Identification and characterization of a second 4,4'-dibenzamido-2,2'-stilbenedisulphonate (DBDS)-binding site on band 3 and its relationship with the anion/proton co-transport function

James M. SALHANY*^{†1}, Karen S. CORDES* and Renee L. SLOAN*

*The Veterans Administration Medical Center, 4101 Woolworth Avenue, Omaha, NE 68105, U.S.A., and [†]Departments of Internal Medicine and Biochemistry and Molecular Biology, University of Nebraska Medical Center, 984510 Nebraska Medical Center, Omaha, NE 68198-4510, U.S.A.

Band 3 mediates both electroneutral AE (anion exchange) and APCT (anion/proton co-transport). Protons activate APCT and inhibit AE with the same pK (~ 5.0). SDs (stilbenedisulphonates) bind to a primary, high-affinity site on band 3 and inhibit both AE and APCT functions. In this study, we present fluorescence and kinetic evidence showing that lowering the pH activates a second site on band 3, which binds DBDS (4,4'-dibenzamido-2,2'-stilbenedisulphonate) independently of chloride concentration, and that DBDS binding to the second site inhibits the APCT function of band 3. Activation of the second site correlated with loss of chloride binding to the transport site, thus explaining the lack of competition. The kinetics of DBDS binding at the second site could be simulated by a slow-transition, two-state exclusive binding mechanism ($R_0 \leftrightarrow T_0 + D \leftrightarrow TD \leftrightarrow RD$, where D represents DBDS, R_0 and T_0 represent alternate conformational states at the second DBDS-binding site, and TD and RD are the same two states with ligand DBDS bound), with a calculated overall K_d of $3.9 \mu M$ and a $T_0 + D \leftrightarrow TD$ dissociation constant of 55 nM .

DBDS binding to the primary SD site inhibited approx. 94 % of the proton transport at low pH ($K_1 = 68.5 \pm 11.8 \text{ nM}$). DBDS binding to the second site inhibited approx. 68 % of the proton transport ($K_1 = 7.27 \pm 1.27 \mu M$) in a band 3 construct with all primary SD sites blocked through selective cross-linking by bis(sulphosuccinimidyl)suberate. DBDS inhibition of proton transport at the second site could be simulated quantitatively within the context of the slow-transition, two-state exclusive binding mechanism. We conclude that band 3 contains two DBDS-binding sites that can be occupied simultaneously at low pH. The binding kinetic and transport inhibition characteristics of DBDS interaction with the second site suggest that it may be located within a gated access channel leading to the transport site.

Key words: anion/proton co-transport, 4,4'-dibenzamido-2,2'-stilbenedisulphonate (DBDS), drug binding to membrane channels, membrane transport, model simulation studies, stopped-flow kinetics.

INTRODUCTION

Band 3 exists in membranes as stable dimers or tetramers of two stable dimers [1–5]. It functions as an AE (anion exchange) transport protein [6], and AE is inhibited completely when SD (stilbenedisulphonate) molecules [7] bind to a primary, high-affinity site on each subunit [6]. SD molecules are allosterically competitive inhibitors with respect to chloride binding to the transport site [8,9]. Band 3 can also co-transport either sulphate and a proton [10] or chloride and a proton [11]. This APCT (anion/proton co-transport) [10–20] can be inhibited by the binding of SD molecules to the primary site [11], thus assigning both AE and APCT functions to band 3. APCT is activated with $pK \sim 5.0$ [12,13], concomitant with a loss of the AE function and of chloride binding to the AE transport site [17,19]. These reciprocal functional transitions appear to involve protonation of Glu-681 [14–16] and interaction with histidine residues [17,18].

Neutralization of the negative charge on Glu-681, by modification of band 3 with WRKB (Woodward's Reagent K/sodium borohydride) [14–16], caused band 3/DBDS complexes (where DBDS stands for 4,4'-dibenzamido-2,2'-stilbenedisulphonate) to undergo a significant conformational change leading to enhancement in the fluorescence quantum yield of bound DBDS molecules and induction of apparent subunit interactions with respect

to DBDS release from the band 3 dimer [21]. These changes occurred at neutral pH, but only when chloride was present. WRKB modification exposed a cryptic chloride-binding modifier site.

