FMA has different orientation in neighboring proteins, so that $\theta > 0°$, otherwise no depolarization would occur in spite of extensive energy transfer (Eqn. 7). As shown by Weber [32], for a random distribution of molecules the probability of transfer, $\bar{n}$ in Eqn. 7, is a linear function of the concentration. It can be shown [32] that the dependence of the polarization on concentration in a two-dimensional system, such as a mem-

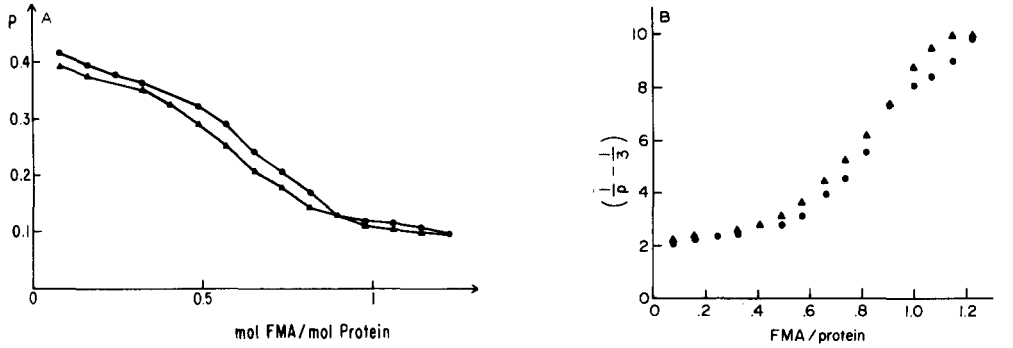


Fig. 7. (A) Fluorescence polarization of FMA in band 3 vesicles. Suspensions of vesicles in 50 mM histidine, pH 7.3, containing either 0.5 (●) or 1 μM (▲) band 3 were divided in 15 test tubes to which increasing amounts of FMA were added. After a minimum of 30 min of incubation at 24°C the suspensions were transferred to cuvettes and the fluorescence polarization was measured. Volume, 2.2 ml; λ_{ex} = 500; λ_{em} = 550 nm. (B) The data in A plotted according to Eqn. 8. See text for discussion.

brane, is given by:

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{\pi R_0^6 C}{2(2a)^4}\right) \tag{8}$$

where C is the number of fluorescent molecules in 1 cm^2 of membrane area and $2a$ is the distance of closest approach, i.e. twice the effective molecular radius. Eqn. 8 predicts that, for a random distribution of molecules, a plot of $(1/p - 1/3)$ vs. concentration should be linear. In contrast the data of Fig. 7A plotted according to Eqn. 8 are nonlinear with a 9—10-fold increase in slope above 50—60% labeling (Fig. 7B). This behavior is different from that expected for depolarization as a function of concentration for a random distribution of monomers. The change in slope in Fig. 7 suggests that the average distance between fluorophores decreases drastically after half of the band 3 proteins are labeled, and thus, that the band 3 monomers are not randomly distributed in these membranes. The behavior in Fig. 7 could be observed if the band 3 proteins existed as dimers in the membrane, since at low concentrations the probability of labeling two monomers in the same dimer would be much lower than the probability of labeling monomers in different dimers. At high concentrations the probability of labeling both monomers in a dimer is high since all monomers are labeled. This model is shown graphically in Fig. 8A in which the fraction of unlabeled, singly labeled, and doubly labeled dimers as a function of the degree of saturation was estimated using a normal distribution, assuming that the probability of labeling is independent of the degree of saturation, i.e. no interaction between sites.

If we assume that the average distance between dimers is too large for energy transfer to occur ($r \geqslant 2R_0$), so that depolarization is only due to transfer between the monomers in each dimer, the change in polarization with degree of labeling can be estimated using Weber's law of addition of polarizations [33]:

$$p = f_1 p_1 + f_2 p_2 \tag{9}$$

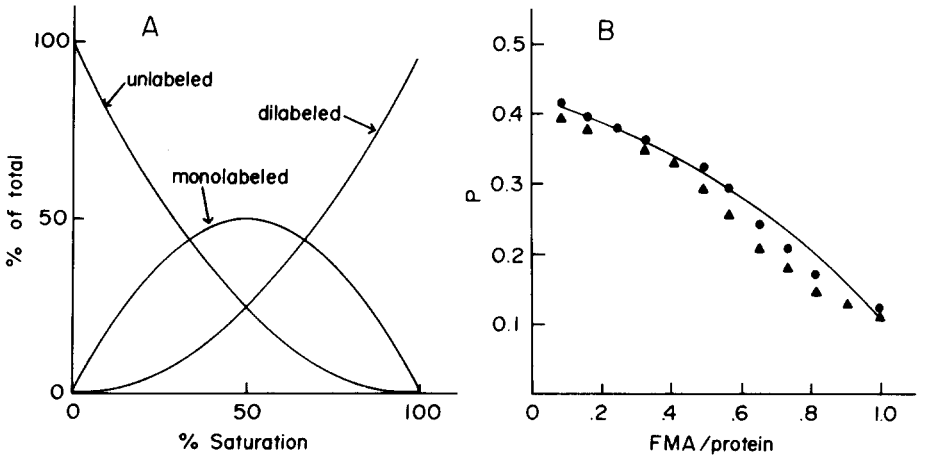


Fig. 8. (A) Theoretical labeling pattern of band 3 dimers as a function of the degree of saturation with FMA. The graphs were estimated from a normal distribution assuming that band 3 is a dimer with 2 SH groups that have equal probability of reaction with FMA. The fractions of unlabeled, singly labeled and doubly labeled dimers are equal to the probability of labeling and are given by $(1-x)^2$, $2(1-x)x$ and x^2, respectively, where x is the degree of saturation, which varies from 0 to 1 when all the SH groups are reacted. Ordinate: percent of dimers that have reacted with 0, 1 or 2 FMA molecules. Abscissa: percent of total sites that have reacted with FMA. (B) Comparison between experimental and predicted depolarization as a function of degree of saturation with FMA. Experimental points are the same as in Fig. 7A. The smooth curve was estimated from Eqn. 9 using $P_1 = 0.42$ and $P_2 = 0.1$ (i.e. the observed polarization at very low FMA concentration and at saturation, respectively); f_1 and f_2 were estimated from $f_1 = A/(A+B)$, $f_2 = 1 - f_1$, where A and B are the fraction of mono- and dilabeled dimers in A, respectively. See text for discussion.

Where p is the observed polarization, f_1 and f_2 are the fractional concentrations ($f_1 + f_2 = 1$), and p_1 and p_2 are the polarizations of the singly labeled and doubly labeled dimers, respectively. Substituting in Eqn. 9 the polarizations measured at low and high saturation for p_1 and p_2, respectively, and the fractional concentrations derived from Fig. 8A, the calculated polarization is plotted as a function of the degree of labeling in Fig. 8B.

The similarity between the calculated (smooth curve) and experimental polarization in Fig. 8B lends support to the idea that band 3 proteins exist as dimers in these membranes. However, in the region between 65 and 90% labeling the observed depolarization is about 10% higher than that predicted by the model. This increased depolarization may indicate that there is some energy transfer between dimers and/or that there exist higher oligomers of band 3 in these membranes.

In order to demonstrate that the observed depolarization at high FMA saturation is due to nonradiative energy transfer, and not to trivial reabsoorption and reemission, the polarization was measured as a function of the excitation wavelength. Weber and Shinitsky [34] have shown that self-energy transfer does not occur when excitation is at the red edge of the absorption band. Fig. 9 shows excitation polarization spectra of two preparations of vesicles labeled at a high (0.66) and a low (0.33) FMA : protein ratio. The depolarization observed in the vesicles labeled at the high FMA : protein ratio disappears at the

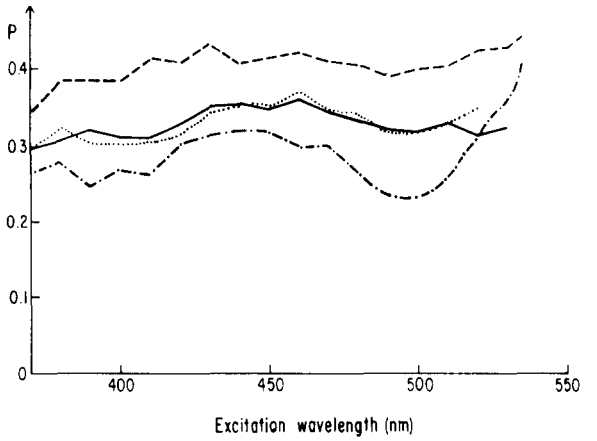


Fig. 9. Effect of degree of saturation and SDS on excitation polarization spectra of FMA in band 3 vesicles. 0.75 and 1.5 μM band 3 vesicles were reacted with 0.5 μM FMA in 50 mM histidine, pH 7.3, for 30 min at 24°C and excitation spectra in the absence and presence of 0.1% SDS were scanned with the polarizer settings described in Materials and Methods. Emission was measured through a Corning CS 2-62 filter. Polarization values were calculated every 5 nm. Mol FMA/mol band 3 = 0.33 (- - - - -); 0.66 (· · · · ·); = 0.33 + 0.1% SDS (· · · · ·); = 0.66 + 0.1% SDS (———).

red edge, confirming the existence of energy transfer. Furthermore, if the vesicles are solubilized in 0.1% SDS the polarization increases for the high FMA : protein vesicles and decreases for the low FMA : protein vesicles to the same final value.

