J. Membrane Biol. 103, 181–189 (1988)

The Journal of
Membrane Biology

Relation Between the Anion Exchange Protein in Kidney Medullary Collecting Duct Cells and Red Cell Band 3

Agnes Janoshazi, David M. Ojcius*, Bruce Kone, Julian L. Seifter, and A.K. Solomon

Biophysical Laboratory, Department of Physiology and Biophysics, Harvard Medical School, and Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115

Summary. A membrane protein that is immunochemically similar to the red cell anion exchange protein, band 3, has been identified on the basolateral face of the outer medullary collecting duct (MCD) cells in rabbit kidney. In freshly prepared separated rabbit MCD cells, M.L. Zeidel, P. Silva and J.L. Seifter (*J. Clin. Invest.* **77**:1682–1688, 1986) found that Cl^-/HCO_3^- exchange was inhibited by the stilbene anion exchange inhibitor, DIDS (4,4'-diisothiocyano-2,2'-disulfonic stilbene), with a K_I similar to that for the red cell. We have measured the binding affinities of a fluorescent stilbene inhibitor, DBDS (4,4'-dibenzamido-2,2'-disulfonic stilbene), to MCD cells in 28.5 mM citrate and have characterized both a high-affinity site (K_1^s = 93 ± 24 nM) and a lower affinity site (K_2^s = 430 ± 260 nM), which are closely similar to values for the red cell of 110 ± 51 nM for the high-affinity site and 980 ± 200 nM for the lower affinity site (A.S. Verkman, J.A. Dix & A.K. Solomon, *J. Gen. Physiol.* **81**:421–449, 1983). When Cl^- replaces citrate in the buffer, the two sites collapse into a single one with K_1^s = 1500 ± 400 nM, similar to the single K_1^s = 1200 ± 200 nM in the red cell (J.A. Dix, A.S. Verkman & A.K. Solomon, *J. Membrane Biol.* **89**:211–223, 1986). The kinetics of DBDS binding to MCD cells at 0.25 μM^{-1} are characterized by a fast process, τ = 0.14 ± 0.03 sec, similar to τ = 0.12 ± 0.03 sec in the red cell. These similarities show that the physical chemical characteristics of stilbene inhibitor binding to MCD cell 'band 3' closely resemble those for red cell band 3, which suggests that the molecular structure is highly conserved.

Key Words kidney · medullary collecting duct · red cell · band 3 · anion exchange protein · stilbene anion exchange inhibitors · DBDS

Introduction

As a final step of urinary acidification, H^+ is secreted in the mammalian medullary collecting duct (MCD) by a H^+-ATPase on the luminal membrane of the MCD cell. For each H^+ secreted, there is an equivalent synthesis, within the MCD cell, of HCO_3^-, which is then returned to the circulation via a Cl^-/HCO_3^- exchange at the basolateral face of this tubular segment (Stone et al., 1983; Koeppen, 1985). Stone et al. (1983) showed that bicarbonate reabsorption in this segment is inhibited by the same stilbene anion exchange inhibitor, SITS (4-acetamido-4'-isothiocyano-2,2'-disulfonic stilbene), that specifically inhibits Cl^-/HCO_3^- exchange in the red cell. Schuster, Bonsib and Jennings (1986) raised monoclonal antibodies in the mouse against the membrane domain of human red cell band 3 and found that it was bound preferentially to the basolateral face of rabbit MCD. Drenckhahn et al. (1985) reported that antibodies, to either the cytoplasmic or the membrane-spanning domain of human (or rat) red cell band 3, bound to the basolateral face of a distinct population of intercalated cells in the MCD. The molecular weight of the immunostained protein was about 120 kD, slightly higher than the 103 kD of human red cell band 3. Subsequently, Zeidel et al. (1986*b*) studied Cl^-/HCO_3^- exchange in separated cells, freshly prepared from the rabbit outer MCD, and found that the exchange was inhibited by DIDS (4,4'-diisothiocyano-2,2'-disulfonic stilbene) with a K_I of 0.5 μM, similar to the K_I of 0.2 μM DIDS for reversible inhibition of human red cell Cl^-/HCO_3^- exchange (Knauf, 1979).

These experiments show that the anion exchange protein on the basolateral face of the MCD cells, which we call 'band 3,' is functionally and immunochemically similar to red cell band 3. However, the lipid and protein microenvironment of the two proteins probably differs substantially and we wanted to know whether these differences, as well as differences in the peptide sequence that had not been probed immunochemically, had a significant effect on the transport and other physical chemical properties of the protein.

* *Present address:* Institut National de la Santé et de la Recherche Médicale, U-91, C.H.U. Henri Mondor, 94010 Creteil, France.

In order to answer this question, we studied the binding affinities and kinetics of a fluorescent analog of DIDS, the stilbene inhibitor DBDS (4,4'-dibenzamido-2,2'-disulfonic stilbene). DBDS increases its fluorescence by more than a factor of two when bound to band 3 so that we could use the fluorimetric stopped-flow method to determine the DBDS binding affinities and kinetics of MCD cell 'band 3' and compare these results with those previously obtained for the DBDS/band 3 reaction in red cell ghosts (Verkman et al., 1983; Dix et al., 1986). The present experiments on MCD cell 'band 3' show that the DBDS binding affinities and kinetics are similar, and possibly identical, in the two cells.

Materials and Methods

Materials

DBDS, synthesized by the method of Kotaki, Naoi and Yagi (1971), was kindly provided through the courtesy of Dr. James A. Dix. H_2-DIDS (4,4'-diisothiocyano-2,2'-dihydrostilbene disulfonate) was obtained from Molecular Probes (Eugene, OR). All other chemicals, obtained from Fisher (Fairlawn, NJ) were reagent grade. Outdated bank blood was kindly supplied by the Children's Hospital (Boston, MA).