The aim of the present study was to determine whether lowering the pH can mimic the effects of WRKB modification and chloride addition on the fluorescence spectral and release kinetic characteristics of DBDS bound to the primary SD site on band 3. The results indicate that lowering the pH does not mimic the WRKB effect observed for DBDS bound to the primary SD site. Instead, lowering the pH activated a second, low-affinity DBDS-binding site on band 3 [22], which we show is an APCT inhibitory site. The DBDS binding kinetic and transport inhibitory characteristics suggest that the second site may be located within a gated access channel leading to the transport site.

EXPERIMENTAL

Materials

In-date erythrocytes were obtained from the Omaha Chapter of the American Red Cross. DIDS (4,4'-di-isothiocyanato-2,2'-stilbenedisulphonate), phosphatidylcholine (L- α -phosphatidylcholine, type III-B from bovine brain) and FITC-dextran were

Abbreviations used: AE, anion exchange; APCT, anion/proton co-transport; BS³, bis(sulphosuccinimidyl)suberate; C₁₂E₈, polyoxyethylene-8-lauryl ether; DBDS, 4,4'-dibenzamido-2,2'-stilbenedisulphonate; DIDS, 4,4'-di-isothiocyanato-2,2'-stilbenedisulphonate; MDB3, membrane domain of band 3; SD, stilbenedisulphonate; WRKB, Woodward's Reagent K/sodium borohydride.

¹ To whom correspondence should be addressed (email jsalhan@attglobal.net).

obtained from Sigma. DIDS and DBDS stock solutions were prepared in water as described in [21,23]. BS³ [bis(sulphosuccinimidyl)suberate] was obtained from Pierce (Rockford, IL, U.S.A.). Buffers used in the DBDS binding reactions of the present study were: 5P(8) (5 mM sodium phosphate, pH 8.0); PBS [150 mM NaCl and 5P(8)]; chloride buffer, (150 mM NaCl, 20 mM Bis-Tris and 20 mM Mes); gluconate buffer (150 mM sodium gluconate, 20 mM Bis-Tris and 20 mM Mes); magnesium chloride buffer (150 mM NaCl, 20 mM Bis-Tris, 20 mM Mes and 10 mM MgCl₂); C₁₂E₈ (polyoxyethylene-8-lauryl ether) buffer (150 mM NaCl, 20 mM Bis-Tris, 20 mM Mes, 25 μ M phosphatidylcholine and 0.1 % C₁₂E₈). pH values are given in the Figure legends except where stated above.

Methods

Unsealed erythrocyte ghosts were prepared by the method described in [21]. Magnesium resealed control ghosts were prepared by lysing washed erythrocytes 1:50 in ice-cold 5P(8) and resealing in magnesium chloride buffer for 1 h at 37°C. MDB3 (membrane domain of band 3) was prepared as described previously [2] from erythrocytes prelabelled with a slight stoichiometric excess of DIDS. The percentage of the band 3 subunit population modified by covalent binding of DIDS was determined as described in [23]. Selective intrasubunit cross-linking of band 3 was performed as described previously [24] using 300 μ M BS³. The extent of intrasubunit cross-linking of band 3 was determined quantitatively: (i) by using an SDS/PAGE assay, where the ability of chymotrypsin to cleave band 3 subunits was tested [24], (ii) by measuring the extent of covalent binding of DIDS to 'lysine A' [23] and (iii) by measuring reversible binding of DBDS to the primary SD site using a stopped-flow fluorescence kinetic assay [25]. DBDS release experiments were performed using the SD replacement reaction [26–28].