Discussion

The main findings in this paper are that FMA specifically labels one reactive sulfhydryl in the band 3 protein without perturbing its transport function and that self-energy transfer between bound FMA molecules is detected by fluorescence polarization measurements. That the observed depolarization is due to self-energy transfer between FMA molecules in close proximity is demonstrated by the fact that it disappears with red edge excitation or by dissolving the membranes in SDS.

These results indicate that the FMA-labeled band 3 polypeptides must be within energy transfer distances in the membrane vesicles. Since energy transfer can occur only over distances smaller than or equal to $2R_0$, an estimate of R_0 gives an upper limit to the separation between the monomers. The main uncertainty in estimating R_0 is the value of the orientation factor κ^2. If the bound FMA molecules were all similarly oriented (such that κ^2 would be larger than 2/3) and with restricted motion, little depolarization would be measured even in the presence of energy transfer, since little loss in orientation between excitation and emission would occur. On the other hand, if the orientations were unfavorable because of slow rotation and symmetry relations between adjacent protein monomers, κ^2 would be less than 2/3 and the distance over which energy transfer can occur would be shorter. Since the polarization in the absence of transfer is high (~0.4) and in the present of maximum transfer is

low (~0.1), indicating different orientations between donors and acceptors, κ^2 should approach the value for random orientation. Therefore, the assumption of $\kappa^2 = 2/3$ is reasonable for distance estimates between sites, giving $R_0 =$ 37.4 Å. These observations indicate that the average separation between the labeled sites in band 3 monomers is no larger than 50 Å (=10% transfer) and most likely of the order of 35 Å or less, since the large depolarization observed suggests that the transfer efficiency may be quite high (⩾50%). This is not unexpected, since the SH groups in band 3 can come sufficiently close to each other to form a disulfide bond in the presence of Cu^{2+} and *o*-phenantroline [15,26,27]. Recently Ross and McConnell [11] reported that no magnetic dipole-dipole interactions were observed in band 3 proteins labeled with a sulfhydryl spin label and reconstituted in phospholipid vesicles, and concluded that the labels must be more than 10—20 Å apart. Their observations are consistent with our findings, since FMA self-energy transfer can occur over longer distances, and may indicate that the average separation between sites is of the order of 30—40 Å, if the different preparations are comparable.

Our data do not allow direct measurements of transfer efficiency since depolarization is a function of both the probability of transfer and the angle between the emission oscillators of the donor and acceptor molecules (Eqn. 7). Thus, a precise distance cannot be determined with the present methods, but should be measurable by more conventional energy transfer experiments using two different probes as donor and acceptor in order to determine transfer efficiencies and relative orientations.

The close proximity between band 3 monomers determined in the present work suggests that the band 3 proteins in these membranes vesicles exist as dimers or higher oligomers. This conclusion is supported by the nonlinearity in Fig. 7B, which indicates that the observed depolarization cannot be due to labeling of randomly distributed free monomers. A simple model in which it is assumed that band 3 is a dimer and energy transfer occurs only within the dimer, but not between dimers, fits the observed depolarization qualitatively (Fig. 8). However, the observed depolarization is about 10% larger than that predicted by the simple model (Fig. 8B), suggesting that some energy transfer also occurs between dimers and/or that there are higher oligomers.

The band 3 proteins have been shown to exhibit both rotational [35] and translational [36] mobility, with diffusion coefficients around 10^{-3}/s and 10^{-11}—10^{-12} cm^2/s at 25°C, in ghosts, respectively. Thus, the mobility of the band 3 proteins is essentially zero within the lifetime of the excited state of FMA (1—2 ns), so that the proximity measured by energy transfer must reflect the distribution of distances in the steady state. The change in polarization with degree of labeling (Fig. 7) is inconsistent with a random distribution of freely diffusing monomers and indicates that all, or at least a large fraction of the monomers are in close proximity to another monomer at any one time. If it is assumed that transient aggregates are formed by random collisions of diffusing monomers, the lowest polarization of the aggregate would be around zero (since the angle between donor and acceptor would have a random value). Then, Weber's addition law (Eqn. 9) predicts that at most 25% of the band 3 proteins in these vesicles can exist as free monomers at any instant owing to the low value (0.1) of the polarization observed at saturation. Thus, if transient

