Source of Transport Site Asymmetry in the Band 3 Anion Exchange Protein Determined by NMR Measurements of External Cl^- Affinity†

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ABSTRACT: Flux measurements indicate that a far greater number of unloaded band 3 anion transport sites face the cytoplasm than face the external medium, but the reason for this striking asymmetry has remained obscure. To resolve this question, we have measured the apparent Cl^- affinity of the transport site of human red blood cell band 3 protein under various conditions by analyzing the ^{35}Cl NMR free induction decay (FID). The $[Cl^-]$ that half-saturates the transport sites with $[Cl_i] = [Cl_o]$ ($K_{1/2}$) in RBC membranes (ghosts) is 46 ± 5 mM at 0°C, while the $K^o_{1/2}$ (for half-saturation with $[Cl_o]$ at constant $[Cl_i]$) of intact cells is 3.2 ± 2.1 mM. When cells were pretreated with EM, an inhibitor of band 3 anion exchange that does not prevent Cl^- binding to the external transport site, $K_{1/2}$ and $K^o_{1/2}$ are 41 ± 14 and 46 ± 12 mM, respectively. The EM-induced increase in $K^o_{1/2}$ with little change in $K_{1/2}$ can be most simply interpreted as meaning that EM abolishes the effects of the translocation rate constants on $K^o_{1/2}$ so that $K^o_{1/2}$ and $K_{1/2}$ of EM-treated cells now both reflect the true dissociation constant for binding of Cl^- to the external transport site, K_o. The fact that K_o for a slowly transported anion, iodide, is nearly the same in EM-treated as in control cells indicates that EM does not significantly affect K_o for chloride. Our results indicate that the true dissociation constants for Cl^- at the inside and outside are very similar but that the rate constant for inward translocation is much larger than that for outward translocation. For this reason, both unloaded and Cl-loaded transport sites are asymmetrically oriented toward the inside, and $K^o_{1/2}$ (in untreated cells) is much lower than K_o.

It is generally agreed that the band 3-mediated anion exchange process can be best described by a ping-pong model (Jennings, 1992; Knauf, 1989; Passow, 1992; Knauf & Brahm, 1989). In the ping-pong kinetic model (Figure 1), band 3 can exist in two distinct conformations. In the E_o form, the transport site faces the extracellular medium, while in the E_i form, it faces the cytoplasm. Substrates such as Cl^- and HCO_3^- can bind to either form of band 3, which results in four different states of the protein, e.g., E_o, E_i, ECl_o, and ECl_i (assuming that Cl^- is the substrate); ECl_o and ECl_i are the states of band 3 with Cl^- bound corresponding to E_o and E_i. The one-for-one tightly coupled exchange is explained by postulating that the conformational change that converts the transport site from facing one side of the membrane to facing the other can only take place in the substrate-bound forms of band 3, e.g., ECl_o and ECl_i.

Four intrinsic parameters are needed to fully describe the ping-pong model (Figure 1). K_o and K_i are dissociation constants for substrate (assumed to be Cl^- if not otherwise stated) binding to outward-facing or inward-facing sites, respectively, while k and k' are rate constants for the conformational change from ECl_i to ECl_o and for ECl_o to ECl_i, respectively. Previous flux studies provided some information about the asymmetry of the band 3-mediated anion exchange system. The asymmetry factor A is defined as $[E_o]/[E_i]$ with $[Cl_i] = [Cl_o]$. In terms of the intrinsic transport parameters, $A = LK_o/K_i$ where $L = k/k'$. The calculated values of A from older studies range from 0.06 to 0.2, with an average value of 0.15 ± 0.09 (*K*nauf & Brahm, 1989). A more recent extensive set of data gives a similar A value of 0.12 ± 0.02 (Gasbjerg & Brahm, 1991).

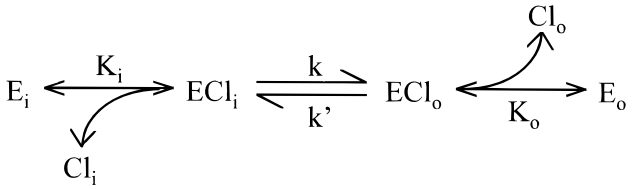

FIGURE 1: Kinetic description of the ping-pong model. E_o and E_i are the forms of band 3 with the transport site facing the extracellular or intracellular space, respectively. ECl_o and ECl_i are the corresponding forms with substrate (Cl^-) bound. K_i and K_o are the dissociation constants for Cl^- binding to E_i and E_o, respectively, and k and k' are the rate constants for the in to out and out to in transitions, respectively.

To determine the reason for the asymmetry, as well as the distribution of band 3 among the different states, it is necessary to know the value of the four intrinsic parameters as well as the inside and outside substrate concentrations. However, none of the four parameters for Cl^- transport has been measured. The difficulty lies in the fact that the measured dissociation constants are strongly influenced by the rate constants, k and k'. The dissociation constants for Cl^- measured by radioactive isotope flux experiments are actually apparent dissociation constants which are functions of both the true dissociation constants, K_o and K_i, and the rate constants.

There are different ways of measuring the apparent K_d, the concentration of Cl^- ([Cl]) required to give half-maximal

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Cl^- exchange. One way is to vary $[Cl^-]$ inside and outside of the cells simultaneously ($[Cl_i] = [Cl_o]$). The K_d measured in this way is called $K_{1/2}$ and is given by (Falke & Chan, 1985)

$$K_{1/2} = \frac{LK_o + K_i}{1 + L} \quad (1)$$

$K_{1/2}$ can be determined from flux measurements in intact cells that have been treated with nystatin to make $[Cl_i]$ equal to $[Cl_o]$ (Knauf & Brahm, 1989; Knauf et al., 1989). In addition, $K_{1/2}$ can also be measured in leaky ghosts by ^{35}Cl NMR (Falke et al., 1984a,b, 1985a). No nystatin treatment is necessary in this case since Cl^- equilibrates instantly across leaky ghost membranes. The values of $K_{1/2}$ measured by NMR are consistent with the values measured by radioisotope flux experiments on intact cells (Funder & Wieth, 1976; Knauf & Brahm, 1989; Fröhlich, 1982; Brazy & Gunn, 1976; Dalmark, 1976).

