^{35}Cl Nuclear Magnetic Resonance Line Broadening Shows that Eosin-5-Maleimide Does Not Block the External Anion Access Channel of Band 3

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ABSTRACT It has been suggested that Lys-430 of band 3, with which eosin-5-maleimide (EM) reacts, is located in the external channel through which anions gain access to the external transport site, and that EM inhibits anion exchange by blocking this channel. To test this, we have used ^{35}Cl nuclear magnetic resonance (NMR) to measure Cl^- binding to the external transport site in control and EM-treated human red blood cells. Intact cells were used rather than ghosts, because in this case all line broadening (LB) results from binding to external sites. In an NMR spectrometer with a 9.4-T magnetic field, red blood cells at 50% concentration (v/v) in 150 mM Cl^- medium at 3°C caused 19.0 ± 1.2 Hz LB. Of this, 7.9 ± 0.7 Hz was due to Cl^- binding to the high affinity band 3 transport sites, because it was prevented by an apparently competitive inhibitor of anion exchange, 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS). The LB was not due to hemoglobin released from the cells, as little LB remained in the supernatant after cells were removed by centrifugation. Saturable Cl^- binding remained in EM-treated cells, although the binding was no longer DNDS-sensitive, because EM prevents binding of DNDS. The lower limit for the rate at which Cl^- goes from the binding site to the external medium is 2.15×10^5 s^{-1} for control cells and 1.10×10^5 s^{-1} for EM-treated cells, far higher than the Cl^- translocation rate at 3°C (about 400 s^{-1}). Thus, EM does not inhibit Cl^- exchange by blocking the external access channel. EM may therefore be useful for fixing band 3 in one conformation for studies of Cl^- binding to the external transport site.

INTRODUCTION

Band 3 is a 101,700-Da integral membrane protein in human erythrocytes. A major function of band 3 is to mediate a tightly coupled one-for-one anion exchange across the membrane (Knauf, 1989; Jennings, 1992a,b). Eosin-5-maleimide (EM) labels Lys-430 of band 3 (Cobb and Beth, 1990) and inhibits the anion exchange process (Nigg et al., 1979). EM labels band 3 with ~1:1 stoichiometry and is widely used as a fluorescence probe for measuring rotational diffusion of band 3 (Cherry, 1979). Covalent reaction of EM with band 3 takes place only after EM is reversibly bound (Liu and Knauf, 1993). The reversible binding also inhibits Cl^- exchange, but the inhibition is partial (Knauf et al., 1993b). Although the bound EM appears to overlap the binding site of some apparently competitive inhibitors such as stilbene disulfonic acids (Cobb and Beth, 1990), the reversible inhibition is non-competitive (Knauf et al., 1993b). In addition, Passow et al. (1992) showed that mutation of the corresponding lysine residue (Lys-449) on mouse band 3 did not prevent Cl^- binding and transport. Therefore, EM does not block transport by binding to the Cl^- transport site.

A different mechanism of EM inhibition was suggested on the basis that external Cl^- ions gain access to the transport site by diffusing through a channel in the band 3 protein (Fig. 1). The external anion access channel was first proposed by Rao et al. (1979) on the basis of fluorescence energy transfer data that show that the stilbene disulfonate binding site, used as a marker for the transport site, is in the interior of the membrane bilayer. The existence of the external anion access channel is also supported by Jennings' (1992c; Jennings et al., 1990) data indicating that sulfate ions traverse part of the transmembrane electric field on their way to the external binding site. Some nuclear magnetic resonance (NMR) data showing that the transport site is protected from proteolysis (Falke et al., 1985b) also suggest that there is an anion access channel. Falke and Chan (1986b) showed that some inhibitors, such as 1,2-cyclohexanedione and dipyridamole, act by blocking this access channel.

Passow et al. (1992) suggested that the residue with which EM reacts might be located in the external anion access channel, based on the fact that Lys-430 is located toward the outer surface of the membrane and there is an overlap between EM and 4,4′-diisothiocyano-dihydrostilbene-2,2′-disulfonate (H_2DIDS, a disulfonic stilbene inhibitor that belongs to the same family of inhibitors as 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS)) binding. Because of this location, they proposed that EM and other reagents that bind to nearby sites might cause "inhibition by binding to access channel and ensuing blockage of access to T" (p. 95) (the transfer site), as shown schematically in Fig. 1. They also included the alternate possibility that EM binding has an allosteric effect on the transport site. Wyatt and Cherry (1992) explained the partial protection of bound EM from fluorescence-quenching agents present in the extracellular medium by proposing that EM is bound in a cleft in

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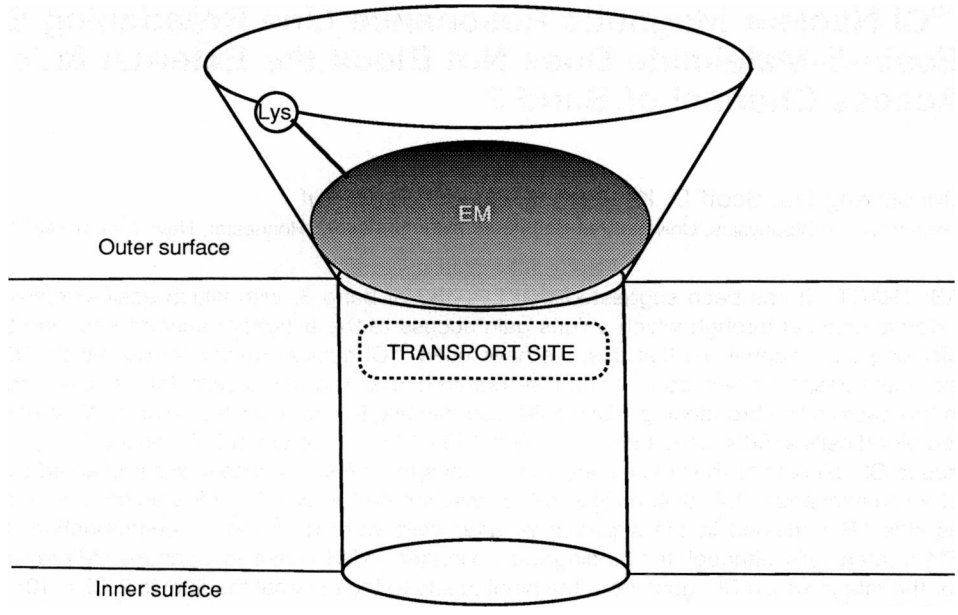

FIGURE 1 Channel blocker model of EM inhibition. The binding of EM blocks the external anion access channel.

band 3, which might correspond to the access channel for external anions. This concept was used to explain the effects of membrane potential on quenching and on EM reaction rate. This model, like that of Passow et al. (1992), implies that EM might block the access channel and thereby prevent external Cl^- from reaching the external anion transport site.