Preparation of MCD Cells and Red Cells

Highly purified suspensions of outer medullary collecting duct cells were prepared from New Zealand white rabbits as described by Zeidel et al. (1986*a*,*b*). The inner stripe of the outer medulla was excised, digested with protease, and centrifuged on continuous (5-46%) Ficoll® gradients at 2300 × *g* for 45 min. A homogeneous population of cells with morphologic and functional characteristics of outer MCD cells was located in the upper two fractions. These cells have a high glycolytic capacity, are rich in carbonic anhydrase, and use H^+-ATPase and Cl^-/HCO_3^- exchange to maintain intracellular pH (Zeidel et al., 1986*a*,*b*; Silva, Klausner, Segall, Rosen, Zeidel & Seifter, *personal communication*). Suspensions were either freshly prepared or had been stored at −20°C until use. The suspension was either washed four times with 10 volumes of 28.5 mM Na citrate (titrated to pH 7.4 at 20°C, with citric acid) or with PBS buffer of the following composition, in mM: 150 NaCl, 5 Na_2HPO_4, pH 7.4, 300 ± 5 mOsm. To reverse any aggregation of the MCD cells, the first wash contained 10 mM EDTA which was omitted in the subsequent washes. The cells were vortexed for 5 min to homogenize the suspension.

For the protein determinations, the MCD cell suspension was sonicated for 5 min in a Branson bath sonicator (Model 12, Shelton, CN) and the protein content measured by the Bradford (1976) method (Pierce Chemical Co, Rockford, IL). Bovine serum albumin (BSA) was used as a standard and the appropriate corrections were made to compensate for the difference between the absorbance of a BSA standard solution and that of an average protein. In a sevenfold replication of one determination at 380 (μg protein) ml^{-1}, the standard deviation was 2.3 (μg protein) ml^{-1}. When all the sources of error are taken into account, particularly the sonication process, there is a much larger standard deviation at the same protein concentration, 50 (μg protein) ml^{-1}. To maximize the sensitivity of this method, the protein concentration was determined by the difference between the absorbance at 595 nm and that at 465 nm. By this means we were able to obtain a sensitivity of 0.5 μg protein (ml supernatant)$^{-1}$ which decreased the error in our measurements for the Scatchard plot by a factor of three.

For red cell experiments, bank blood, outdated by no more than 2 weeks, was used. After aspiration of plasma and buffy coat, the blood was washed three times with PBS buffer. Osmolality was determined with a Fiske Model OS osmometer (Uxbridge, MA).

Equilibrium Binding of DBDS to MCD Cells and Red Cells

For the Scatchard plots, known concentrations of DBDS were added to MCD cells (0.36–0.38 (mg protein) ml^{-1}) suspended in 28.5 mM Na citrate, unless otherwise specified. DBDS binding was measured by fluorescence enhancement, excited at 350 nm (resolution 4 nm) and monitored between 400–600 nm (resolution 4 nm) in a single beam spectrofluorimeter (Photon Technology International, Princeton, NJ). The spectrofluorimeter output was interfaced on-line to our computer (p-System, Infinity Computer Corp, Worcester, MA). In order to maximize our accuracy, it was necessary to correct for the small fluctuations in lamp intensity and photomultiplier amplification, etc., which varied from day to day, so each set of measurements was normalized to the fluorescence intensity of 1 μM DBDS at 420 nm, measured at the same time.

Centrifugation Method

After 25-min incubation with known DBDS concentrations at 22–25°C, the MCD cell suspension was centrifuged at 5000 × *g* for 20 min. $[DBDS]_{free}$ in the supernatant was measured by fluorescence enhancement, based on the assumption that no bound DBDS (either to protein or lipid) is in the supernatant; $[DBDS]_{bound}$ was taken as the difference between $[DBDS]_{total}$ and $[DBDS]_{free}$. A total of 71 data points were determined in two series of measurements and a standard curve of fluorescence enhancement against $[DBDS]_{free}$ was made for each series. K_1^s, K_2^s, n, the number of binding sites, and c, the bound/free ratio for the nonspecific binding sites were determined as described in the Appendix.

Two different methods were used to show that the supernatants were free of protein or bound DBDS. The supernatant protein concentration was found (by the Bradford method) to be <0.5 μg ml^{-1} in MCD cell preparations in which DBDS was omitted. On the assumption that any supernatant binding sites, such as lipids, are not saturated, we added known concentrations of DBDS to the supernatant, but could detect no additional fluorescence enhancement. Our detection limit of 5 nM $[DBDS]_{free}$ is set by the noise level of the spectrophotometer, which means that the errors in the Scatchard plot increase sharply from 2% at the higher concentrations up to 20% at concentrations <50 nM. The effect of this detection limit is to cause an underestimation of $[DBDS]_{free}$, which means that we cannot compensate for very small adventitious fluorescence enhancements in the supernatant. In consequence $[DBDS]_{bound}$ is slightly overestimated in the centrifugation method, which leads to a small shift in the Scatchard plot.

Fluorescence Enhancement Method

Known concentrations of DBDS were added to MCD cell suspensions and the fluorescence enhancement was measured after 25-min incubation, as described above. The measured fluorescence unit f, which contains contributions from both free and bound DBDS, as well as light scattering, self quenching and the inner filter effect, is given by:

$$f = Q(\alpha B + \beta F + S) \quad (1)$$

in which B and F are bound and free [DBDS]. Q is the correction for the inner filter and self-quenching effects, S is the light-scattering of MCD cells and α and β are the fluorescence intensities per bound and free [DBDS]. S was determined from a direct measurement of the fluorescence intensity of an MCD cell suspension in the absence of DBDS. Q and β were determined from the fluorescence intensity of $[DBDS]_{free}$ by a quadratic fit to the equation, $f = \beta F + Q\beta F^2$. The values of K_1^s, K_2^s, α, n and c were determined as given in the Appendix.