If $[Cl_i]$ is kept constant and only $[Cl_o]$ is varied, a totally different apparent K_d, $K^o_{1/2}$, is measured. $K^o_{1/2}$ is given by (Liu et al., 1996b)

$$K^o_{1/2} = \frac{LK_o}{1 + L + \frac{K_i}{[Cl_i]}} \quad (2)$$

$K^o_{1/2}$ includes a term in $[Cl_i]$ as well as L. This is due to the translocation of the Cl^--bound forms of band 3 which re-equilibrates the system between inward- and outward-facing conformations. Similarly, another apparent K_d, $K^i_{1/2}$, can be obtained by keeping $[Cl_o]$ constant and varying $[Cl_i]$. The measurements of $K^o_{1/2}$ and $K^i_{1/2}$ are possible because the net Cl^- flux in red cells is less than $^1/_{10^4}$ of the flux caused by anion exchange (Passow, 1992), which makes it possible to keep the Cl^- concentration constant at one side of the membrane for a reasonable period of time while varying the Cl^- concentration at the other side of the membrane.

In this study, we measured Cl^- dissociation constants under different conditions by ^{35}Cl NMR. The NMR binding assay is based on the studies of Falke et al. (Falke et al., 1984a,b, 1985a,b; Falke & Chan, 1985), except that, instead of using a line broadening (LB)[1] assay as in previous studies, we fitted the ^{35}Cl free induction decay (FID) signal to a biexponential function (Price et al., 1991; Liu et al., 1996). The slow and fast relaxation rates arise from the central and satellite transitions of the ^{35}Cl nuclear spin, respectively (Bull, 1972). We have shown previously that the different relaxation properties of central and satellite transitions result from Cl^- binding to the band 3 transport site (Liu et al., 1996). Since our measurements were done at a relatively high field strength where the biexponential character of the FID seems more prominent, we used biexponential fits to determine the Cl^- relaxation properties to avoid systematic errors which would be caused by assuming a Lorentzian line shape (corresponding to a single-exponential FID).

[1] Abbreviations: RBC, red blood cell; FID, free induction decay; LB, line broadening; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; EM, eosin-5-maleimide; 150 KH, 150 mM KCl, 24 mM sucrose, 20 mM HEPES [*N*-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid], and 5 mM glucose, pH 6.9 at room temperature with KOH; 5P8, 5 mM NaH_2PO_4, 130 μM dithiothreitol, and 10 μM phenylmethanesulfonyl fluoride at pH 8 with NaOH; S:N, signal-to-noise ratio.

Another difference in the present NMR study is the use of intact cells instead of leaky or resealed ghosts as in previous studies. As pointed out by Falke et al. (1984b), the high hemoglobin content of red cells makes the intracellular ^{35}Cl NMR signal decay much faster than the extracellular signal (Riddell & Zhou, 1995; Liu et al., 1996). Thus, only the very beginning part of the FID has a significant contribution from intracellular ^{35}Cl, and the intensity of the intracellular signal decays to a negligible level after the first 2−3 ms of acquisition. This simplifies the system because, with the proper signal acquisition parameters, only Cl^- binding to externally accessible sites is observed. Another advantage is that both $K_{1/2}$ and $K^o_{1/2}$ can be measured in intact cells, but it is impossible to determine $K^o_{1/2}$ in leaky ghosts.

We have previously shown that eosin-5-maleimide (EM) does not block the external anion access channel of band 3 (Liu et al., 1995), even though it nearly completely inhibits Cl^- exchange (Liu & Knauf, 1993). This means that EM may prevent the band 3 conformational change while leaving the transport site accessible for Cl^- binding. We exploited this property of EM and measured the apparent K_d values of EM-treated cells under symmetric ($[Cl_o] = [Cl_i]$) and asymmetric conditions ($[Cl_i]$ is constant). The theory predicts that, if the translocation is prevented, the difference between $K_{1/2}$ and $K^o_{1/2}$ will vanish. Furthermore, if EM does not significantly alter the Cl^- binding affinity of the external transport site, the K_d of EM-treated cells provides an accurate measurement of the true dissociation constant of the outward-facing transport site of band 3, K_o.

EXPERIMENTAL PROCEDURES

Cell Preparation. Red blood cells were obtained from freshly drawn blood, donated by apparently healthy volunteers. Heparin was added as an anticoagulant. The cells were washed at least three times with 150 KH. For each wash, the cells were centrifuged for 5 min at 2000 rpm using a Sorvall GLC-1 table-top centrifuge; 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.

Preparation of Leaky Ghost Membranes. Leaky ghosts were prepared essentially as described by Falke et al. (1984a). Red cells prepared as described above were brought to 50% hematocrit (v/v). The cells were lysed at 0−4 °C by mixing with 5P8 at a 1:30 cell to medium volume ratio. The suspension was centrifuged at 12 000 rpm in a Sorvall SS-34 rotor for 10 min. The supernatant was removed by aspiration, and the dense pellet underlying the membranes at the bottom of the tube was also removed. The leaky ghost membranes were washed at least three times in 150 KH before the membranes were pooled and weighed in one tube. The suspension was well mixed before being distributed equally by weight to multiple tubes. The ghost membranes in each tube were washed at least twice in medium with the desired Cl^- concentration. Finally, the total weight of the ghost suspension in each tube was adjusted to the same value by adding appropriate media (to make the concentration of ghost membranes in each sample the same) before the

samples were transferred into the NMR tubes for measurement. The membranes were stored on ice and were used within 6 days of preparation.

^{35}Cl NMR Spectroscopy. NMR measurements were carried out on a 9.4 T (400 MHz for protons) Bruker/GE (Fremont, CA) Omega NMR spectrometer as described previously (Liu et al., 1995), except that 256 or 512 points were collected for each scan. The number of scans averaged varied according to the concentration of Cl^- and the line width of the sample. The typical range was 1000−10000 scans.