The binding of ^{35}Cl to macromolecules can be detected by NMR, because the spectral linewidth of bound Cl^- is much greater than that of free Cl^- in solution. In our case, the free Cl^- in solution is always in great molar excess relative to the bound Cl^-, and the free Cl^- exchanges with bound Cl^-. The ^{35}Cl spectra we observe are actually the signals of free Cl^- in solution with its linewidth broadened because of the exchange of free Cl^- with Cl^- in the bound state. The line broadening (LB) of the NMR spectrum caused by binding to a macromolecule is proportional to the ratio of bound to total Cl^- concentration, which is given by Falke et al. (1984a,b):

$$LB = \alpha * P_B = \alpha \frac{[Cl]_B}{[Cl]_T} = \alpha \frac{[X]}{[Cl] + K_d} \qquad (1)$$

where $[Cl]_B$ is the bound Cl^- concentration and $[Cl]_T$ is the total Cl^- concentration, which is nearly identical to the Cl^- concentration of the medium, [Cl], under the conditions where $[Cl]_T \gg [Cl]_B$. K_d is the dissociation constant of the binding site, [X] is the concentration of the binding site, which is proportional to cell concentration, α is a proportionality constant, and P_B is the probability of finding Cl^- in the bound state. In our study, LB was calculated by subtracting buffer linewidth from total linewidth of the sample.

NMR provides a way to measure the binding of substrate even when the translocation is inhibited. Thus, NMR is particularly useful to study the effect of inhibitors on the binding of substrate, whether the exchange is stopped or not (Falke and Chan, 1986a,b,c). This is a great advantage over the radioactive tracer flux method normally used to study the anion transport system, and makes it possible to investigate the mechanism of irreversible EM inhibition in detail, because binding of Cl^- to the transport site can be measured even when transport is completely inhibited by covalent reaction with EM. As pointed out by Falke and Chan (1986a,b,c), either a channel blocker type inhibitor or a transport site inhibitor (competitive inhibitor) will eliminate the LB caused by Cl^- binding to the transport sites. On the contrary, a translocation inhibitor, which inhibits by preventing the transporting conformational change of band 3, will not eliminate transport site LB.

In this study, we measured the LB of EM-treated intact red blood cells at various Cl^- concentrations. EM treatment did not significantly decrease total LB of the red cells, and the LB was still sensitive to changes in Cl^- concentration, indicating a saturable binding site. We have also tested the effect of a competitive substrate, iodide, on the Cl^- LB of control and EM-treated cells. The results also support the idea that the transport site is still accessible to external medium after EM reacts with band 3.

Finally, we estimated the Cl^- exchange rate between medium and the binding sites. The difference between control and EM-treated samples was small, demonstrating that EM could not inhibit Cl^- transport by slowing down the Cl^- exchange between the transport site and the external medium. A preliminary report of some of these results has appeared in abstract form (Liu et al., 1994).

MATERIALS AND METHODS

Cell preparation

Red blood cells were obtained from freshly drawn blood, donated by apparently healthy volunteers. Heparin was added as anticoagulant. The

cells were washed at least 3 times with 150 KH (150 mM KCl, 24 mM sucrose, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 5 mM glucose). For each wash, the cells were centrifuged for 5 min at 7,000 rpm; then the supernatant and buffy coat were removed by aspiration. The cells were then subjected to different treatments as described in the text and figure legends. The cells were stored on ice before NMR measurement. The pH of all media used in the experiments was adjusted to 6.9 at room temperature. Most of the measurements were completed within 36 h after the blood had been drawn.

^{35}Cl NMR spectroscopy

Most of the NMR measurements were performed on a Bruker/GE Omega NMR spectrometer (Fremont, CA) operating at a frequency of 39.2 MHz for ^{35}Cl (400 MHz for protons). A 10 mm diameter broad-band tunable probe was used. Temperature of the sample was controlled by flow of nitrogen or air regulated at 3 or 20°C, respectively, over the sample. Some of the experiments were performed on a 2 T superconductor magnet using a Bruker/GE CSI II NMR spectrometer/imager operating at ^{35}Cl resonance frequency 7.9 MHz (85 MHz for protons). Experiments were done at room temperature using a probe with a 12 mm diameter radio frequency coil.

For both spectrometers, the sweep width was 2000 Hz, and there were 256 points collected for each scan. The signal averages varied according to the concentration of Cl^- and LB of the sample. The typical range was 100 to 1000 scans requiring 14.8–148 s, respectively. An extra line broadening of 10 Hz was added to all spectra to improve signal/noise ratio. The original data set was zero-filled to 8192 data points before Fourier transformation. The linewidth was calculated by fitting the spectrum to a Lorentzian function after removal of any baseline offset. Shimming was done on the water proton signal of buffer. Solvent proton-linewidth was 3–5 Hz for buffer and 12–15 Hz for red cell samples.

Treatment with inhibitors

The treatment with EM (Molecular Probes, Inc., Eugene, OR) was carried out in the dark at 37°C in 150 KH buffer (pH = 6.71 at 37°C). To obtain more than 99% inhibition of Cl^- exchange, the cells were treated with 300 μM EM in 150 KH at 10% hematocrit for 2 h (Liu and Knauf, 1993).

To achieve nearly complete binding of band 3 with DNDS, enough DNDS was added to the samples to achieve more than 98% inhibition of Cl^- exchange. Based on the measurement that ID_{50} for DNDS is 4.11 μM in 150 mM Cl^- medium (Knauf et al., 1993b), the final concentration of DNDS in the extracellular volume was at least 400 μM. After adding DNDS, the samples were usually measured within 8 h to avoid lysis that might make the LB abnormally large. Treatment with other inhibitors is described in the figure legends.

Nystatin treatment

To vary the Cl^- concentration both outside and inside the cells, nystatin was used to increase the cation permeability of the membrane, so that cations and Cl^- could equilibrate as previously described (Knauf et al., 1989; Knauf and Brahm, 1989). The cells were loaded with medium containing 10, 20, 50, 100, or 200 mM Cl^- by incubating with 30 μg/ml nystatin at 0°C for 10 min. When the osmolarity difference between the starting and final media was large, several steps were used to bring the Cl^- concentration down. In order to avoid severe lysis, we always kept the ratio of osmolarity between two consecutive treatments <1.6. After each treatment, the samples were spun down and the supernatant was removed by aspiration. After the nystatin treatment, the cells were washed at least five times in the final medium to remove nystatin. There was a 5-min incubation at room temperature before each wash.

^{36}Cl flux experiments

To measure the effect of inhibitors on Cl^- exchange, cells were loaded with 2.0 μCi/ml ^{36}Cl (ICN Radiochemicals, Irvine, CA) by incubation at 25% hematocrit for 5 min at room temperature. Then the samples were stored on ice before measurement of Cl^- exchange as described by Dalmark and Wieth (1972).

Data analysis

The NMR data were processed with the spectrometer software. The radioactive isotope flux data were analyzed by using an Excel (Microsoft, PC version) spreadsheet. The Cl^- exchange rate constant was determined from a least-squares fit of the data to a two-compartment isotope exchange model (Gunn and Fröhlich, 1989).