Kinetics of DBDS Binding to MCD Cells and Red Cells

For the binding kinetics experiments, the MCD cell concentration was 0.02–0.2 mg ml^{-1}, for suspensions in either the 28.5 mM citrate solution, or in PBS. The MCD cell solution was mixed with an equal volume of 1–10 μM DBDS, in the same buffer, in a stopped-flow apparatus (Model SFA-11, Hi-Tech Scientific, Salisbury, Wiltshire, England). This apparatus consisted of two hand-driven syringes which rapidly drove 0.2 ml of solution through a mixing chamber into an observation cuvette in the light path of our single-beam spectrofluorimeter. The path length from the mixing chamber to the observation port, together with the time required to replace the solution in the observation chamber, gave an effective dead time of 40 msec, to which must be added an additional 10 msec for delays in the trigger circuit. DBDS binding was measured by fluorescence enhancement, excited at 350 nm (resolution 4 nm), path length 10 mm, and monitored at 427 nm (resolution 8 nm, path length 2 mm). The spectrofluorimeter output was interfaced on-line to our computer, whose data acquisition board (Lab Master, Scientific Solutions Inc., Solon, OH) provided a 30-kHz conversion. 1000 data points, spaced between 2–10 msec, were collected for each run and 3–5 runs were averaged for each data point. The data were usually fitted by nonlinear least-squares to a single exponential, except that, occasionally, there was a measurable slow component of small amplitude with a time constant one order of magnitude higher. We do not know the cause of this second exponential and have neglected it except that, when it is detectable, the data have been fitted to two exponentials which were then deconvoluted so that the fast time constant, which is the one we use, could be determined accurately.

In the red cell experiments, the cell solution contained 0.5% washed red cells in PBS. The signal was small, due to the high absorbance of hemoglobin in our 300–450 nm observation range, so that it was necessary to average 50–100 runs for each data point.

Experiments with H_2-DIDS

Approximately 0.1 mg total protein/ml of MCD cells were reacted with 1 μM H_2-DIDS for 30 min (37°C) in PBS and centrifuged. The cells were washed twice with saline buffer containing 0.5% bovine serum albumin (BSA) to remove unreacted H_2-DIDS and twice with saline buffer to remove BSA. [This is a modification of the procedure for red cells by Ramjeesingh, Gaarn and Rothstein (1980).]

Results and Discussion

Equilibrium Binding

Our experiments are based on the premise that the DBDS fluorescence enhancement that we observe is the result of binding to MCD cell 'band 3' and that there is relatively little nonspecific binding. Since DIDS, which is known to bind to red cell band 3 with high specificity (Lepke et al., 1976) reacts covalently with the membrane, covalent DIDS binding should suppress DBDS fluorescence enhancement in MCD cell 'band 3,' as it does in the red cell (Verkman et al., 1983). Figure 1 shows, not only that covalent binding of 1 μM H_2-DIDS suppresses DBDS fluorescence enhancement, but also that fluorescence enhancement in H_2-DIDS-treated cells does not increase with [DBDS], as it would if any nonspecific DBDS binding sites remained. Thus, there are no measurable nonspecific DBDS binding sites that do not react with H_2-DIDS.

Since the yield of MCD cells was low, the maximal amount of pooled fresh separated MCD cells on any single day virtually never exceeded 10 mg, and averaged $\approx$5 mg. In order to avoid problems of biological and preparative variability, it was necessary to complete an entire series of measurements on a single pooled sample. When a series of measurements required more than a single day's supply of cells, we pooled samples from several day's runs, which meant that the cells had to be frozen. Scatchard plots can be made by either fluorescence enhancement of an entire cell suspension or by centrifugation. Fluorescence enhancement requires a relatively small number of cells but evaluates the number of binding sites in fluorescence units, rather than giving them directly in nanomolar units. In the centrifugation method the supernatant is separated, which permits measurement of the number of sites in nanomolar units, but more cells are required so that this method can only be applied to pooled frozen cells.

The initial set of experiments was carried out in 28.5 mM citrate buffer, since citrate is a nontransportable anion in the red cell. Thus our results were not affected by the transport kinetics of Cl^- and could be compared with our previous results in red cell ghosts (Verkman et al., 1983) measured under the same conditions. Figure 2 shows that the bind-

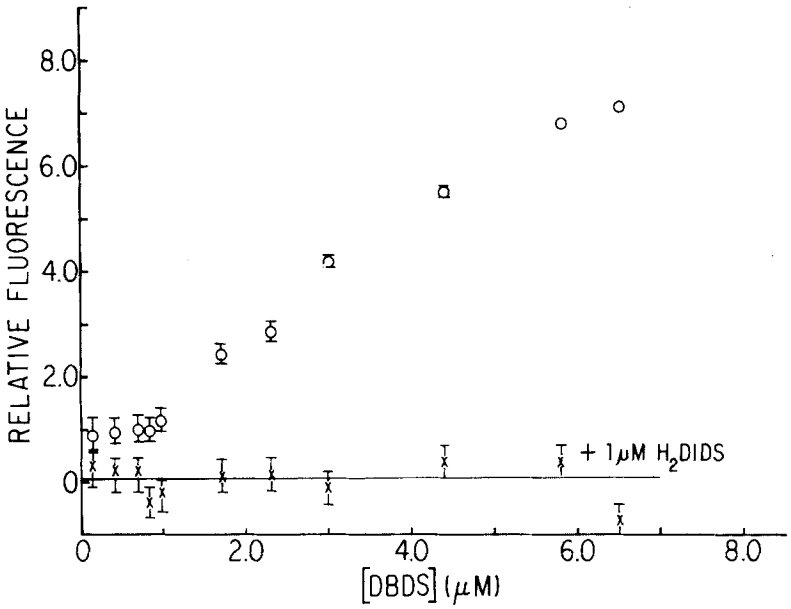

Fig. 1. Effect of covalent H_2-DIDS reaction on DBDS/'band 3' binding in MCD cells. The upper (control) curve shows the relative fluorescence (in fluorescence units, *see* Materials and Methods) of DBDS binding to 0.1 mg ml^{-1} MCD cells in 28.5 mM citrate, pH 7.4, 23°C. The contribution of free DBDS has been subtracted and the curve has been corrected for self-quenching and inner filter effects. The bottom curve shows the relative fluorescence after pretreatment with 1 μM H_2-DIDS, as described in Materials and Methods