Carbonic anhydrase-free resealed ghosts containing FITC-dextran were prepared from both control and BS³-modified erythrocytes using the column chromatographic method described in detail by Lepke et al. [19]. These ghosts were washed twice with 150 mM NaCl, 5 mM Mes and 5 mM Bis-Tris (pH 7.1) and brought to 50 % haematocrit and kept on ice. The 150 mM NaCl, 5 mM Mes and 5 mM Bis-Tris buffer system was adjusted to various pH values by mixing a stock buffer (pH 7.1) with a pH 2.9 stock of the same buffer at 8°C. The pH values used in the plots represent the pH values of various buffers measured at 8°C. The proton influx experiment was performed using a PerkinElmer 650-40 fluorescence spectrophotometer equipped with a water-jacketed cuvette holder attached to a temperature-controlled water bath. A digital thermometer was inserted periodically, to monitor the temperature in the cuvette. The cuvette was filled with 0.95 ml of the cold flux buffers at various pH values. A 50 μ l volume of the FITC-dextran resealed ghosts at 50 % haematocrit (pH 7.1, on ice) was added to the 0.95 ml volume of the flux buffers at various pH values with a plastic mixing dasher, thus establishing an inward pH gradient. The final haematocrit was 2.5 %. In studies where DIDS or DBDS was present, these reagents were added to the flux buffers at the various pH values before the addition of the FITC-dextran resealed ghosts. The strip chart recorder time base was started simultaneously with mixing of the resealed ghosts. Intracellular acidification was followed by observing the decrease in fluorescence of intracellular FITC-dextran using an excitation wavelength of 492 nm, and an emission wavelength of 517 nm.

The kinetic data were analysed quantitatively [21] and model simulation studies were performed as described in [29].

Figure 1 Schematic diagram of the experimental approach used in the present study

See the Experimental section for a description.

RESULTS

Preliminary considerations

Figure 1 illustrates the experimental procedure used. DBDS release from and binding to the second site are first identified and characterized and they are assigned to the outer surface of MDB3. The ability of DBDS binding to the second site to inhibit the APCT function of band 3 is then tested. We used the Staros cross-linking reagent (BS³) to block the primary SD site on band 3 [30–32] (Figure 1). Such intrasubunit cross-links can be inserted selectively by using micromolar levels of BS³ [24]. The possibility that the APCT function of band 3 would be preserved after BS³ cross-linking of the primary SD site follows from the studies of Jennings et al. [33].

pH dependence of DBDS release from isolated human erythrocyte membranes (ghosts)

Lowering the pH caused the time course for DBDS release to convert from monophasic to biphasic (Figure 2). Biphasic kinetics developed independent of chloride (results not shown), in contrast with WRKB-modified band 3 [21]. The slow phase was approximately two orders of magnitude slower (Figure 2B) than the slow phase for DBDS release from WRKB-modified band 3 [21]. Lowering the pH caused the development of a larger fraction of slow phase (Figure 2B) than that observed for WRKB band 3 measured in chloride (~71 % versus 50 %) [21]. Figures 3(A) and 3(B) show that ΔF_{total} for DBDS release titrated with an apparent pK of approx. 5, independently of whether chloride was present or absent. This contrasts with our work using WRKB-modified band 3 at neutral pH, where enhancement of DBDS fluorescence occurred only when chloride was present [21]. It is clear that the appearance of the slow phase of the reaction (Figures 3C and 3D, open circles) accounted for the entire increase in fluorescence

Figure 2 The pH dependence of the DBDS/DIDS replacement reaction

Unsealed ghosts (4% haematocrit) and DBDS (6.76 μM) in gluconate buffer were mixed in the stopped-flow apparatus with 14 μM DIDS that was prepared in chloride buffer at various pH values and 25°C. The excitation wavelength was set at 335 nm and fluorescence was observed through a 415 nm cutoff filter. The time courses were fitted using either a single [$\Delta F = \Delta F_{\text{total}} \exp(-kt)$] or a double [$\Delta F = \Delta F_{\text{fast}} \exp(-k_1 t) + \Delta F_{\text{slow}} \exp(-k_2 t)$] exponential function. (A) The time course at pH 7 followed single exponential kinetics with the following values for the constants: $\Delta F_{\text{total}} = 0.11 \pm 0.001$ and $k = 0.94 \pm 0.02 \text{ s}^{-1}$. (B) The time course at pH 4 followed double exponential kinetics with the following values for the constants: $\Delta F_{\text{fast}} = 0.12 \pm 0.01$, $k_1 = 0.04 \pm 0.006 \text{ s}^{-1}$ and $\Delta F_{\text{slow}} = 0.29 \pm 0.006$, $k_2 = 0.002 \pm 0.001 \text{ s}^{-1}$.