RESULTS AND DISCUSSION

Characteristics of the intact cell system

Cl^- can bind to both the inside and the outside of the red cell membrane. In leaky red cell ghosts, binding sites on both sides of the membrane give rise to line broadening (Falke et al., 1984a,b). Under normal conditions, the numbers of band 3 in the inward- and outward-facing conformations are different, and the ^{35}Cl relaxation properties in these two types of binding sites might also be different. To selectively detect LB effects of the outward-facing conformation, we used intact cells instead of red cell ghost membranes. Intact red blood cells have a very high concentration of hemoglobin, which binds Cl^- and greatly broadens the NMR spectral linewidth for intracellular Cl^- (Forsen and Lindman, 1981). The effect of line broadening inside the cells is such that the Cl^- signal from inside the cell decays before data acquisition begins, and the observed signal is entirely from extracellular ^{35}Cl (Falke et al., 1984b). Furthermore, the Cl^- exchange rate across the cell membrane is much slower than the relaxation rate of the extracellular ^{35}Cl NMR signal (Brahm, 1977), which ensures that the exchange rate of extracellular Cl^- with intracellular Cl^- does not contribute to our LB signal. To verify this, we used a noncompetitive inhibitor, niflumic acid, that inhibits Cl^- transport but does not prevent binding of Cl^- to the transport site (Falke and Chan, 1986c). The LB of niflumic acid-treated cells did not decrease compared with that of control (data not shown), indicating that no significant portion of the LB is related to transport of Cl^- across the membrane. Thus, with intact cells, only the external Cl^- binding sites account for the LB observed. This system has an obvious advantage over using ghost membranes if one wishes to see whether an inhibitor blocks the external access channel.

For quantitative analysis of the data, it is usual to assume that the number of binding sites is far less than the number of free anions (Bull, 1972). In our studies, the lowest Cl^- concentration used was 10 mM. At 50% hematocrit, the band 3 protein concentration is about 1.66×10^{-2} mM (expressed in terms of mol/l of extracellular solution). Thus, the Cl^- concentration is 500 times greater than the number of sites, so the assumption is valid even for the lowest Cl^- concentration used.

Relation of LB to hematocrit

To get an appreciable LB signal, we need to use a high cell concentration (~50%). The cell volume must be excluded in the data analysis because the signal from chloride inside the cells is not observed. Fig. 2 shows the relation between LB and cell concentration expressed in terms of hematocrit, h (volume of cells/volume of suspension). $[Cl]_B$ in Eq. 1 is proportional to h and $[Cl]_T$ is proportional to $(1 - h)$, so $h/(1 - h)$ is proportional to $[Cl]_B/[Cl]_T$. LB is proportional to $h/(1 - h)$ at low hematocrit as expected from Eq. 1, but the LB levels off when $h > 0.5$ (data not shown).

This effect is explained by the fact that the shape of the NMR spectrum is not a perfect Lorentzian function. Under this circumstance, the single Lorentzian fit to the spectra is only an approximation, and the spectra are better represented by two Lorentzian functions that are superimposed but have different linewidths. Thus, the free induction decay signal can be better fitted by a biexponential function (Price et al., 1991; Liu, 1995). The two components arise from separate transitions within the 3/2 spin system (Bull, 1972) and will be discussed in detail in a future publication. At lower hematocrit, the difference between the linewidths for the two components is small enough that the signal can be well approximated by a single component. At higher hematocrit, the difference becomes larger and the single exponential approximation leads to an underestimate of LB and to a distortion of the linear relationship at high hematocrit. This effect does not exist in DNDS-treated cells at 20°C, which indicates different properties of the transport site and the low affinity DNDS-insensitive site (see below).

In practice, we kept the hematocrit as consistent as possible for the samples that were compared with each other to avoid using a correction curve that might introduce an additional systematic error. The small variations of hematocrit around 50% were corrected by an empirical equation in which $h/(1 - h/3)$ instead of $h/(1 - h)$ is used, because this gives the most linear result.

Separation of low affinity and high affinity sites

Besides the transport sites on band 3 protein that can bind Cl^-, there are other sites on the surface of the membrane that can also bind Cl^- and give rise to LB. According to Eq. 1, the total LB can be written as:

$$LB_{Total} = \alpha_1 \frac{[X_1]}{[Cl] + K_{dl}} + \alpha_2 \frac{[X_2]}{[Cl] + K_{d2}}. \quad (2)$$

Subscript 1 stands for the parameters for transport sites and subscript 2 for those corresponding to other sites. The transport sites have a higher affinity for Cl^-, and the LB due to Cl^- binding to the transport sites can be eliminated by adding DNDS, which behaves like a competitive inhibitor of band 3-mediated Cl^- exchange (Fröhlich, 1982). DNDS also binds with lower affinity to a noncompetitive inhibitory site (S. J. Liu, E. A. Ries, and P. A. Knaupf, submitted for publication; Knauf et al., 1993a). This binding cannot take place when the higher affinity competitive site is occupied, so under most conditions DNDS will be bound to the noncompetitive inhibitory site in only a small fraction of the total number of band 3 molecules.

The DNDS-insensitive LB comes from Cl^- binding to low affinity sites ($K_d \gg 0.5$ M) (Falke et al., 1984a,b). This K_d is much larger than the physiological Cl^- concentration, so $K_{d2} \gg [Cl]$ in Eq. 2. Thus Eq. 2 can be simplified as:

$$LB_{Total} \approx \alpha_1 \frac{[X_1]}{[Cl] + K_{d1}} + \alpha_2 \frac{[X_2]}{K_{d2}} = \alpha_1 \frac{[X_1]}{[Cl] + K_{d1}} + LB_{DNDS}. \quad (3)$$

Because the term for DNDS-insensitive LB is independent of [Cl], and hematocrit is constant (i.e., $[X_2]$ is constant), this term is represented by a constant, LB_{DNDS}. Thus, the difference between LB for control and DNDS-treated sam-