Treatment with Inhibitors and Nystatin. Treatment with inhibitors and nystatin (Sigma, St. Louis, MO) were as previously described (Liu et al., 1995, 1996).

Changes in the Cellular and Medium Chloride Concentration in Low-Cl^- Media. The intracellular Cl^- concentration was determined as described previously (Knauf & Brahm, 1989; Knauf & Mann, 1986; Knauf et al., 1989). The cells were incubated with ^{36}Cl and [^{3}H]sucrose in 150 KH medium at 37 °C for 10 min to allow Cl^- equilibration. The hematocrit was made very high (75%) to mimic the conditions of the NMR protocol. The Cl^- ratio ($[Cl_i]$:$[Cl_o]$) in the 150 KH medium was taken as the control. After the incubation in 150 KH, an equal volume of 0 KH [0 mM KCl, with 20 mM HEPES and sufficient sucrose (0−140 mM) to keep the osmolality equal to that of 150 KH] was mixed with the concentrated cells. The Cl^- ratio was measured at different time points after mixing. Except for the control sample, the extracellular Cl^- concentration was also determined by radioactivity.

Data Analysis. The NMR data were processed with the spectrometer software (Bruker Omega). The magnitude of the FID signal was calculated and transferred to a fitting routine. The first two or three data points (corresponding to approximately the first 2 ms after the 90° pulse) were discarded to minimize the effect of receiver recovery as well as to avoid any residual contribution of intracellular Cl^-. The biexponential fitting was done by assuming that the ratio of the amplitudes of the rapidly decaying (fast) component and slowly decaying (slow) component of transverse magnetization is 3:2, as predicted by theory (Bull, 1972). From the T_2 of the fast (T_{2f}) and slow (T_{2s}) components, two corresponding line widths, LB_f $[1/(\pi T_{2f})]$ and LB_s $[1/(\pi T_{2s})]$, can be calculated.

In order to calculate Cl^- dissociation constants, a series of LB_f values at different Cl^- concentrations was measured and plotted against $[Cl^-]$. The data were fitted to the function $LB_f = C/([Cl^-] + K_d) + LB_{low}$. LB_{low} is the portion of line broadening caused by low-affinity sites and is the plateau of the curve at high $[Cl^-]$. K_d is the dissociation constant and can be $K_{1/2}$, $K^o_{1/2}$, or K_o, depending on the conditions of the experiment. C is a constant which is determined by α_o (an intrinsic constant related to the relaxation characteristics of Cl^- in the outward-facing binding site and the rate of exchange between bound Cl^- and free Cl^-), α_i (corresponding constant for Cl^- binding to inward-facing sites), E_T (total concentration of band 3), and L (k/k'). The exact form of C depends on which kind of experiment is being performed. In experiments measuring $K_{1/2}$ in intact cells, $C = \alpha_o E_T L/(1 + L)$. If the system is leaky ghosts, $C = (\alpha_o L + \alpha_i)E_T/(1 + L)$. When the $K^o_{1/2}$ is being measured in intact cells, $C = \alpha_o E_T L/(1 + L + K_i/[Cl_i])$. The data were subjected to a three-parameter unweighted nonlinear least-squares fit to determine K_d, C, and LB_{low} by the program Origin (Microcal).

RESULTS AND DISCUSSION

$K_{1/2}$ in Control Intact Cells and Leaky Ghosts. $K_{1/2}$ ($[Cl^-]$ half-saturation with $[Cl_i] = [Cl_o]$) has been extensively studied. Several NMR measurements on leaky ghost membranes by Falke et al. give an average $K_{1/2}$ value of 80 ± 30 mM (Falke et al., 1984a,b, 1985a,b). Falke and Chan (1985) also reported a $K_{1/2}$ value of 60 ± 10 mM measured with crushed ghosts. Glibowicka et al. reported a $K_{1/2}$ of 60 ± 9 mM, on the basis of NMR measurements on resealed ghosts (Glibowicka et al., 1988). Earlier data from flux experiments with intact red cells gave $K_{1/2}$ values ranging from 39 ± 4 mM (Fröhlich, 1982) to 65 ± 5 mM (Brazy & Gunn, 1976; Dalmark, 1976); the most recent value is 50 ± 10 mM (Gasbjerg & Brahm, 1991). Given the relatively large error in NMR experiments, it was generally considered that values of $K_{1/2}$ measured by these two different methods are the same, as predicted by the ping-pong model. We measured $K_{1/2}$ in leaky ghosts and intact cells by NMR. Unlike the LB assay in previous NMR studies, however, we used a two-component model to fit the FID signal so we get two, instead of one, calculated line broadenings, LB_s and LB_f (see Experimental Procedures). LB_f is the LB calculated from the fast component and is more sensitive to changes in $[Cl^-]$; therefore, the dependence of LB_f on $[Cl^-]$ was used to calculate $K_{1/2}$.

According to the ping-pong model, the $K_{1/2}$ measured by NMR in ghosts or intact cells should be the same regardless of whether only the outward-facing sites are visible (as in intact cells) (Falke et al., 1984b) or both outward- and inward-facing sites (as in leaky ghosts) are visible (Falke & Chan, 1985). This prediction has been verified, at least approximately, by comparison of previous NMR measurements on leaky ghosts with flux measurements on intact cells under similar conditions (Falke et al., 1984a,b; Falke & Chan, 1985). Our NMR $K_{1/2}$ values for ghosts and intact cells are also consistent with this prediction. Figure 2a shows the result of such a measurement in leaky ghosts. $K_{1/2}$ was calculated to be 46 ± 5 mM at 0 °C with glutamate replacing Cl^- to maintain the ionic strength. $K_{1/2}$ was also measured with intact red cells (Figure 2b). In this case, nystatin was used to make $[Cl_i]$ and $[Cl_o]$ equal and no replacement anion was used, so the ionic strength varied with $[Cl^-]$. The $K_{1/2}$ value based on data from four different experiments was 44 ± 14 mM. This represents the first measurement of $K_{1/2}$ by NMR in intact red cells. The value is very close to that obtained with leaky ghosts (Figure 2a), despite the fact that for the intact cells the ionic strength was not kept constant.