Figure 3 The pH dependence of the change in fluorescence amplitude for the DBDS/DIDS replacement reaction

Reactions were performed as described in the legend to Figure 2. When chloride was present (A, C), the DBDS-ghost suspensions of Figure 2 were prepared in gluconate buffer and mixed in the stopped-flow apparatus with 14 μM DIDS in chloride buffer. When chloride was absent (B, D), DBDS-ghost suspensions in gluconate buffer were mixed with 14 μM DIDS in the same respective gluconate buffers. (A, B) The pH dependence of ΔF_{total} ; (C, D) plots of pH dependence versus the amplitudes for the fast (●) and slow (○) phases. The fluorescence amplitudes for ΔF_{total} and ΔF_{slow} increase with decreasing pH value, yielding titrations with pK values close to 5.0. The pK values were determined by fitting data using the equation

$$\Delta F = \{[L_1 + L_2 10^{(pH - pK)}] / [10^{(pH - pK)} + 1]\}$$

where L_1 and L_2 are the values of ΔF at the upper and lower limits of the titration curve. The specific pK values were, for ΔF_{total} , 5.23 ± 0.15 (A) and 4.85 ± 0.16 (B), and for ΔF_{slow} , 5.01 ± 0.16 (C, ○) and 4.46 ± 0.26 (D, ○). The amplitude of the fast phase (● in C and D) essentially did not vary over the pH range of these experiments.

Figure 4 The pH dependence of the fast phase apparent rate constant for DBDS release from band 3

●, Values of the rate constants from experiments where DBDS-ghost complexes in gluconate buffer were mixed with DIDS in chloride buffer as described in the legend to Figure 2 (75 mM NaCl + 75 mM sodium gluconate after mixing); ○, values of rate constants for the fast phase from experiments where ghosts were first suspended in chloride buffer at various pH values to which DBDS was then added, with the reaction being initiated by mixing with DIDS in the same chloride buffer, at the pH values indicated (150 mM NaCl); □, values of rate constants for reactions performed entirely in gluconate buffer. There was no significant titration for the fast phase rate constant determined in the absence of chloride (□). The pK values with chloride present were determined to be 4.97 ± 0.14 (●) and 4.96 ± 0.19 (○).

amplitude of the overall reaction process. The amplitude of the fast phase was essentially independent of pH over the range studied (Figures 3C and 3D, closed circles).

Effect of lower pH on the chloride-induced acceleration in the rate of release of DBDS from band 3

Chloride binding to the transport site causes a 16–20-fold increase in the rate constant for DBDS release when measured at neutral pH [8,27,28]. Results in Figure 4 show that lowering the pH from neutrality caused the rate constant for the fast phase to decrease significantly when measured in chloride (open and closed circles). The pK values were the same for premixed (Figure 4, open circles) and non-premixed (closed circles) samples. The difference in apparent rates between the two curves is solely due to the difference in chloride concentration [27] and not due to the method of chloride addition. There was no significant titration of the fast phase rate constant for DBDS release when chloride was absent (Figure 4, open squares), and the rate constants at pH 4 were the same, no matter how chloride was added.

DIDS prelabelling of band 3 selectively eliminates the fast phase in the DBDS/DIDS reaction measured at low pH

Band 3 was prelabelled with DIDS, which is known to eliminate the binding of DBDS at neutral pH [27]. The DBDS release reaction became monophasic at pH 4, through the elimination of the fast phase (results not shown). This indicates that the slow phase (Figure 2B) arises from DBDS binding to a second site.

Stopped-flow dilution-jump experiments show that the slow phase arises from a DIDS-independent, dilution-induced release of DBDS from the second binding site

Dilution of DBDS-ghost complexes in the stopped-flow reaction at pH 6.5 did not cause DBDS to dissociate from band 3 (Figure 5A). The DBDS concentration used in Figure 5 was approx. 3.4 μM after the mixing. However, at pH 4, there was a very slow exponential loss of DBDS fluorescence (Figure 5A). The rate constant and amplitude of the reaction in the absence of DIDS (Figure 5A; $k = 4.4 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$ and $\Delta F_{\text{total}} = 0.32 \pm 0.004$)

Figure 5 Stopped-flow dilution-jump experiments for the pH dependence of DBDS release from DBDS-ghost complexes