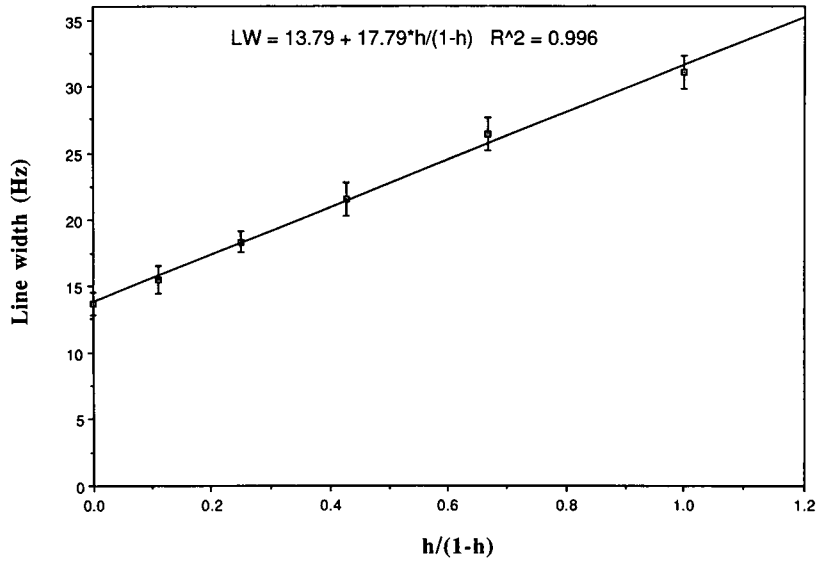

FIGURE 2 Linewidth of red blood cell samples with different hematocrits (h) is plotted as a function of $h/(1 - h)$. The result is consistent with the prediction that the Cl^- signal inside the cells is invisible. Measurements were done on the low field (2 T) spectrometer at room temperature. Cells were suspended in 150 KH medium. Three or four values from different experiments were averaged to give each point. Cells were not necessarily from the same donor, so the error bars (SD) include variation among different donors.

ples, $LB_{Total} - LB_{DNDS}$, gives the LB due to Cl^- binding to transport sites (Eq. 3).

Table 1 gives LB of control and DNDS-treated cells in media containing 150 or 50 mM Cl^- at 3 or 20°C. Although the DNDS-sensitive LB in Hz is similar at different temperatures, the buffer linewidth increases from 13.1 ± 0.7 Hz to 19.9 ± 0.4 Hz when the temperature changes from 20°C to 3°C and the DNDS-insensitive LB also increases. Therefore, the DNDS-sensitive LB (at 150 mM Cl^-) represents a larger portion of the total linewidth at 20°C (33%) than at 0°C (21%).

The effect of cell lysis

As stated above, the high concentration of hemoglobin inside the cells suppresses the chloride signal from inside and simplifies the system. On the other hand, the hemoglobin released from lysed cells can cause additional line broadening of the Cl^- outside the cells. This effect could interfere with our observations of LB associated with the transport site.

When we measured the LB of supernatant from the cell samples, however, the average LB of supernatant from control cells was 3.2 ± 1.2 Hz, whereas that from DNDS-treated cells was 4.0 ± 1.5 Hz. The additional DNDS-sensitive LB caused by cell lysis is thus −0.8 ± 1.9 Hz. This number is not significantly different from 0 and is comparable to the total experimental standard error, ~1 Hz. In our studies, therefore, we did not correct the LB for cell lysis. However, measures have been taken to reduce cell lysis in several ways, such as reducing the time each sample has to wait before NMR measurement and handling samples with care. Because lysis increases with time, we designed our experiments so that all NMR measurements can be done within 36 h from the time the blood is drawn. Low temperature reduces cell lysis, so the samples were kept on ice as long as possible before the NMR measurements.

LB in EM-treated cells comes from both high and low affinity sites

Fig. 3 shows the inhibition of Cl^- exchange resulting from covalent reaction of band 3 with EM. Under the conditions of our experiments, the flux was inhibited more than 99%. Liu and Knauf (1993) observed that the residual flux could be further inhibited by treatment with 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), an inhibitor that is mutually exclusive with EM (i.e., both cannot be bound at the same time) so it can only react with band 3 molecules that have not been labeled by EM. The residual flux is only 0.13% after further treatment with DIDS (Liu and Knauf, 1993). This suggests that the ~1% residual flux in Fig. 3 represents unreacted band 3 and that once band 3 is labeled by EM, it is inhibited nearly completely. Because the fluctuation of the DNDS-sensitive NMR LB signal due to experimental error is in the range of 10% of the measured value, the 1% unreacted band 3 should not significantly affect our measurements.

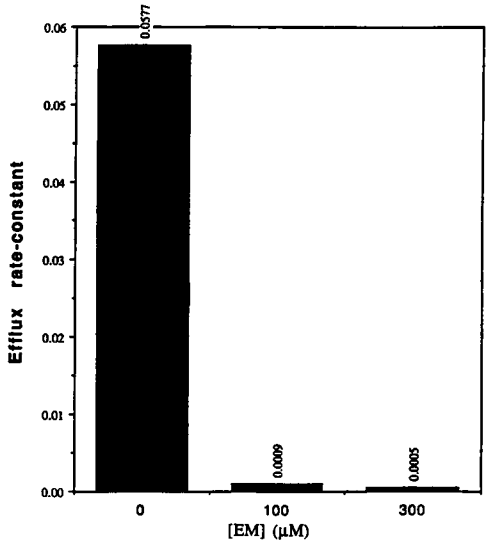

FIGURE 3 Effect of EM treatment on Cl^- efflux measured by ^{36}Cl exchange rate constant. Cells were pretreated with 300 or 100 μM EM at 10% hematocrit at 37°C for 2 h and then washed at least three times with 150 KH to remove unreacted EM. Fluxes were done at 0°C in 150 KH medium.

Although the labeling of band 3 with EM dramatically decreases Cl^- flux (Fig. 3), the ^{35}Cl NMR LB of EM-treated cells is not significantly decreased compared with that of control cells (Table 1). It is obvious that the LB of EM-treated cells is significantly greater than the LB caused only by low affinity sites, i.e., the LB with DNDS present. This throws doubt on the suggestions that EM inhibits by blocking the external anion access channel, because such blockage should eliminate LB due to the transport sites. However, this observation alone cannot prove that transport

TABLE 1 Comparison of control, EM, and DNDS LB at 3 and 20°C in 150 and 50 mM Cl^- medium

	150 mM Cl^-			50 mM Cl^-		
Temperature	Control	EM	DNDS	Control	EM	DNDS
3°C	19.0 ± 1.2	16.2 ± 2.5	11.1 ± 0.7	19.5 ± 0.7	18.1 ± 0.6	10.6 ± 1.2
20°C	14.8 ± 0.6	13.3 ± 1.0	5.2 ± 0.8	16.0 ± 1.7	16.3 ± 0.5	6.5 ± 1.2

LB is measured in Hz. Each value represents the average and standard deviation for three to five samples. Control stands for the samples without any inhibitor treatment. EM refers to cells that were treated with 300 μM EM for 2 h at 37°C followed by three to five washes to remove unreacted EM. DNDS stands for samples containing 500 μM DNDS. All samples were adjusted to 50% hematocrit before NMR measurement.

sites are still accessible to free Cl^- in the external medium after the band 3 is labeled by EM. The same experimental result might also be obtained if EM increases the non-transport-site-related LB just enough to compensate for the decrease in LB due to EM blocking the anion access channel.