The larger standard deviation in intact cell experiments was due to reduced signal intensity caused by the decrease in the observable (extracellular) Cl^- signal at the high hematocrit used. The problem becomes worse at lower Cl^- concentrations, and more scans are required to get a reasonable S:N. Cell lysis caused by mechanical stress during sample preparation may also contribute to the larger standard deviation. The samples with intact cells were handled carefully, and the period between completion of sample preparation and data acquisition was kept at a minimum to keep the level of cell lysis low enough (less than 1% of total cells) so that the extra LB introduced by

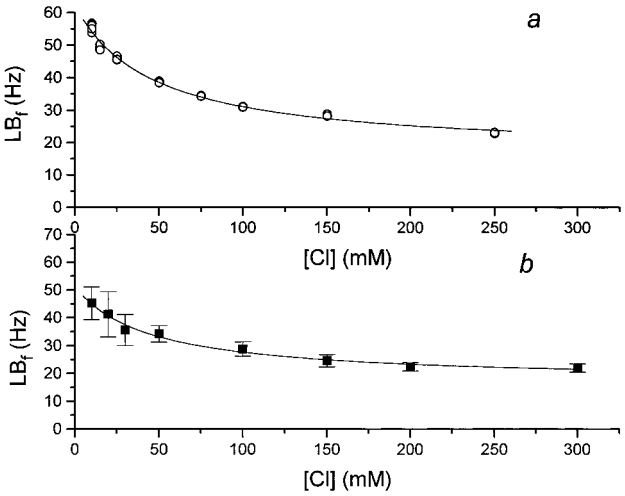

FIGURE 2: LB_f values of untreated control leaky ghosts (a) and intact cells (b) are plotted against Cl^- concentration. LB_f was calculated from the fast component of the biexponentially decaying FID signal, as described in Experimental Procedures. The data were fitted to the function $LB_f = C/([Cl^-] + K_d) + LB_{low}$. In this case, K_d is the apparent dissociation constant, $K_{1/2}$. For leaky ghosts (a), $K_{1/2} = 46 \pm 5$ mM, $C = 2100 \pm 200$ Hz·mM, and $LB_{low} = 17 \pm 1$ Hz. For intact cells (b), $K_{1/2} = 44 \pm 14$ mM, $C = 1500 \pm 400$ Hz·mM, and $LB_{low} = 17 \pm 2$ Hz. Error bars represent the SD from four or five experiments.

cell lysis, determined by examining the LB of the supernatant of samples of intact cells, was smaller than 10% of the total LB. We also tested the [Cl] dependence of supernatant LB and found no observable correlation between the LB caused by hemoglobin in the supernatant and [Cl] (data not shown). Because the extra LB caused by cell lysis in different samples was very similar, the major effect of cell lysis is the overestimation of the low-affinity LB (LB_{low}), while the effect on the high-affinity LB is minimal (Liu et al., 1995). Thus, the influence of cell lysis on the K_d measurement is small. Actually, the principal impact of cell lysis on the quality of our data is not mainly due to the extra LB caused by hemoglobin released from lysed cells but rather due to the restraint on the number of scans for each sample. Since the level of cell lysis is time-dependent, a lower S:N ratio has to be accepted in order to keep the acquisition time short, and this problem is more pronounced for samples with a low $[Cl^-]$ and hence a low S:N.

Although our results agree approximately with previous NMR and radioactive flux measurements, our $K_{1/2}$ is somewhat smaller than the values of Falke et al. (Falke & Chan, 1985; Falke et al., 1984a). The major cause of the small discrepancy is probably the use of a different spectator anion,[2] glutamate instead of citrate. In fact, when we used the same replacement anion as Falke et al. (citrate), the measured $K_{1/2}$ in ghosts was 70 ± 11 mM, significantly larger

[2] Falke et al. (1984a) showed that at low field strength (8.8 MHz ^{35}Cl NMR frequency) the ^{35}Cl NMR resonance fits very well to a Lorentzian down to 20% of peak height, thereby demonstrating that their technique of measuring line width at half-maximal peak height was appropriate. Even at low field strength (8.384 MHz), we observed two relaxation rates (non-Lorentzian behavior), consistent with observations by Price et al. (1991), but this should have little effect on the line widths as measured with the technique of Falke et al. It is critical, however, that the two-component assay for the data from the high-field spectrometer be used. When we applied the conventional LB method to analyze our NMR data, the LB appeared to be very insensitive to the changes of $[Cl^-]$ and the calculated $K_{1/2}$ was in the range of hundreds of millimolar.

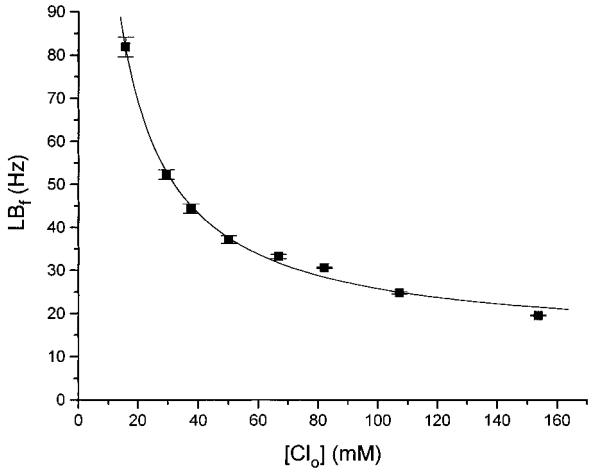

FIGURE 3: NMR measurement of $K^o_{1/2}$ in intact cells. The *x*-axis is the Cl^- concentration of the extracellular medium. The Cl^- concentration inside of the cells was kept constant at 150 mM. Sucrose was used to balance the osmotic pressure when $[Cl_o]$ was reduced. Error bars represent the SD. The equation used is the same as that described in Figure 2, except that $K_{1/2}$ is replaced with $K^o_{1/2}$. The value of $K^o_{1/2}$ measured from these data is 3.2 ± 2.1 mM. *C* and LB_{low} are 1300 ± 200 Hz·mM and 13 ± 2 Hz, respectively.