DBDS-ghost complexes were prepared in gluconate buffer exactly as described in the legend to Figure 2. These samples were mixed 50:50 in the stopped-flow apparatus at 25 °C with chloride buffer (no DIDS), at the same respective pH values as the DBDS-ghost suspensions. The kinetics were measured as described in the legend to Figure 2, and the data were analysed using the single exponential function in the legend to Figure 2. (A) Time courses for dilution-jump experiments of DBDS-ghost mixtures at pH 6.5 and 4.0. The values of the constants for the time course at pH 4.0 were $\Delta F_{\text{total}} = 0.32 \pm 0.004$ and $k = 4.4 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$. (B) Plot of the ΔF_{total} values from the fits versus the measured pH after mixing for the dilution-jump experiment ($pK = 5.4 \pm 0.3$).

were equivalent to the same parameters observed for the slow phase in the presence of DIDS (Figure 2B; $k_s = 2 \pm 1 \times 10^{-3} \text{ s}^{-1}$ and $\Delta F_{\text{slow}} = 0.29 \pm 0.006$). The rate constant for DBDS release in the dilution-jump experiment at pH 4 was one order of magnitude slower than the rate constant for DBDS release from the primary site (fast phase in Figure 2B) measured at the same pH. When the dilution-jump experiment was performed at various pH values, we obtained the same type of titration curve (Figure 5B) as that observed for the slow phase in the DBDS/DIDS replacement reaction (Figure 3), with both titrations having the same apparent pK .

Additional control experiments

The experiments presented above indicate that DBDS binding to the second site is reversible. Control experiments suggest that formation of the second site itself is also reversible. We lowered the pH of a ghost suspension to 4.0, and incubated it for 20 min at room temperature (23 °C). We then returned the suspension to pH 7.0. There was no evidence for the existence of the second DBDS-binding site. In addition, it should be noted that DBDS does not partition into the lipid bilayer of the erythrocyte membrane [34], indicating that the interactions of DBDS that we observed most probably occurred with erythrocyte membrane protein (see below).

Kinetics of DBDS binding to DIDS-prelabelled unsealed and resealed ghosts

Mixing of DIDS-prelabelled unsealed ghosts with DBDS at pH 4 caused a large, gradual increase in DBDS fluorescence (Figure 6A). The kinetics were monophasic and exponential. Mixing resealed ghosts in the ratio 50:50 with 20 μM DBDS at pH 7 in the stopped-flow apparatus produced no signal change, whereas at pH 4, a slow, exponential increase in fluorescence was observed (Figure 6B). This result suggests that the second DBDS-binding site is located on the outer surface of the membrane.

The rate of DBDS binding to DIDS-prelabelled unsealed ghosts at pH 4.0 and 25 °C decreased as a function of increase in DBDS concentration (Figures 6C and 6D). $K_{1/2}$ (the apparent half-effect constant for the decrease in the rate constant with increasing DBDS concentration) was 0.6 μM . Such a kinetic behaviour is reported relatively rarely in the biochemical literature [35–39].

Figure 6 Kinetics of DBDS binding to DIDS-prelabelled unsealed and resealed ghosts

(A) Unsealed ghosts were prepared and prelabelled with DIDS and suspended up to a maximum value of 25 ml ($\sim 2 \mu\text{M}$ band 3) in chloride buffer at the final measured pH of 4. These suspensions were then mixed 50:50 in the stopped-flow apparatus with 20 μM DBDS or with the same buffer without DBDS. Kinetic measurements were taken at 25 °C. The time course for the reaction was fitted using a single exponential (Figure 2) with $\Delta F_{\text{total}} = -1.32 \pm 0.003$ and $k = 7.6 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$. (B) Mg^{2+} resealed ghosts were prepared and prelabelled with DIDS. The ghosts were then divided into two portions, and one portion was washed with magnesium chloride buffer at pH 7 and the other with the same buffer at pH 4. These ghosts were suspended up to a maximum value of 25 ml ($\sim 2 \mu\text{M}$ band 3) in their respective buffers, and mixed in the stopped-flow apparatus with 20 μM DBDS prepared in the same respective buffers. The time course at pH 4 was analysed using a single exponential function (Figure 2): $\Delta F_{\text{total}} = -0.29 \pm 0.003$, $k = 10 \pm 0.4 \times 10^{-3} \text{ s}^{-1}$. (C) Semi-logarithmic plot of the normalized time course of DBDS binding to DIDS-prelabelled unsealed ghosts in chloride buffer at pH 4 and 25 °C. The time courses were well-represented by a single exponential function with the following constants: $k = 14 \pm 0.2 \times 10^{-3} \text{ s}^{-1}$ (1.7 μM DBDS) and $k = 7.3 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$ (12.7 μM DBDS). (D) Plot of the observed rate constant versus DBDS concentration (measured spectrophotometrically) for DBDS binding to DIDS-prelabelled unsealed ghosts suspended in chloride buffer (pH 4). The rate constant decreased with increase in DBDS concentration and could be represented by the following empirical equation: $k_{\text{obs}} = k^{\text{H}} + k^{\text{L}} \{K_{1/2}/([D] + K_{1/2})\}$, where k^{H} is the limiting value of k_{obs} at high DBDS concentration, with k^{L} being the limiting rate constant as the concentration of DBDS approaches zero. The curve drawn through the data is from the fit using the latter equation with $k^{\text{H}} = 5.6 \pm 0.4 \times 10^{-3} \text{ s}^{-1}$, $k^{\text{L}} = 27.5 \pm 9.5 \times 10^{-3} \text{ s}^{-1}$ and $K_{1/2} = 0.6 \pm 0.3 \mu\text{M}$.