To distinguish these alternatives, we need to know how much of the total LB of EM-treated cells is due to binding to the transport site. Unfortunately, with EM-treated cells we could not separate the two kinds of LB by using DNDS as with control cells, because EM and DNDS are mutually exclusive (Knauf et al., 1993b). We therefore used a different approach in which we changed the Cl^- concentration at both sides of the membrane and measured the LB of EM-treated cells (Fig. 4). As in control cells, a portion of the total LB of EM-treated cells decreases with increasing Cl^- concentration. If the LB of EM-treated cells were totally due to low affinity sites, the LB would be almost completely insensitive to Cl^- concentration (Eq. 3). By fitting the total LB of EM-treated cells to Eq. 3, we can separate the LB of EM-treated cells into two components, one for high affinity sites and one for low affinity sites (Fig. 4, *A* and *B*), just as in untreated control cells. LB_{DNDS} represents the LB of low affinity sites and is calculated to be 6.8 ± 1.8 Hz at 20°C and 9.7 ± 1.6 Hz at 3°C, not significantly different from the measured DNDS-insensitive LB for control cells, which is 6.3 ± 1.0 Hz (20°C) or 9.9 ± 2.1 Hz (3°C). This indicates that low affinity site LB does not change after EM treatment and that high affinity sites are present even though the flux is inhibited by more than 99%. This result strongly suggests that Cl^- can still bind to the transport site after EM labeling.

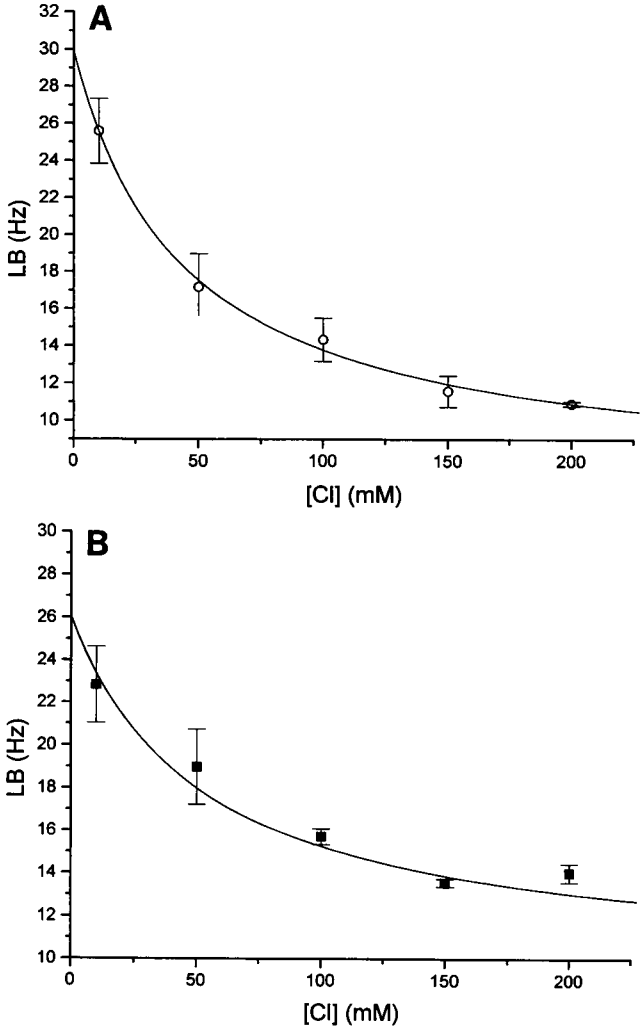

FIGURE 4 Effect of Cl^- concentration on LB for EM-treated cells. The hematocrit was 50%. Cells were treated with nystatin to bring $[Cl_i]$ equal to $[Cl_o]$. Error bars represent SD. The data were fitted to the function $LB_{Total} = \alpha[X]/([Cl] + K_d) + LB_{DNDS}$ (Eq. 3) by a nonlinear least-squares fitting, with each point weighted according to the reciprocal of its SD squared (instrumental weighting). The K_d estimated from this set of data is 44 ± 26 mM for 20°C (*A*) and 52 ± 30 mM for 3°C (*B*). The LB_{DNDS} was calculated to be 6.8 ± 1.8 Hz (*A*) and 9.7 ± 1.6 Hz (*B*). All data were collected with blood from a single donor.

As a reversible inhibitor, EM binds five times more strongly to the E_o form of band 3, with the transport site empty and facing outward, than to the E_i form, with the transport site facing inward (Knauf et al., 1993b). Because most of the unloaded transport sites face inward under normal conditions (Knauf and Brahm, 1989), one might therefore expect EM treatment to increase the fraction of band 3 molecules with the transport site facing outward, and therefore to increase the LB, which should be proportional to the number of outward-facing sites under our conditions.

The failure to observe an increase in LB could be explained in several ways. First, we do not know the relative number of inward- (ECl_i) and outward-facing (ECl_o) Cl-loaded transport sites, nor do we know anything about the preference of EM for ECl_o versus ECl_i. With 150 mM Cl^- present, most of the sites will be in the Cl-loaded form, so no predictions about the effects of EM on site orientation can be made under these conditions. Second, upon covalent reaction EM might alter the site orientation. Third, EM might alter the α factor for Cl^-, which could have a compensatory effect on the LB. As we will discuss below, the rate of exchange between free and bound Cl^- at the transport site is slower in EM-treated cells, which will result in a decreased value of α. Falke and Chan (1986c) observed that niflumic acid, a translocation inhibitor that also prefers E_o over E_i by about a factor of 5, also did not increase the LB caused by intact cells. They interpreted this as probably indicating that niflumic acid simply raises the free energy of the transition state between the ECl_i and ECl_o forms, thereby preserving the original orientation of transport sites. This interpretation may also apply to the situation with EM, but other possibilities cannot be ruled out without further information.

Iodide competes with chloride in EM-treated cells

Although the presence of EM prevents many inhibitors of band 3, such as DNDS, from binding, a smaller molecule, e.g., a substrate of the anion exchanger, might still bind to

the transport site, if EM really does not block access to this site. We used iodide as a competitive anion substrate and measured the effect of iodide on the LB of chloride. The results are shown in Fig. 5. Iodide competes with both DNDS-sensitive (control − DNDS) and DNDS-insensitive (DNDS) sites, because LB from both components decreases when iodide is present. With the assumption that the low affinity site LB was the same for control and EM-treated cells at each I^- concentration, the portion of LB corresponding to the DNDS-sensitive LB in EM-treated cells (EM − DNDS) also decreases when the iodide concentration is increased from 0 to 62 mM. This is similar to the situation in control cells, and it indicates that I^- competes with Cl^- for high affinity sites in both control and EM-treated cells, further reinforcing the idea that the external access channel is not blocked by EM.