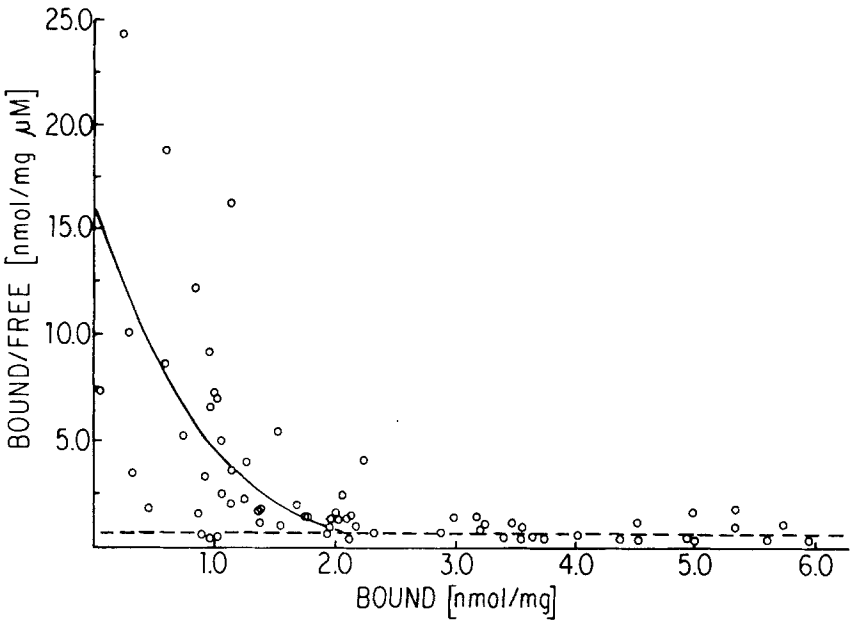

Fig. 2. Scatchard plot of DBDS binding to MCD cell 'band 3' measured by the centrifugation method. Bound is $[DBDS]_{bound}$ (nmol (mg protein)$^{-1}$) and Free is $[DBDS]_{free}$ (μM). The solid line is a two-site fit, drawn as described in the text, and the dashed line is the nonspecific binding, calculated as described in the text. Each of the 71 points is an average of 3–5 runs

ing curve obtained by the centrifugation method on frozen MCD cells is characterized by two binding sites, which are also evident in measurements by the fluorescence method in both fresh and frozen cells, as shown in Figs. 3 and 4. To facilitate comparison between MCD cell 'band 3' and red cell band 3, we have fitted our binding data to the sequential binding model used by Verkman et al. (1983) to fit their red cell data (*see* further discussion in the Appendix). Verkman et al. used the F-test to show that the sequential binding model fits the red cell data significantly better than a single-site model and we would expect the same conclusion to apply to MCD cells since the scatter of the data is similar.

The data obtained by the centrifugation method in Fig. 2 were fit to a two-site Scatchard plot by nonlinear least-squares using the method given in the Appendix. The high-affinity apparent dissociation constant, $K_1^s = 93 \pm 24$ nM ($n_1 = 1.0 \pm 0.1$ nmol (mg protein^{-1})) and the low-affinity apparent dissociation constant, $K_2^s = 430 \pm 260$ nM ($n_2 = 1.0 \pm 0.1$ nmol (mg protein)$^{-1}$), as shown in Table 1. These data agree with those obtained by fluorescence en-

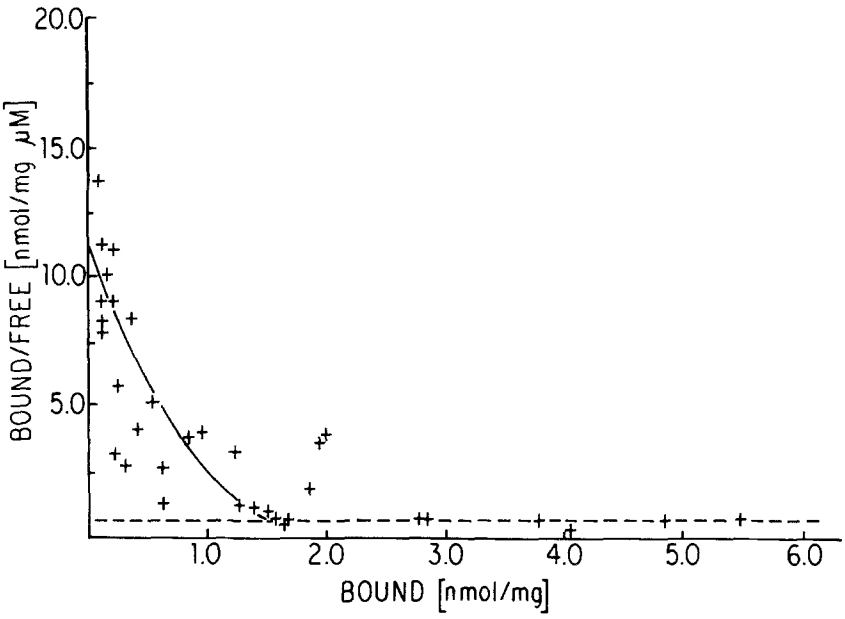

Fig. 3. Equilibrium binding of DBDS to MCD cell 'band 3' measured by the fluorescence method. The solid and dashed lines are drawn as described in the legend to Fig. 2. Each of the 36 points is the average of three independent measurements