There is one classical example in the membrane transport literature where a similar behaviour was observed for the binding of a substrate analogue to the glucose transporter [40].

The second DBDS-binding site is located on isolated DIDS-prelabelled MDB3

DIDS-prelabelled MDB3 was prepared in C_{12}E_8 buffer containing 25 μM phosphatidylcholine [2,3]. Isolated MDB3 is particularly stable when prepared in the presence of phospholipids [3], and the addition of both SD and phospholipids to MDB3 increased stability significantly [4]. Finally, other work [5] has established that the latter type of MDB3 preparation is stable over a very broad pH range, down to pH values well into the range of the experiments described below.

A very slight signal change was observed on mixing DIDS-MDB3 with DBDS at pH 7, whereas mixing DBDS with protein-free detergent/PC mixtures in stopped-flow experiments at low pH did not produce a change in DBDS fluorescence. In

Figure 7 The pH dependence of DBDS binding to the second site on band 3 derived from erythrocytes modified with 300 μM BS³

Erythrocytes were treated with 300 μM BS³ and unsealed ghosts were isolated as described in the Experimental section. The reactions were studied in 150 mM NaCl, 20 mM Mes and 20 mM Bis-Tris at various pH values. The concentrations of DBDS and band 3 before the mix were 20 and 3.2 μM respectively. **(A)** Effect of pH on the time course of DBDS binding to BS³-modified band 3 at 25 °C, where the primary SD-binding site is blocked in all copies of band 3. There was no significant reaction of DBDS with band 3 at pH 6.5 ($\square$). Lowering the pH to 3.8 ($\bullet$) increased substantially the amplitude of the reaction, indicating increased binding of DBDS. **(B)** Semi-logarithmic plot of the normalized time course for the reaction of DBDS with BS³-modified band 3 at pH 3.8 and 25 °C ($\bullet$) or at 8 °C ($\diamond$). The reactions were biphasic and the data were fitted using the equation: $\Delta F/\Delta F_{\text{total}} = (\Delta F_{\text{fast}}/\Delta F_{\text{total}}) \exp(-k_1 t) + (\Delta F_{\text{slow}}/\Delta F_{\text{total}}) \exp(-k_s t)$. The following results were obtained at 25 °C: $\Delta F_{\text{fast}}/\Delta F_{\text{total}} = 0.742 \pm 0.007$; $k_1 = 0.035 \pm 0.001 \text{ s}^{-1}$; $\Delta F_{\text{slow}}/\Delta F_{\text{total}} = 0.26 \pm 0.008$; $k_s = 0.0054 \pm 0.0001 \text{ s}^{-1}$. The following results were obtained at 8 °C: $\Delta F_{\text{fast}}/\Delta F_{\text{total}} = 0.37 \pm 0.01$; $k_1 = 0.034 \pm 0.002 \text{ s}^{-1}$; $\Delta F_{\text{slow}}/\Delta F_{\text{total}} = 0.63 \pm 0.01$; $k_s = 0.0053 \pm 0.0001 \text{ s}^{-1}$. **(C)** The pH dependence of the amplitude of ΔF_{total} for the reaction of BS³-modified band 3 with DBDS at various pH values ($\text{pK} = 4.1 \pm 0.05$).

contrast, decreasing the pH induced a large kinetic relaxation comparable with that shown in Figure 6(A) (J. M. Salhany, K. S. Cordes and R. L. Sloan, unpublished work). The change in total reaction fluorescence gave an apparent pK of 4.37 ± 0.15 , which was somewhat lower than that observed with unsealed ghosts (Figures 3 and 5B). We conclude that the second DBDS-binding site seen on the outer surface of the DIDS-prelabelled red cell membranes (Figure 6B) is located on DIDS-prelabelled MDB3.