The rate of Cl^- exchange between medium and transport site

Falke et al. (1985a) pointed out that the $^{35}Cl/^{37}Cl$ LB ratio provides some information about the exchange rate of Cl^- between the binding sites and the external medium. The exchange of Cl^- between a binding site and medium can be slow, intermediate, or rapid compared with chloride T_2 in the bound environment. The ^{35}Cl to ^{37}Cl LB ratio is close to 1 for the slow exchange condition, while it is 1.6 for rapid exchange. The ratios for the LB from different types of sites are different. The Cl^- in free solution gives a ^{35}Cl to ^{37}Cl linewidth ratio close to 1.6, which is the limit for the extreme fast exchange condition. The ratio for the high affinity sites indicates that the exchange between free Cl^- in the medium and Cl^- bound to the high affinity sites is near the slow exchange limit (Falke et al., 1985a).

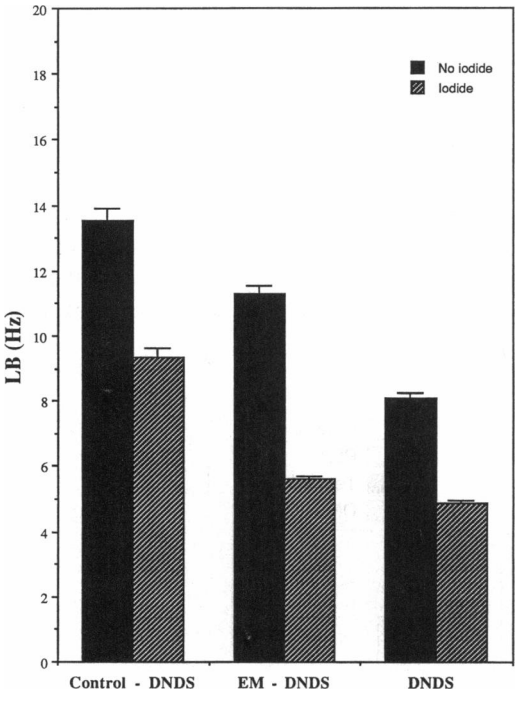

FIGURE 5 Iodide as a competitive anion in control and EM-treated cells. The medium with iodide is 100 mM KCl, 62 mM KI, and 20 mM HEPES; the medium without iodide is 100 mM KCl, 124 mM sucrose, and 20 mM HEPES. The experiment was performed at 20°C. "DNDS" indicates the LB of cells exposed to 500 μM DNDS in the medium with (Iodide) or without iodide (No iodide). "Control − DNDS" is obtained by subtracting LB of DNDS-treated cells from the total LB of control cells, which indicates the DNDS-sensitive LB. Assuming that EM does not affect the low affinity site LB, we subtracted the LB of DNDS-treated cells from total LB of EM-treated cells to obtain the result designated "EM − DNDS". It shows that the portion of LB in EM-treated cells that corresponds to DNDS-sensitive sites was also reduced by adding iodide. Note that this donor has a higher than average LB for the low affinity sites.

We measured the ^{35}Cl to ^{37}Cl DNDS-sensitive LB ratio with control cells and observed that the ratio was 1.1 (200 mM [Cl^-], 3°C). Based on the assumption that the low affinity site LB is the same for both control and EM cells, the ratio for EM-treated cells was calculated to be 1.0 (200 mM [Cl^-], 3°C). Thus, the exchange in EM-treated cells is even closer to the slow exchange limit than in control cells.

If the exchange of free Cl^- with bound Cl^- at the transport sites is in the slow exchange limit, the LB is proportional to the rate of exchange of bound Cl^- with Cl^- in the solution. We can thus estimate the dissociation rate constant, k_{off}, if we know the probability that Cl^- is in the bound state, P_B (Falke et al., 1985a). The equation is:

$$k_{off} = \pi \times LB/P_B. \quad (4)$$

If we use the maximal value for P_B, assuming that all band 3 molecules have Cl^- bound, we get a lower limit for k_{off}, as shown in Table 2. The k_{off} values estimated for control and EM-treated cells are very similar and much too large for the exchange process to limit the transport rate. For example, at 3°C in 150 KH medium, the translocation rate is 400 s^{-1} (Brahm, 1977). To get 99% inhibition by slowing down the exchange of Cl^- between medium and transport sites, the k_{off} should be $<4\ s^{-1}$ instead of the value $1.10 \times 10^5\ s^{-1}$ calculated from the LB data. Therefore, it is impossible for EM to inhibit Cl^- transport by slowing down (blocking) the exchange of Cl^- between the external medium and the transport sites.

Application of intact cell NMR to other inhibitors

The methods discussed here can be used to study the effect of any inhibitor on Cl^- binding to the outward-facing site. Fig. 6 shows an experiment with DNDS and two other inhibitors, tetrathionate and pyridoxal-5-phosphate (P5P). It was shown previously that P5P could react both reversibly and irreversibly with band 3 and inhibit anion transport (Bar-Noy and Cabantchik, 1990; Salhany et al., 1987). In this experiment, both inhibitors were used as reversible inhibitors. The decrease of transport site LB is consistent with the decrease of Cl^- flux (Fig. 7), which indicates that they both inhibit either by blocking the external access channel or by competing with Cl^- for the transport site. When the cells are pretreated with EM, none of the inhib-

TABLE 2 Dissociation rate constants for control and EM-treated cells at 3°C

Cl Concentration (mM)	Control LB (Hz)	EM LB (Hz)	P_B ([P]/[Cl])	Control $k_{off} \geq$	EM $k_{off} \geq$
50	9.74	10.08	3.32E − 04	9.20E + 04	9.55E + 04
100	7.77	6.02	1.66E − 04	1.47E + 05	1.14E + 05
150	7.57	3.86	1.11E − 04	2.15E + 05	1.10E + 05
200	5.79	4.30	8.30E − 05	2.19E + 05	1.63E + 05

Cell concentration was 50% hematocrit. "Control LB" was calculated by subtracting DNDS line width from total line width of corresponding control cells. "EM LB" was obtained by subtracting LB_{DNDS} (calculated in figure 4 B) from total line width of corresponding EM-treated cells. [P] is the estimated band 3 concentration, which is 16.6 μM at 50% hematocrit. k_{off} was estimated from Eq. 4.

itors can decrease LB. This indicates that the binding sites of all three inhibitors overlap with that of EM.

CONCLUSIONS

Previously we have argued that Lys-430, the residue with which EM reacts, is not located at the external transport site (Liu and Knauf, 1993). This was based on evidence that reversible inhibition by EM is noncompetitive (Knauf et al., 1993b) and that EM acts as an affinity label, reacting covalently only after binding reversibly to band 3 (Liu and Knauf, 1993). This evidence, however, could not rule out the possibility that there is a change of conformation after EM reaction, such that covalently bound EM overlaps the transport site. Indeed, there is some evidence for a conformational change, because covalent reaction with EM causes much greater inhibition than does reversible binding (Knauf et al., 1993b; Liu and Knauf, 1993). The present data show that Cl^- binding to the external transport site persists even after covalent reaction with EM, thereby conclusively demonstrating that intact Lys-430 is not required for Cl^- binding. This conclusion is in accord with a previous mutagenesis study by Passow et al. (1992).