aggregates are formed by random collisions between free monomers, they must exist for long times compared with the mean collision time in order to be the preponderant (>75%) fraction of the population in the steady state. From the above diffusion coefficient and the protein content of our vesicles we estimate a mean collision time between monomers in the range of 10—100 ms, which sets a lower limit to the lifetime of the dimers. This is much longer than the turnover time of anion exchange in red cells, which is of the order of microseconds [1,3,35], and is consistent with the idea that the transporting species is a dimer of band 3 [4]. However, comparisons between red cells and the vesicles used in this work must be made with caution since the fraction of functional proteins, their turnover, and their state of aggregation may be altered by the procedures used in the preparation of the vesicles.

The present experiments indicate that the band 3 proteins are in close interaction even in the nanosecond time scale. If the properties of the vesicle preparations used in this work are comparable to those of ghosts, our observations confirm previous suggestions that band 3 exists as dimers or tetramers, based on crosslinking experiments [15,26,27,37], electron microscopy [38], and molecular size estimates in detergent extracts [7,37,39], and exclude the possibility that the dimers are formed only in the presence of a crosslinker, or are aggregates formed by detergent solubilization. However, although the functional characteristics of the anion transport system in these vesicles are very similar to those in intact red blood cells [14], suggesting that the purification procedures do not alter significantly the properties of band 3, the possibility that protein aggregation occurs during isolation of the vesicles cannot be excluded.

The evidence for specific reaction with an SH group arises from the approximate 1 : 1 stoichiometry between FMA and band 3, and the prevention or reversal of the reaction by dithiothreitol or Cu^{2+}-phenanthroline. Although the location of this SH group cannot be determined directly from our experiments, the slower kinetics of the reaction in 'sealed' vs. 'leaky' vesicles suggest that the rate-limiting step in the former is translocation of FMA across the membrane. Since these vesicles are apparently 'right side out' [14] the most likely location of this group is on the inside surface, as previously determined in red cell ghosts [26]. The lack of effects of DNDS or ANS on the rate of FMA reaction suggests that FMA is not transported by the anion carrier.

Thus far, we have observed no significant spectral or kinetic changes in FMA fluorescence caused by the inhibitor DNDS or by the presence or absence of the substrates Cl^- and SO_4^{2-}, that may reflect conformational changes in the protein. Although it is possible that conformational changes do not occur, it is also likely that they may not be detectable even if present, either because FMA is not sensitive enough or because its binding site is too distant from the functional regions of the protein.

Proteolytic dissections with trypsin in ghosts [26] and inside-out vesicles [40] have shown that the reactive SH group is on a 40 000 dalton soluble segment of the band 3 protein, near the NH_2-terminal end. Since the transport function of band 3 appears to be in a membrane-bound 17 000 dalton segment remaining after proteolysis [40] the SH group may be too distant to sense conformational changes at the transport sites.

We must point out that although the anion transport system in these vesicles is functional as shown by the flux studies (Figs. 1, 2, 5 and ref. 14) the fraction of functional proteins has not been determined. Thus, it is possible that only a small fraction of the proteins in these vesicles are functional, and any functionally related conformational changes could be obscured by a large background of labeled, but non-functional proteins. Further work is necessary to clarify this point.

The present results suggest that FMA may be a useful probe to study the structure and dynamics of the band 3 protein with the following advantages: (a) The reaction is fast and a single site per monomer is labeled. Thus, no long incubation periods, high concentrations of probe, nor dialysis or washing of excess material are required. (b) The large fluorescence changes that occur upon reaction of FMA with the protein allow direct fluorimetric determination of the rate and stoichiometry of labeling, and may be used to detect conformational transitions. (c) The labeled protein is still functional in anion transport, since no significant changes in SO_4^{2-} fluxes are observed in vesicles labeled with saturating FMA concentrations (Fig. 5). (d) FMA is an excellent energy transfer acceptor for blue-emitting fluorophores. Thus, mapping of relevant sites up to 70 Å away from the FMA site should be possible from energy transfer measurements.

Since a variety of anion transport inhibitors like SITS, DIDS, ANS, dipyridamole and pyridoxal phosphate fluoresce in the blue, it may be possible to estimate the distance between their sites of interaction and the SH group labeled by FMA from energy transfer experiments.

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