than the value with glutamate (46 ± 5 mM) and closer to the values of Falke et al. (80 ± 30 mM). The fact that K_d is lower with glutamate replacement than with citrate suggests that, at least at 0 °C (pH 7.2), glutamate competes less effectively with Cl^- and is therefore a better replacement anion to maintain constant ionic strength, as previously reported by Jennings (1989). Because glutamate appears to be a better "spectator" anion than citrate, our lower value of $K_{1/2}$ (46 ± 5 mM) is probably more reliable than those obtained in the presence of citrate (Falke & Chan, 1985; Falke et al., 1984a). Our value is also close to 48 ± 19 mM, the average of $K_{1/2}$ values measured by flux experiments [reviewed by *K*nauf and Brahm (1989)]. The most recent flux measurements by Gasbjerg and Brahm (1991) gave a $K_{1/2}$ value of 50 ± 10 mM, almost identical to our NMR value. The agreement of our NMR measurements of $K_{1/2}$ with these values from flux experiments provides evidence that our modified NMR method is valid for measuring dissociation constants for Cl^- binding to band 3 in leaky ghosts and intact cells.

Measurement of $K^o_{1/2}$ by NMR. A variation of the protocol that we used to measure $K_{1/2}$ with intact red blood cells can be used to measure $K^o_{1/2}$ by NMR. We kept $[Cl_i]$ constant at 150 mM in intact red cells and used sucrose to balance the osmolality decrease caused by lowering $[Cl_o]$. Measurements of the time dependence of the extracellular Cl^- signal intensity show no significant change in $[Cl_o]$ (data not shown), so the $[Cl^-]$ gradient across the membrane is maintained long enough for the NMR measurements. Figure 3 shows the plot of LB_f of 50% cells as a function of $[Cl_o]$. The value of $K^o_{1/2}$ is 3.2 ± 2.1 mM. Typical values of $K^o_{1/2}$ measured under similar conditions in previous flux experiments are 3.0 ± 0.4 mM (Gunn & Fröhlich, 1979) and 2.2 ± 0.3 mM (Gasbjerg & Brahm, 1991) in intact red cells and 2.6 ± 0.4 mM in resealed ghosts (Gasbjerg & Brahm, 1991). The similarity of our $K^o_{1/2}$ value to those measured by flux experiments further demonstrates the validity of the NMR method.

The asymmetry factor A can be calculated from measurements of $K_{1/2}$ and $K^o_{1/2}$ (Knauf & Brahm, 1989). The NMR

Table 1: Calculated Values of L, A, and K_i Using Measured $K_{1/2}$, $K^o_{1/2}$ and K_o Values[a]

			K_o					
			30	35	40	45	50	55
		L	0.152	0.128	0.110	0.097	0.086	0.078
	40	K_i	41.5	40.6	40.0	39.5	39.1	38.8
		A	0.110	0.110	0.110	0.110	0.110	0.110
		L	0.157	0.132	0.113	0.100	0.089	0.080
$K_{1/2}$	45	K_i	47.4	46.3	45.6	45.0	44.6	44.2
		A	0.100	0.100	0.100	0.100	0.100	0.100
		L	0.162	0.136	0.117	0.102	0.091	0.082
	50	K_i	53.2	52.0	51.2	50.5	50.0	49.6
		A	0.091	0.091	0.091	0.091	0.091	0.091

[a] The value of $K^o_{1/2}$ was set at 3.2 mM in the calculation. K_o was chosen from 30 to 55 mM at 5 mM intervals which covers the range of K_o according to our data (46 ± 12 mM). The range of $K_{1/2}$ is chosen from 40 to 50 mM which approximates the range of $K_{1/2}$ values obtained in leaky ghosts (46 ± 5 mM). The units for K_i, K_o, and $K_{1/2}$ in the table are millimolar.

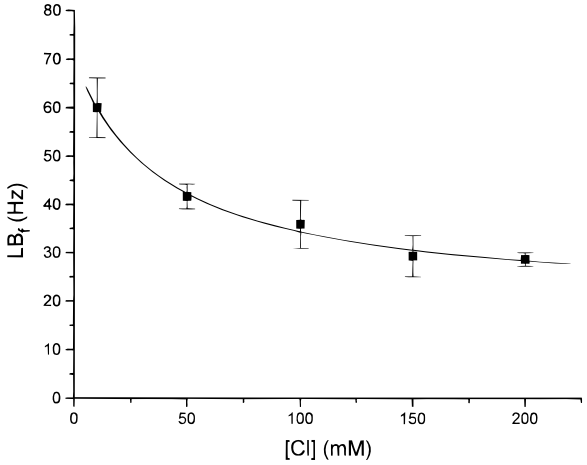

FIGURE 4: Measurement of $K_{1/2}$ in EM-treated intact cells. Data points represent averages from multiple experiments involving two or three different blood donors. Error bars are standard deviations. Cells were pretreated with nystatin to make $[Cl_i]$ and $[Cl_o]$ equal. No replacement anion was used in this case. The equation used is the same as that described in Figure 2. Error bars represent the SD. The calculated $K_{1/2}$, C, and LB_{low} are 41 ± 14 mM, 2000 ± 700 Hz·mM, and 20 ± 2 Hz, respectively. In this case, $C = \alpha_o E_o$, where E_o is the total concentration of band 3 with the transport site facing outward.

measurements give an A value of about 0.1 (Table 1), indicating about 10 times as many inward-facing as outward-facing unloaded transport sites, in good agreement with isotope flux measurements of this parameter (Knauf & Brahm, 1989; Gasbjerg & Brahm, 1991). Thus, the NMR data, like earlier flux measurements, indicate asymmetry in the intrinsic parameters, K_i, K_o, k, and k', but they do not tell us whether the asymmetry is primarily due to a difference between K_i and K_o or between k and k'.