Earlier reports (Wyatt and Cherry, 1992; Passow et al., 1992) suggested that EM may inhibit Cl^- flux by restricting Cl^- access to the band 3 transport site (Fig. 1). Our results indicate that Cl^- can still bind to the transport site after the cells are labeled by EM, demonstrating that a different mechanism must be responsible for transport inhibition. Evidence suggesting that EM does not directly block Cl^- binding to the inward-facing site of band 3 is provided by the fact that EM binds to Lys-430 (Cobb and Beth, 1990), which is located toward the outer surface of the membrane, and that EM is not accessible to fluorescence-quenching agents from the inside of the cells (Wyatt and Cherry, 1992). Although the possibility that Cl^- binding at the inward-facing site is inhibited by an allosteric effect cannot be strictly excluded, it seems most probable that transport inhibition occurs because EM does not allow band 3 to

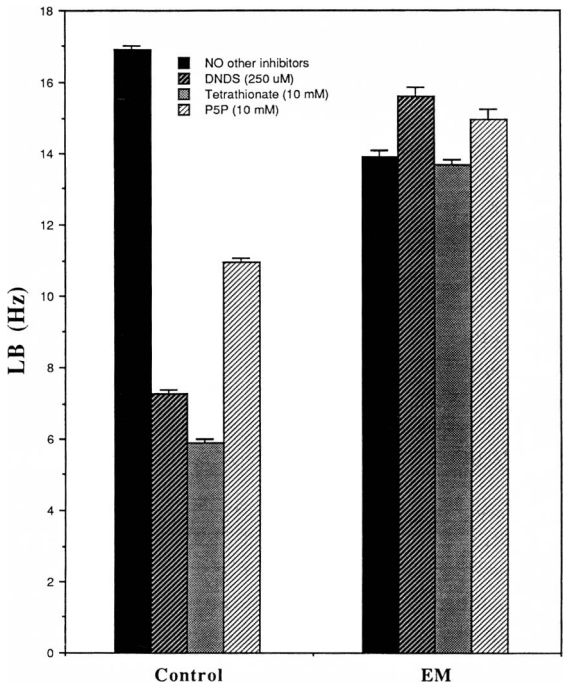

FIGURE 6 Application of intact cell ^{35}Cl NMR to other inhibitors. Data show the effects of three inhibitors, DNDS, P5P, and tetrathionate, on LB of control and EM-pretreated cells. The fact that P5P does not totally inhibit transport LB indicates a lower affinity compared with tetrathionate, whose K_d is no more than 500 μM (Deuticke et al., 1978). For this figure and for Fig. 7, concentrations are expressed per total volume of cell suspension.

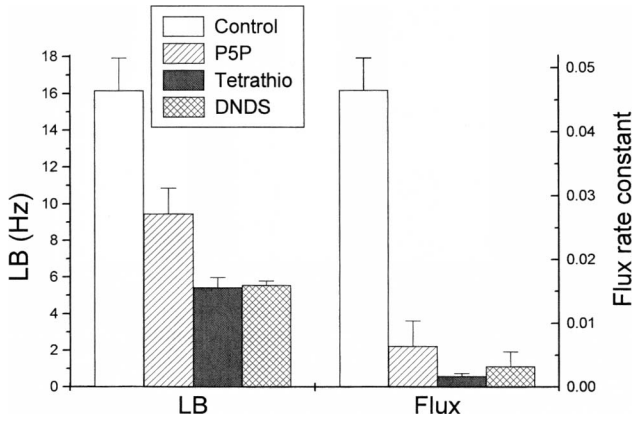

FIGURE 7 Effect of 10 mM P5P on LB and flux rate constant. ^{35}Cl NMR spectral linewidths were measured at 20°C for control cells, 10 mM pyridoxal-5-phosphate-treated cells (P5P), 10 mM tetrathionate-treated cells (Tetrathio) and 250 μM DNDS-treated cells (DNDS). LB was calculated by subtracting buffer linewidth from the total linewidth of the corresponding samples. The hematocrit was 50% for all samples. Flux rate constant was measured by ^{36}Cl exchange at 0°C as described in Materials and Methods. Error bars indicate SD of three to five samples.

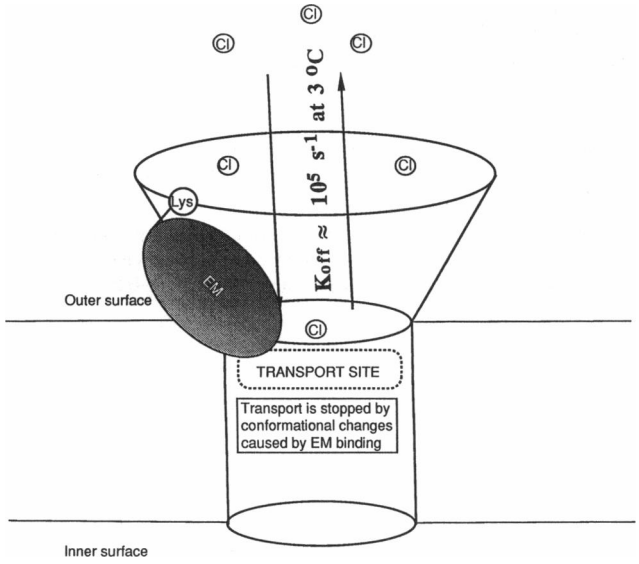

FIGURE 8 Proposed model for EM inhibition. The EM molecule does not block the anion access channel (substrate can still bind to the transport site), but the covalent reaction of EM with band 3 prevents the protein from undergoing the translocating conformational change, which is the rate-limiting step in anion transport.

alternate between the outward- and the inward-facing conformations (Fig. 8). By freezing the band 3 conformation, EM may provide a way to measure the true binding affinity of Cl^- to the outward-facing transport site, because under these conditions the effects of the transporting conformational change on the apparent affinity (Fröhlich and Gunn, 1986) are eliminated.

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REFERENCES

Bar-Noy, S., and Z. I. Cabantchik. 1990. Transport domain of the erythrocyte anion exchange protein. *J. Membr. Biol.* 115:217–228.

Brahm, J. 1977. Temperature-dependent changes of chloride transport kinetics in human red cells. *J. Gen. Physiol.* 70:283–306.

Bull, T. E. 1972. Nuclear magnetic relaxation of spin-3/2 nuclei involved in chemical exchange. *J. Magn. Reson.* 8:344–353.

Cherry, R. J. 1979. Rotational and lateral diffusion of membrane proteins. *Biochim. Biophys. Acta.* 559:289–327.

Cobb, C. E., and A. H. Beth. 1990. Identification of the eosinyl-5-maleimide reaction site on the human erythrocyte anion-exchange protein: overlap with the reaction sites of other chemical probes. *Biochemistry.* 29:8283–8290.

Dalmark, M., and J. O. Wieth. 1972. Temperature dependence of chloride, bromide, iodide, thiocyanate and salicylate transport in human red cells. *J. Physiol.* 224:583–610.