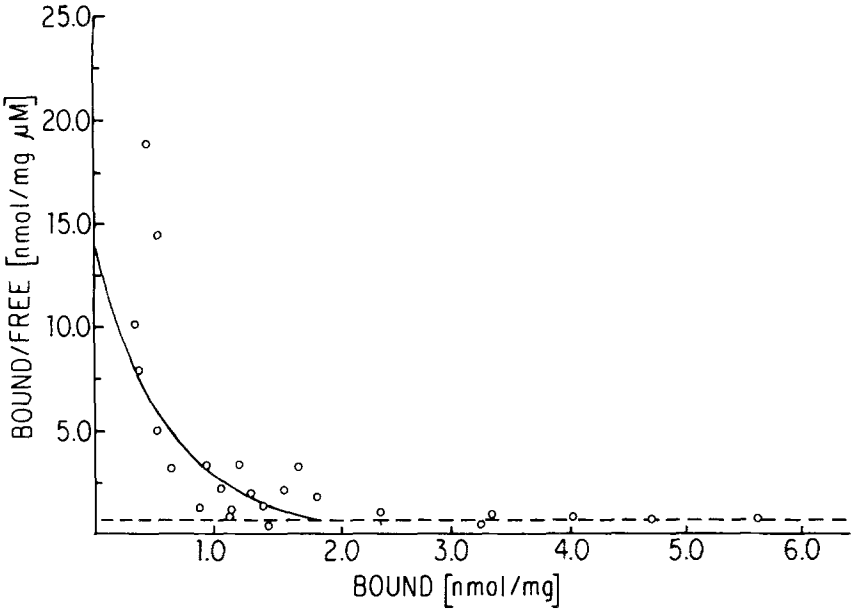

Fig. 4. Equilibrium binding of DBDS to MCD cell 'band 3' measured by the fluorescence method using freshly prepared MCD cells. The fitting process is as described under Fig. 2 and each of the 28 points is the average of three runs. K_1^s = 86 ± 12 nM; K_2^s = 570 ± 210 nM and n = 0.7 ± 0.1 nmol (mg protein)$^{-1}$

hancement, K_1^s = 93 ± 13 nM (n_1 = 0.8 ± 0.1 nmol (mg protein)$^{-1}$) and K_2^s = 610 ± 180 nM (n_2 = 0.8 ± 0.1 nmol (mg protein)$^{-1}$), also shown in Table 1. Freezing the MCD cells does not appear to affect the equilibrium binding of DBDS, as can be seen by a comparison of Fig. 3 with Fig. 4 whose dissociation constants are given in the legend. Table 1 also shows that the binding affinities, obtained from the Scatchard plots in 28.5 mM citrate buffer of MCD cell 'band 3,' agree well with those observed previously in the red cell ghost, which provides strong support for the view that the DBDS binding sites in MCD cells closely resemble those of red cell band 3.

The relationship between the DBDS binding sites in red cells and band 3 can be probed further by replacing the nontransportable citrate in the suspension medium with 130 mM Cl^-. Under these conditions, Dix et al. (1986) showed that the two red cell DBDS binding sites, which are observed at 28.5 mM citrate, collapse into a single site at 130 mM Cl^-. As Fig. 5 shows, a similar behavior is observed by the fluorescence method in MCD cells, whose single binding site has K_1^s = 1500 ± 400 nM (n_1 = 1.3 ± 0.2 nmol (mg protein)$^{-1}$), in good agreement with the red cell, as shown in Table 1. Since the principal function of red cell band 3 is Cl^-/HCO_3^- exchange,

Table 1. Affinities for DBDS binding to MCD Cells[a]

	K_1^s (nM)	n_1 (nmol(mg protein)$^{-1}$)	K_2^s (nM)	n_2 (nmol(mg protein)$^{-1}$)
Centrifugation method (28.5 mM citrate)				
MCD cells	93 ± 24	1.0 ± 0.1	430 ± 260	1.0 ± 0.1
Red cell ghosts	110 ± 51	1.6 ± 0.2	980 ± 200	1.6 ± 0.2
Fluorescence enhancement method (28.5 mM citrate)				
MCD cells	93 ± 13	0.8 ± 0.1	610 ± 180	0.8 ± 0.1
Red cell ghosts	65 ± 8	1.6 ± 0.2	820 ± 100	1.6 ± 0.2
Experiments at 130 mM Cl^-				
MCD cells	1500 ± 400	1.3 ± 0.4		
Red cell ghosts	1200 ± 200	3.3 ± 0.3		

[a] Red cell ghost data at 28.5 mM citrate from Verkman et al. (1983); red cell ghost data at 130 mM Cl^- from Dix et al. (1986).

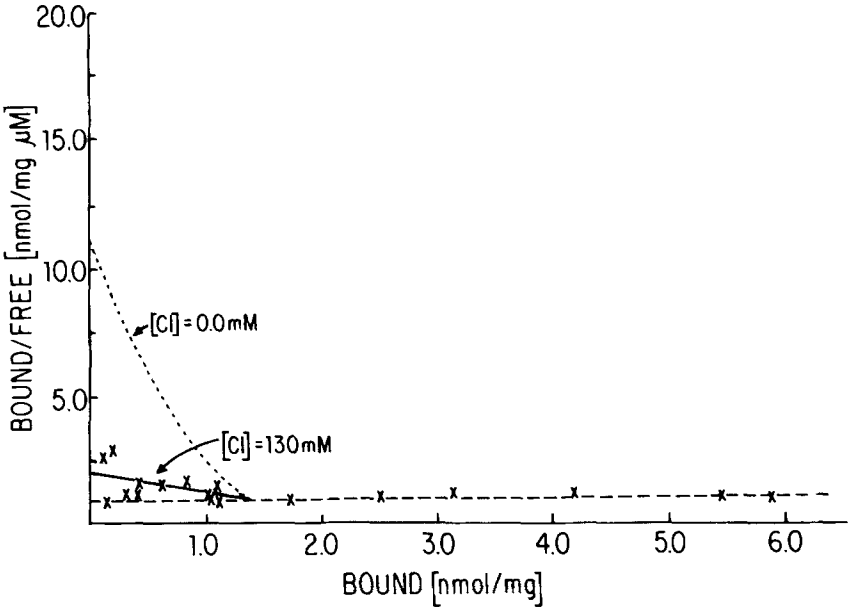

Fig. 5. Effect of Cl^- on DBDS binding to MCD cell 'band 3' measured by the fluorescence method. 0.357 mg MCD cell protein ml^{-1} were incubated for 25 min at 23°C, pH 7.4 in 160 mM ionic strength buffer containing 130 mM NaCl, and the balance Na citrate. The dotted curve for $[Cl^-] = 0$ is taken from Fig. 3 and that for $[Cl^-] = 130$ mM was obtained by a nonlinear least-squares fit to a single-site Scatchard plot with $K^s = 1500 \pm 400$ nM and $n = 1.3 \pm 0.4$ nmol (mg protein)$^{-1}$. The correction for nonspecific binding sites (dashed line) was taken from the $[Cl^-] = 0$ data. Each of the 18 points is the average of three runs

the observation that the replacement of citrate by Cl^- produces virtually identical results in the DBDS binding affinities provides additional strong support for the view that closely similar anion exchange mechanisms are expressed in both red cells and MCD cells.