$K_{1/2}$ and $K^o_{1/2}$ of EM-Treated Intact Cells. To determine the intrinsic constants, we need to measure the Cl^- affinity under conditions where the exchange flux is stopped so that the transport sites cannot reorient but Cl^- can still bind to the transport site. In EM-treated cells, Cl^- exchange is nearly completely stopped (Liu & Knauf, 1993). Nevertheless, EM-treated cells still exhibit LB from a high-affinity band 3 Cl^--binding site (Liu et al., 1995), and EM does not abolish a double-quantum-filtered NMR signal arising from Cl^- binding to the transport site (Liu et al., 1996). The $K_{1/2}$ of EM-treated intact cells, measured by the biexponential method, was 41 ± 14 mM (Figure 4).

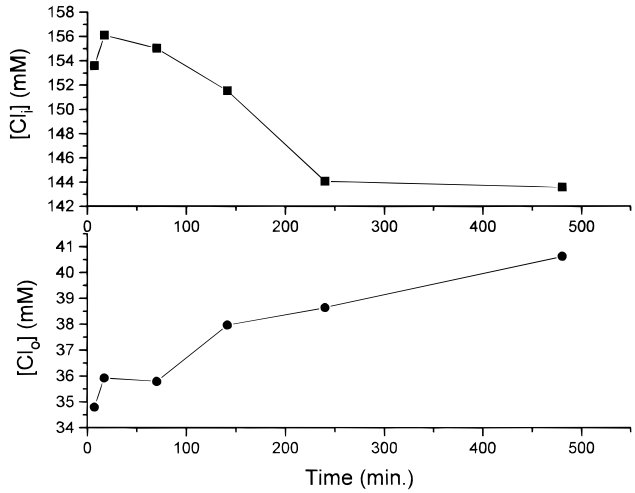

FIGURE 5: Time-dependent changes in $[Cl_i]$ and $[Cl_o]$ for EM-treated cells in low-$[Cl_o]$ medium. The starting condition is 150 mM Cl_i and 35 mM Cl_o. During the 8 h period, the change of the Cl^- concentration both inside and outside is gradual and slow.

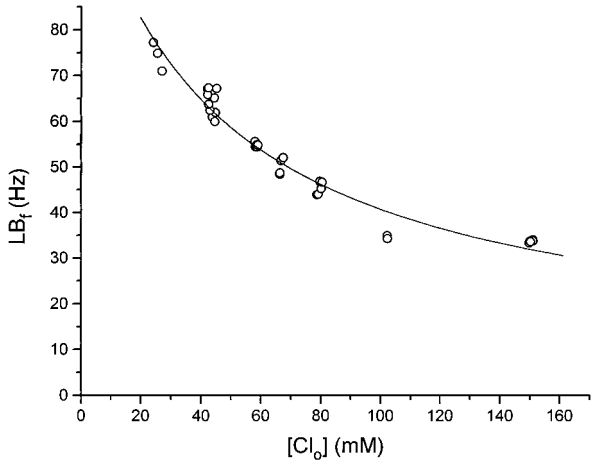

FIGURE 6: Measurement of $K^o_{1/2}$ of in EM-treated intact cells. Potassium glutamate was used to replace external KCl when $[Cl_o]$ was reduced. $[Cl_i]$ was kept constant at 150 mM. The data were analyzed as described in Experimental Procedures. The equation used is the same as that described in Figure 2. The calculated $K^o_{1/2}$, C, and LB_{low} are 46 ± 12 mM, 5000 ± 1000 Hz·mM, and 6.4 ± 5.0 Hz, respectively.

To measure $K^o_{1/2}$ in EM-treated cells, we first had to ensure that, when $[Cl_i] > [Cl_o]$, the net Cl^- efflux from EM-treated cells is very slow, as in control cells (Jennings, 1992; Passow, 1992). To test this, the intracellular and extracellular Cl^- concentrations of EM-treated cells were measured under the same conditions that were used for the NMR experiments (Figure 5). During an 8 h period, $[Cl_o]$ increased less than 20% while $[Cl_i]$ remained at around 150 mM, with less than an 8% decrease. Given the fact that NMR measurement of each sample was usually completed within 1 h, the Cl^- concentration gradient is maintained long enough for the NMR measurements. This verifies that the change in the Cl^- concentration gradient across the membrane due to Cl^- leakage is negligible under the conditions of our $K^o_{1/2}$ measurement.

As can be seen in Figure 6, $K^o_{1/2}$ of EM-treated cells with sucrose to balance the osmolality was 46 ± 12 mM. Another experiment in EM-treated cells with glutamate to balance

the osmolality gave a $K^o_{1/2}$ value of 40 ± 17 mM. In contrast to the value of the untreated control cells, the $K^o_{1/2}$ values in EM-treated cells were extremely close to $K_{1/2}$ (41 mM).

The similar $K_{1/2}$ and $K^o_{1/2}$ values are exactly what the ping-pong model predicts if EM stops anion translocation and fixes the number of band 3 molecules in each conformation while allowing external Cl^- binding to the outward-facing transport site of band 3. Regardless of whether only $[Cl_o]$ or both $[Cl_i]$ and $[Cl_o]$ are varied, the $[Cl_o]$ dependence of the NMR LB with intact red cells reflects the binding of external Cl^- to outward-facing transport sites, whose distribution is not affected by the change of the transmembrane Cl^- gradient (in contrast to untreated cells, where there is interconversion between the ECl_o and ECl_i forms). This makes the two conditions used for measuring $K_{1/2}$ and $K^o_{1/2}$ essentially identical for EM-treated cells; in both cases, one is measuring the binding of external Cl^- to a fixed number of outward-facing transport sites.