Deuticke, B., M. von Bentheim, E. Beyer, and D. Kamp. 1978. Reversible inhibition of anion exchange in human erythrocytes by an inorganic disulfonate, tetrathionate. *J. Membr. Biol.* 44:135–158.

Falke, J. J., and S. I. Chan. 1986a. Molecular mechanisms of band 3 inhibitors. 1. Transport site inhibitors. *Biochemistry.* 25:7888–7894.

Falke, J. J., and S. I. Chan. 1986b. Molecular mechanisms of band 3 inhibitors. 2. Channel blockers. *Biochemistry.* 25:7895–7898.

Falke, J. J., and S. I. Chan. 1986c. Molecular mechanisms of band 3 inhibitors. 3. Translocation inhibitors. *Biochemistry.* 25:7899–7906.

Falke, J. J., K. J. Kanes, and S. I. Chan. 1985a. The kinetic equation for the chloride transport cycle of band 3. A ^{35}Cl and ^{37}Cl NMR study. *J. Biol. Chem.* 260:9545–9551.

Falke, J. J., K. J. Kanes, and S. I. Chan. 1985b. The minimal structure containing the band 3 anion transport site. A ^{35}Cl NMR study. *J. Biol. Chem.* 260:13294–13303.

Falke, J. J., R. J. Pace, and S. I. Chan. 1984a. Chloride binding to the anion transport binding sites of band 3. A ^{35}Cl NMR study. *J. Biol. Chem.* 259:6472–6480.

Falke, J. J., R. J. Pace, and S. I. Chan. 1984b. Direct observation of the transmembrane recruitment of band 3 transport sites by competitive inhibitors. A ^{35}Cl NMR study. *J. Biol. Chem.* 259:6481–6491.

Forsen, S., and B. Lindman. 1981. Ion binding in biological systems as studied by NMR spectroscopy. *Methods Biochem. Anal.* 27:289–486.

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

Fröhlich, O., and R. B. Gunn. 1986. Erythrocyte anion transport: the kinetics of a single-site obligatory exchange system. *Biochim. Biophys. Acta.* 864:169–194.

Gunn, R. B., and O. Fröhlich. 1989. Methods and analysis of erythrocyte anion fluxes. *In* Methods in Enzymology, Vol. 173. S. Fleischer and B. Fleischer, editors. Academic Press, Inc., San Diego, California. 54–80.

Jennings, M. L. 1992a. Cellular anion transport. *In* The Kidney: Physiology and Pathophysiology. D. W. Seldin and G. Giebisch, editors. Raven Press, New York. 113–145.

Jennings, M. L. 1992b. Anion transport proteins. *In* The Kidney: Physiology and Pathophysiology. D. W. Seldin and G. Giebisch, editors. Raven Press, New York. 503–535.

Jennings, M. L. 1992c. Characteristics of the binding site for extracellular substrate anions in human red blood cell band 3. *Ann. N. Y. Acad. Sci.* 84–95.

Jennings, M. L., M. Allen, and R. K. Schulz. 1990. Effect of membrane potential on electrically silent transport: potential-independent translocation and asymmetric potential-dependent substrate binding to the red blood cell anion exchange protein. *J. Gen. Physiol.* 96:991–1012.

Knauf, P. A. 1989. Kinetics of anion transport. *In* The Red Cell Membrane. B. U. Raess and G. Tunnicliff, editors. Humana Press, Clifton, N. J. 171–200.

Knauf, P. A., and J. Brahm. 1989. Functional asymmetry of the anion exchange protein, capnophorin: effects on substrate and inhibitor binding. *In* Methods in Enzymology, Vol. 173. S. Fleischer and B. Fleischer, editors. Academic Press, New York. 432–453.

Knauf, P. A., E. A. Ries, L. A. Romanow, S. Bahar, and E. S. Szekeres. 1993a. DNDS (4,4′-dinitro-stilbene-2,2′-disulfonate) does not act as a purely competitive inhibitor of red blood cell band 3-mediated anion exchange. *Biophys. J.* 64:A307 (Abstr.).

Knauf, P. A., L. J. Spinelli, and N. A. Mann. 1989. Flufenamic acid senses conformation and asymmetry of human erythrocyte band 3 anion transport protein. *Am. J. Physiol.* 257:C277–C289.

Knauf, P. A., N. M. Strong, J. Penikas, R. B. Wheeler Jr., and S. J. Liu. 1993b. Eosin-5-maleimide inhibits red cell Cl^- exchange at a noncompetitive site that senses band 3 conformation. *Am. J. Physiol.* 264: C1144–C1154.

Liu, D. 1995. Detection of Cl^- binding to band 3 by double-quantum-filtered ^{35}Cl NMR. *Biophys. J.* 68:A420.(Abstr.)

Liu, D., S. Kennedy, and P. A. Knauf. 1994. ^{35}Cl NMR line-broadening (LB) shows that eosin-5-maleimide (EM) does not block the external anion access channel of band 3. *Biophys. J.* 66:A337.(Abstr.)

Liu, S. J., and P. A. Knauf. 1993. Lys-430, site of irreversible inhibition of band 3 Cl^- flux by eosin-5-maleimide, is not at the transport site. *Am. J. Physiol.* 264:C1155–C1164.

Nigg, E., M. Kessler, and R. J. Cherry. 1979. Labeling of human erythrocyte membranes with eosin probes used for protein diffusion measurements. Inhibition of anion transport and photo-oxidative inactivation of acetylcholinesterase. *Biochim. Biophys. Acta.* 550:328–340.

Passow, H., S. Lepke, and P. G. Wood. 1992. Exploration of the mechanism of mouse erythroid band 3-mediated anion exchange by site-directed mutagenesis. *In* The Band 3 Proteins: Anion Transporters, Binding Proteins and Senescent Antigens. E. Bamberg and H. Passow, editors. Elsevier Science Publishers, Amsterdam.

Price, W. S., P. W. Kuchel, and B. A. Cornell. 1991. A ^{35}Cl and ^{37}Cl NMR study of chloride binding to the erythrocyte anion transport protein. *Biophys. Chem.* 40:329–337.

Rao, A., P. Martin, R. A. F. Reithmeier, and L. C. Cantley. 1979. Location of the stilbene disulfonate binding site of the human erythrocyte anion-exchange system by resonance energy transfer. *Biochemistry.* 18: 4505–4516.

Salhany, J. M., P. B. Rauenbuehler, and R. L. Sloan. 1987. Characterization of pyridoxal 5′-phosphate affinity labeling of band 3 protein. Evidence for allosterically interacting transport inhibitory subdomains. *J. Biol. Chem.* 262:15965–15973.

Wyatt, K., and R. J. Cherry. 1992. Effects of membrane potential on band 3 conformation in the human erythrocyte membrane detected by triplet state quenching experiments. *Biochemistry.* 31:4650–4656.