There may be a difference, however, in the number of binding sites per mg membrane protein. Superficially, Table 1 shows that the total number of DBDS binding sites/mg protein is similar in MCD and red cells, but the preparations are very different. The red cell measurements were made on white ghosts in which the hemoglobin and other intracellular proteins have been washed away. Band 3 comprises 25% of the red cell membrane protein (Guidotti, 1972) and one DBDS binds to each band 3 with a site density of 3.2 (nmol DBDS sites)/(mg membrane protein). Our experiments with MCD cells are carried out on a preparation of whole cells which, though relatively pure, contains significant fractions of other material, but the site density of 1.4 (nmol DBDS site)/(mg total cell protein) is comparable. It may well be that the site density of stilbene binding sites in kidney cells is much greater than in the red cell since Talor et al. (1987) report H_2-DIDS site densities of 11 and 32 (nmol H_2-DIDS sites)/(mg membrane protein) for luminal and basolateral membranes, respectively, of bovine kidney cortex. Our MCD cell site density would be comparable to theirs if the membrane proteins com-

prised ≈4–13% of the total protein in our preparation.[1]

Binding Kinetics

We have measured the time course of DBDS binding to fresh MCD cells by the stopped-flow method and find in the typical experiment shown in Fig. 6, that there is a fast component with a rate constant of 7.1 ± 0.2 sec^{-1} ([DBDS] = 4 μM). We have modelled the first steps of the reaction as a bimolecular association, too fast for us to measure, followed by a conformational change, according to Eq. (2) following the model of Verkman et al. (1983) in the red cell,

$$\text{DBDS} + \text{protein} \overset{K_1}{\leftrightarrow} \text{DBDS-protein} \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} \text{DBDS-protein}^* \qquad (2)$$

in which 'protein' denotes the MCD binding protein and * denotes the DBDS/protein complex following its conformation change. It is possible to determine K_1 and k_2 by plotting τ against $[\text{DBDS}]^{-1}$ according to Eq. (3) (given by Verkman et al., 1983):

$$\tau = K_1/(k_2[\text{DBDS}]_{\text{total}}) + 1/k_2 \qquad (3)$$

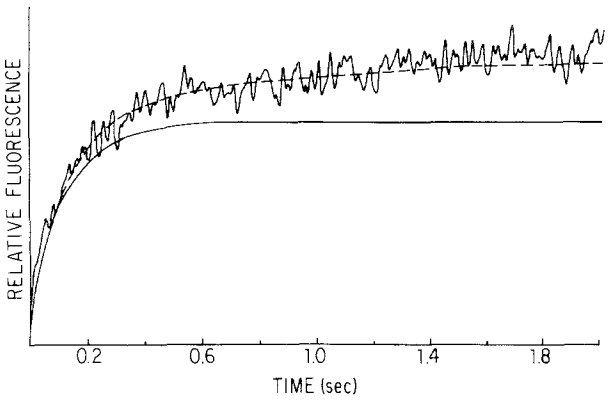

Fig. 6. Time course of 4 μM DBDS binding to 'band 3' in frozen MCD cells in PBS. The data have been fitted to a double exponential (dashed line) which has been deconvoluted to give the fast exponential (full line) with $1/\tau = 7.1 \pm 0.2$ sec^{-1}, as described in Materials and Methods

provided $k_2 \gg k_{-2}$ and $[\text{DBDS}]_{\text{total}} \gg [\text{protein}]_{\text{total}}$. A linear fit of τ to $[\text{DBDS}]^{-1}_{\text{total}}$ gives k_2 = 1/intercept, K_1 = slope/intercept. Using Eq. (A3) in the Appendix, $k_{-2} = k_2/[(K_1/K_1^s) - 1]$. Since the 0.5 to 5 μM DBDS used in our experiments is ≫ than the 1.4 nmol DBDS sites/(mg total cell protein) in suspensions of <0.4 mg MCD cells/ml and since (as will be shown in Table 3) $k_2 \gg k_{-2}$, the conditions for Eq. (3) are satisfied.

Table 2 shows the results of the experiments to determine τ in MCD cells in PBS as a function of $[\text{DBDS}]^{-1}$. For comparative purposes, we have made measurements on red cells with the results given in Table 2. Comparison of the binding kinetics of DBDS shows that the results for MCD cell 'band 3' are very close to those for the human red cell.

We have determined the dependence of τ on $[\text{DBDS}]^{-1}$ at 28.5 mM citrate and observed the linear relationship shown in Fig. 7, which fits Eq. (2) very well. The values of k_2 and K_1 obtained from the slope and intercept can be combined with K_1^s to give k_{-2} and K_2, as shown in Table 3. The observation that the value of 2.1 μM for K_1, the true dissociation constant for the bimolecular association in MCD cells, is very much greater than the apparent dissociation constant, K_1^s = 0.09 μM means that the initial binding is very weak and clearly illustrates the effect of the conformational change in locking the bound DBDS into its site on MCD cell 'band 3.' This is a consequence of the fact that k_2 for the conformational change is 9.9 sec^{-1}, about twenty times greater than the reverse rate constant, k_{-2} which = 0.5 sec^{-1}. In other words, the first binding step may not be particularly specific, but once the protein recognizes how well the ligand fits its site, it

[1] Since the site density is so high, we have carefully considered the possibilities for experimental error in either of the two numbers which comprise the site density, the ratio of the total number of DBDS sites to the total amount of protein in the sample. The total number of DBDS sites is obtained by difference between the initial and final $[\text{DBDS}]_{\text{free}}$ in the supernatant. Control experiments confirm that there has been no loss of DBDS from our initial supernatant solution as, for example, by adsorption to the walls of the pipette or test tube, which would serve to increase the apparent number of sites. We have determined the DBDS extinction coefficient in our initial supernatant solution to be 28.5 ± 2.5 mM^{-1} cm^{-1} at 350 nm and 49.6 ± 1.9 mM^{-1} cm^{-1} at 336 nm in good agreement with the values of 32.1 mM^{-1} cm^{-1} at 350 nm and 50.0 mM^{-1} at 336 nm, given for DBDS by Rao et al. (1979).