In other words, in EM-treated cells, the observed values of $K_{1/2}$ and $K^o_{1/2}$ are nearly identical because they are both measurements of K_o, the true Cl^- dissociation constant for outward-facing band 3 transport sites, which has never been measured before due to the rapid transport of Cl^-. A remaining problem, however, is the fact that the K_o of EM-labeled band 3 may not necessarily be the true K_o of untreated band 3, because the EM treatment might have altered the affinity for Cl^-.

Measurement of the I^- Affinity of the Outward-Facing Transport Site in EM-Treated Cells. Like Cl^-, I^- can be transported by band 3 in red blood cells, but the transport rate for I^- is about 260 times slower than that of Cl^- at 0 °C and pH 7.4 (Dalmark & Wieth, 1972). This provides a way to determine the dissociation constant for I^- binding to the outward-facing transport site, K_o^I, by measuring the competitive effect of different external I^- concentrations on the Cl^- flux (Milanick & Gunn, 1986; Knauf et al., 1986; Liu et al., 1996a). Practically, $1/J$ (where J is the flux of Cl^-) vs $[I_o]$ is plotted at two different Cl_o concentrations with a constant $[Cl_i]$ (Knauf & Brahm, 1989). The x-value of the intersection point of the two lines with different Cl_o concentrations gives $-K_o^I$. Flux experiments gave values for K_o^I of 0.83 ± 0.11 mM (sucrose substitution) (Liu et al., 1996a) and 2.0 ± 0.3 mM (citrate−sucrose substitution) (Milanick & Gunn, 1986) at 0 °C.

Figure 7 shows the result of a NMR experiment on EM-treated cells with external I^- as a competitive anion. The external I^- and Cl^- concentrations were changed simultaneously so that the sum of the two was kept constant to maintain a constant osmolality and ionic strength. The relation between $[I^-]$ and LB was fitted to a function predicted by the ping-pong model (see the Figure 7 legend). The K_d for I^- of EM-treated cells measured by NMR is 1.1 ± 0.2 mM, which is very close to the K_o^I of untreated control cells measured by fluxes. This result shows that the external I^- affinity is not substantially altered in EM-treated cells. This makes it unlikely that EM treatment causes any pronounced changes in the Cl^- affinity, which in turn suggests that the K_d values for Cl^- that we measured in EM-treated cells are probably very close to the true Cl^- dissociation constant for the outward-facing transport site of band 3, K_o. Although we cannot strictly exclude the possibility that EM treatment only alters the Cl^- affinity while having no effect on the I^- affinity, this alternative is very improbable given the fact that I^- and Cl^- compete for the external Cl^- transport site. Furthermore, even if the structural perturbation caused by EM were sufficiently unusual to cause different effects on two very similar anions, the chance that the altered K_d would be equal to the $K_{1/2}$ of control cells is exceedingly small.

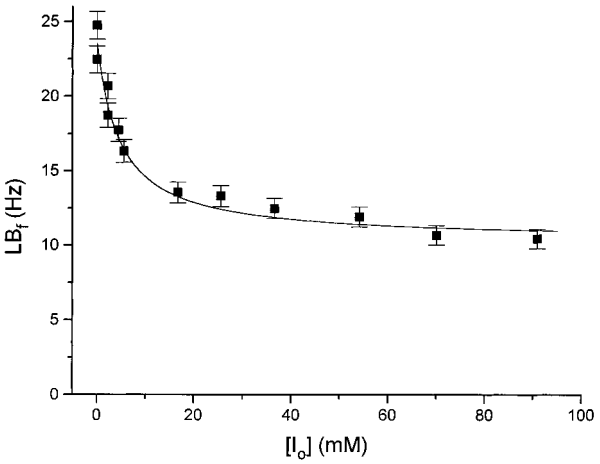

FIGURE 7: Dissociation constant for external I^- of EM-treated cells. In this experiment, $[I^-]$ and $[Cl^-]$ in the extracellular medium were changed simultaneously, keeping their sum constant at 150 mM to maintain equal osmolality. LB_f measured as described in Experimental Procedures was plotted against $[I_o]$. Error bars represent the SD. The function used to fit the data is $LB_f = C/[150 - [I_o] + K_o(1 + [I_o]/K_o^I)] + LB_{low}$, which is a modified version of the function described in Experimental Procedures. LB_f is in hertz, and $[I_o]$ is in millimolar. K_o is the dissociation constant for Cl^- binding to the outward-facing transport site, and K_o^I is the corresponding dissociation constant for I^-. A four-parameter nonlinear fit was performed first to determine the value of K_o. The value of K_o is 46.4 mM, which is virtually the same as the dissociation constants of EM-treated cells ($K_{1/2}$ and $K^o_{1/2}$ in Figures 4 and 6) within the experimental error. Because of this, we fixed the value of K_o at 46.4 mM and used a three-parameter fit to determine the rest of the parameters in the equation. The dissociation constant for I^-, K_o^I, is calculated to be 1.1 ± 0.2 mM. The calculated values of C and LB_{low} are 2600 ± 100 Hz·mM and 10.4 ± 0.4 Hz, respectively.