Protein concentration was determined by the Bradford method, as described in Materials and Methods. In order to make sure that the Bradford method gave the same results as the Lowry method, another member of the laboratory assayed a sample by the Lowry method and obtained a value of 0.78 mg ml^{-1}, as compared to the value of 1.03 ± 0.07 mg ml^{-1} he obtained by the Bradford method (using an ovalbumin standard); our independent assay by the Bradford method gave a value of 1.17 ± 0.09 mg ml^{-1} (using a bovine serum albumin standard). The possibility existed that micelle formation might prevent access of the reagent to all the amino acids in the sample, which would decrease the apparent protein concentration. Consequently, we arranged for an amino acid analysis after total combustion of one of our samples, which yielded a value of 1.44 mg ml^{-1}, in very good agreement with our determination, by the Bradford method, of 1.56 ± 0.14 mg ml^{-1} on the same sample.

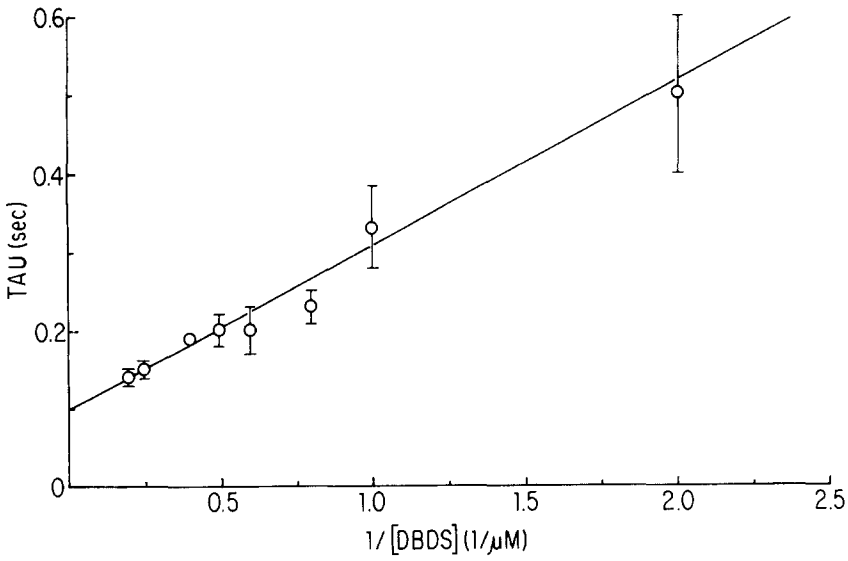

Fig. 7. Dependence of the fast component of τ on $[DBDS]^{-1}$ in MCD cells (0.1 [mg MCD cell protein] ml^{-1}) in 28.5 mM citrate at 23°C, pH 7.4. The line has been drawn according to Eq. (3) by least-squares with $K_1 = 2.1 \pm 0.3$ μM and $k_2 = 9.9 \pm 0.9$ sec^{-1}

Table 2. Concentration dependence of DBDS binding kinetics to MCD cells and red cell preparations

	τ (sec)	τ (sec)	τ (sec)	τ (sec)
$(DBDS\ conc)^{-1}$	0.25 μM^{-1}	0.5 μM^{-1}	1.0 μM^{-1}	2.0 μM^{-1}
MCD cells[a]	0.14 ± 0.03(5)	0.21 ± 0.01(3)	0.24 ± 0.02(4)	
Red cells[a]	0.12 ± 0.03	0.14 ± 0.05	0.18 ± 0.05	0.25 ± 0.07

[a] Expts. in PBS. Number of expts. in parentheses. Errors are SD.

Table 3. Kinetic and equilibrium constants for DBDS/band 3 binding in red cells and MCD cells

Constant	Units	Red cell ghosts	MCD cells[a]
K_1	μM	3.0 ± 1.4	2.1 ± 0.5
k_2	sec^{-1}	4.0 ± 1.5	9.9 ± 0.8
k_{-2}	sec^{-1}	0.09 ± 0.02	0.5 ± 0.3
K_2		44 ± 20	22 ± 9
K_3	μM	0.9 ± 0.1	0.6 ± 0.2
K_1^s	μM	0.065 ± 0.008	0.09 ± 0.01
K_2^s	μM	0.8 ± 0.1	0.6 ± 0.2

[a] Data obtained by the fluorescence enhancement method.

snaps across to its new configuration. Examination of Table 3 shows that an entirely analogous situation obtained for red cell band 3.

Thus, both the binding affinities determined from the Scatchard plot and the binding kinetics show that the physical chemical characteristics of stilbene inhibitor binding to MCD cell 'band 3' closely resemble those for red cell band 3. This means that differences between red cell and MCD cell, in either the peptide sequence or in the microenvironment of the anion exchange protein, have very little effect on inhibitor binding, and by extension on the anion transport process itself, which strongly suggests that the molecular structure is highly conserved.

This work was supported in part by National Institutes of Health grant GM 34099. This work was done during the tenure of a research fellowship to AJ from the American Heart Association, Massachusetts Affiliate. The MCD cells were kindly supplied through the courtesy of Dr. Patricio Silva and Mr. Robert Fuhro of the Renal Division of the Beth Israel Hospital, for whose generous help we are most grateful.

References

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254

Dix, J.A., Verkman, A.S., Solomon, A.K. 1986. Binding of chloride and a disulfonic stilbene transport inhibitor to red cell band 3. *J. Membrane Biol.* **89:**211–223

Drenckhahn, D., Schluter, K., Allen, D.P., Bennett, V. 1985. Colocalization of band 3 with ankyrin and spectrin at the basal membrane of intercalated cells in the rat kidney. *Nature (London)* **230:**1287–1289

Guidotti, G. 1972. The composition of biological membranes. *Arch. Int. Med.* **129:**194–201

Knauf, P.A. 1979. Erythrocyte anion exchange and the band 3 protein: Transport kinetics and molecular structure. *Cur. Top. Membr. Transp.* **12:**251–363

Koeppen, B.M. 1985. Conductive properties of the rabbit outer medullary collecting duct: Inner stripe. *Am. J. Physiol.* **248:**F500–F506

Kotaki, A., Naoi, M., Yagi, K. 1971. A diaminostilbene dye as a hydrophobic probe for proteins. *Biochim. Biophys. Acta* **229:**547–556

Lepke, S., Fasold, H., Pring, M., Passow, H. 1976. A study of the relationship between inhibition of anion exchange and binding to the red blood cell membrane of 4,4′-diisothiocyano stilbene-2,2′-disulfonic acid (DIDS) and its dihydro derivative (H_2DIDS). *J. Membrane Biol.* **29:**147–177

Ramjeesingh, M., Gaarn, A., Rothstein, A. 1980. The location of a disulfonic stilbene binding site in band 3, the anion transport protein of the red blood cell membrane. *Biochim. Biophys. Acta* **599:**127–139

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

Schuster, V.L., Bonsib, S.M., Jennings, M.L. 1986. Two types of collecting duct mitochondria-rich (intercalated) cells: Lectin and band 3 cytochemistry. *Am. J. Physiol.* **251:**C347–C355

Stone, D.K., Seldin, D.W., Kokko, J.P., Jacobson, H.R. 1983. Anion dependence of rabbit medullary collecting duct acidification. *J. Clin. Invest.* **71:**1505–1508

Talor, Z., Gold, R.M., Yang, W.C., Arruda, J.A.L. 1987. Anion exchanger is present in both luminal and basolateral renal membranes. *Eur. J. Biochem.* **164:**695–702

Verkman, A.S., Dix, J.A., Solomon, A.K. 1983. Anion transport inhibitor binding to band 3 in red blood cell membranes. *J. Gen. Physiol.* **81:**421–449

Zeidel, M.L., Silva, P., Seifter, J.L. 1986*a*. Intracellular pH regulation and proton transport by rabbit medullary collecting duct cells: Role of plasma membrane H^+-ATPase. *J. Clin. Invest.* **77:**113–120

Zeidel, M.L., Silva, P., Seifter, J.L. 1986*b*. Intracellular pH regulation in rabbit renal medullary collecting duct cells: Role of chloride : bicarbonate exchange. *J. Clin. Invest.* **77:**1682–1688

Received 22 December 1987; revised 1 March 1988

Appendix

We have used the sequential binding model for DBDS binding to red cell band 3 (Verkman et al., 1983) to fit the Scatchard plot of our MCD data. Verkman et al. made a careful comparison of the sequential binding model with another model in which binding to the sites is entirely independent; they found that the points for the sequential model fit the data much better ($\chi^2 = 1.9$) than for the independent site model ($\chi^2 = 6.2$). We have not made such a comparison, but have chosen the same sequential model Verkman et al. used in order to facilitate the comparison of MCD cell and red cell results. Equation (2) in the text is based on a bimolecular association, too fast for us to measure, followed by a conformational change, as follows,

$$\text{DBDS} + \text{protein} \overset{K_1}{\leftrightarrow} \text{DBDS-protein} \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} \text{DBDS-protein}^* \qquad (2)$$

in which 'protein' denotes the MCD binding protein and * denotes the DBDS/protein complex following its conformation change. The next step in the sequential model is

$$\text{DBDS} + \text{DBDS-protein}^* \overset{K_3}{\leftrightarrow} (\text{DBDS})_2\text{-protein}^*. \qquad (A2)$$

The two observed sequential binding affinities, K_1^s and K_2^s, are related (*see* Verkman *et al.*, 1983) to the constants in Eqs. (2) and (A2) by the following,

$$K_1^s = K_1/(1 + K_2) \qquad (A3)$$

$$K_2^s = K_3/(1 + 1/K_2) \qquad (A4)$$

in which $K_2 = k_2/k_{-2}$. The Scatchard binding data is obtained by fitting the data to the following curve, as described below:

$$B/F = ((K_2^s + F)\gamma n)/(K_1^s K_2^s + 2K_2^s F + F^2) + \gamma c \qquad (A5)$$

in which B is $[\text{DBDS}]_{\text{bound}}$, F, $[\text{DBDS}]_{\text{free}}$ and c is the B/F ratio for the nonspecific binding sites. γ is the conversion factor from fluorescence units to nmol and is 1.0 in the centrifugation method, and $n = n_1 + n_2$, in which n_1 and n_2 are the number of high-affinity and low-affinity sites, taken to be equal, according to the sequential binding model. The Scatchard binding data for the centrifugation method are then obtained by a nonlinear four-parameter fit to the data for K_1^s, K_2^s, n and c.

To fit the data from the fluorescence enhancement experiments, Eq. (1) from the text was substituted into Eq. (A5) and the fluorescence intensity of a mixture of $\text{DBDS}_{\text{bound}}$ and $\text{DBDS}_{\text{free}}$ was used to make a five-parameter fit to the substituted equation to obtain K_1^s, K_2^s, α (required for Eq. (1) in the text), γn and γN. K_1^s and K_2^s are in nanomolar units, but n (the number of binding sites) and c (the B/F ratio for the nonspecific binding sites) are in fluorescence units. To obtain γ, which is necessary to convert from fluorescence units to nmol, we use the value of c previously determined by the centrifugation method and assumed that this parameter was the same in the fluorescence enhancement measurements.