Recently, Pan and Cherry (1995) have measured the ability of iodide to quench the fluorescence of EM in ghosts from EM-labeled red cells. On the basis that the quenching saturates with increasing $[I^-]$, they calculate an iodide dissociation constant for the site that is involved in quenching of 23 mM at 3 °C. Because DIDS at high concentrations ($K_d = 36\ \mu$M at 14 °C) seems to compete with I^-, they argue that the quenching is due to I^- binding to the external transport site. However, disulfonic stilbenes such as DIDS probably bind, at least with low affinity, to sites other than the external transport site (Knauf et al., 1993; Aranibar et al., 1994; Liu et al., 1996b). This, together with the fact that the I^- dissociation constant is more than 20 times higher than the K_o^I value from flux measurements (Liu et al., 1996a; Pan & Cherry, 1995), suggests that the binding site involved in quenching is different from the external transport site. The data in Figure 7, which indicate that, even in EM-treated cells, the NMR-observable high-affinity Cl^- binding site has a much higher I^- affinity ($K_d \sim 1$ mM) than the fluorescence quenching site, argue strongly against the possibility that the binding site described by Pan and Cherry is actually the external transport site. A more likely possibility is that it might be an external inhibitory (modifier) site, which binds I^- with a lower affinity [$K_d \sim 60$ mM (Dalmark, 1976), with recent measurements (Knauf & Spinelli, 1995) suggesting a

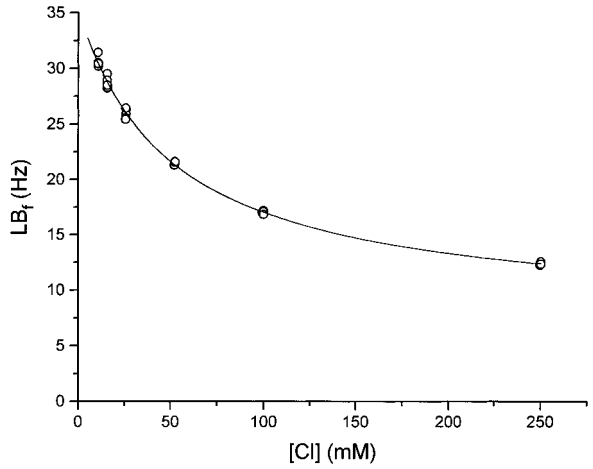

FIGURE 8: $K_{1/2}$ of leaky ghosts from EM-treated cells. Cells were first treated with EM and then lysed and made into leaky ghosts as described in Experimental Procedures. Potassium glutamate was used to maintain the ionic strength constant at 250 mM. A three-parameter fit as described in Experimental Procedures was used to calculate the $K_{1/2}$. The equation used is the same as that described in Figure 2. The calculated $K_{1/2}$, C, and LB_{low} are 51 ± 3 mM, 1400 ± 80 Hz·mM, and 7.7 ± 0.4 Hz, respectively.

lower K_d value, closer to that for the fluorescence quenching site].

Calculation of L, K_i, and A from the Measured Values of $K_{1/2}$, K_o, and $K^o_{1/2}$. Given the measured K_o, $K^o_{1/2}$, and $K_{1/2}$, eqs 1 and 2 can be solved because only two unknowns remain, L and K_i. L and K_i were calculated using combinations of the largest and smallest possible values of $K^o_{1/2}$ and $K_{1/2}$ given by the experimental data (Table 1). The average value of L is 0.11 ± 0.03, and the average value of K_i is 46 ± 5 mM. From the maximum flux (Gasbjerg & Brahm, 1991), the calculated values of k and k' are 490 and 4500 s^{-1}, respectively.[3] This enables us to answer the following old question about the ping-pong model. What causes the asymmetry of the system? Since it is clear from our results that $K_o \approx K_i \approx K_{1/2}$ and L is much less than 1, the asymmetry is caused by the difference in the translocation rates, k and k', while the dissociation constants, K_o and K_i, are very similar.

To test this conclusion, we have measured the $K_{1/2}$ of EM-treated ghost membranes (Figure 8). This measurement alone will not help in determining the intrinsic transport parameters because the LB of leaky ghosts comes from binding to both the inner and outer surface, so the relation of [Cl^-] and LB of EM-treated leaky ghosts in general is too complicated to predict without further information. However, since we know from our measurements that K_o and K_i are very similar, we can predict for this special case that the value of $K_{1/2}$ from EM-treated ghosts should also be close to K_o and K_i. Indeed, the value of $K_{1/2}$ is 51 ± 3 mM, which provides another piece of evidence to support our conclusions.

Our data, however, do not agree with Bjerrum's work (1992), which is based on a ping-pong model involving an exofacially deprotonatable reciprocating anion binding site. From his pK values and apparent dissociation constants measured under neutral or alkaline conditions, Bjerrum calculated that for the neutral state 2.5 mM < K_o < 9 mM and K_i > 38 mM. Obviously, our measured K_o does not fit this indirectly estimated value. Bjerrum's calculations assume that (1) the system is unlikely to function in a metastable situation and (2) the change in pK, when binding a Cl^-, is comparable to that observed from I^- binding. At this point, the inconsistency of our results and Bjerrum's calculations suggests that some modifications are needed in the simple ping-pong, single-titratable carrier model or that the assumptions made in Bjerrum's calculations are not entirely correct.

[3] Under the condition of $[Cl_i] = [Cl_o]$, the maximum flux (J_{max}) is $kE_T/(1 + L)$, where E_T is the total number of band 3 per cell.

CONCLUSION

Biexponential analysis of the high-field ^{35}Cl NMR signal was used to study Cl^- binding to band 3 transport sites. For most experiments, intact cells were used to allow selective observation of the effect of Cl^- binding to the outward-facing transport sites. $K_{1/2}$ was measured in both intact cells and ghost membranes. Using intact cells, we also measured $K^o_{1/2}$ by NMR for the first time. Both $K_{1/2}$ and $K^o_{1/2}$ are consistent with the previously reported values measured by either flux or NMR.

Approximately equal values of $K_{1/2}$ and $K^o_{1/2}$ were obtained when the NMR protocols were applied to EM-treated cells, which indicates that EM prevents Cl^- translocation and allows measurement of the true outward-facing transport site dissociation constant, K_o. The K_o^I value for EM-treated cells is the same as that measured from flux experiments on untreated cells, which provides strong evidence that EM does not alter K_o. From these data, we conclude that the asymmetric distribution of inward- and outward-facing unloaded band 3 sites is caused by the difference of translocation rates k and k', while the dissociation constants, K_o and K_i, are very similar. This implies that both the unloaded and Cl-loaded transport sites are asymmetrically distributed, in favor of inward-facing sites. These results also permit calculation of the distribution of band 3 among its various conformations under any given conditions in Cl^- medium at 0 °C (Knauf & Brahm, 1989).

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