DBDS binding to the second binding site on BS³-modified band 3

The primary SD site was blocked by the covalent intrasubunit cross-linking by BS³ [24]. All primary sites were blocked (J. M. Salhany, K. S. Cordes and R. L. Sloan, unpublished work). In Figure 7(A, open squares), we see that there was no binding of DBDS to band 3 when BS³-modified unsealed ghosts were studied at pH 6.5, in contrast with control ghosts where a large signal change is observed at this pH (results not shown). Lowering the pH activated the binding of DBDS to unsealed ghosts derived from BS³-modified cells (Figure 7A, closed circles). However, the kinetics of DBDS binding to the second site were biphasic for BS³-modified ghosts at pH 3.8 and 25 °C (Figure 7B, closed circles). BS³-modified band 3 showed 74 % fast phase and 26 % slow phase. The rate constant for the slow phase was similar to the rate constant for the binding of 10 μM DBDS to DIDS-prelabelled band 3 (Figure 6D). In addition, the pK for DBDS binding to the second site on BS³-modified band 3 ($\text{pK} = 4.1 \pm 0.05$) was approx. 0.5–1 pH unit lower (Figure 7C) than the pK for DBDS binding to the second site when DBDS is bound to the primary SD site (Figures 3 and 5B). Finally, we measured the kinetics of DBDS binding to the second site in BS³-modified band 3 at 8 °C (Figure 7B, open diamonds). Lowering the temperature yielded a kinetic time course that was still biphasic, with rate constants that did not change significantly. However, the percentages of the two phases did change (fast phase = 37 % and slow phase = 63 %).

pH dependence of proton influx into control and BS³-modified resealed ghosts measured in the absence and presence of various SD molecules

The rate of intracellular acidification was determined by observing fluorescence quenching of FITC-dextran contained within car-

bonic anhydrase-free resealed ghosts [19]. Figure 8(A) shows typical time courses for the normalized change in fluorescence after changing the extracellular pH for control resealed ghosts from 7.1 to 6, 5 or 3.5. Monophasic exponential kinetics was observed at physiological sodium chloride concentrations, in agreement with the literature [19].

The observed pK for proton uptake by control resealed ghosts was 4.77 ± 0.17 (Figure 8B, closed circles). Figure 8(B) also shows that the addition of 20 μM DIDS (open squares) or 20 μM DBDS (open circles) to control ghosts inhibited the rate of proton transport virtually completely, in agreement with the literature [19]. Such an inhibition by SD molecules indicates that the assay we used [19] is specific for the APCT function of band 3 [10,11].

The results presented in Figure 8(C, closed circles) show that cross-linking of the primary SD-binding site by reacting intact cells with 300 μM BS³ did not inhibit proton transport. The presence of proton transport activity for BS³-modified cells contrasts significantly with the effect of binding of DBDS or DIDS to the primary SD site (Figure 8B). Indeed, when measured in the absence of SD molecules, the main difference between control (Figure 8B, closed circles) and BS³-modified ghosts (Figure 8C, closed circles) was that the apparent pK for proton transport by BS³-modified cells was approx. 1 pH unit lower than control, and the velocities were somewhat faster. Addition of 20 μM DBDS to BS³-modified cells inhibited proton transport (Figure 8C, open circles), despite the fact that the primary SD site was blocked on all copies of band 3 (Figure 7A, open squares).

DBDS inhibition of proton influx into control versus BS³-modified FITC-dextran resealed ghosts measured at constant pH

Figure 9(A) shows a plot of the fractional proton transport activity (k/k_0) as a function of DBDS concentration measured after changing the extracellular pH from 7.1 to 3.6, using control (open circles) or BS³-modified (closed circles) resealed ghosts (note the difference in the abscissal scales for the two types of cells). Data in Figure 9(B) are relative Dixon plots which illustrate graphically the wide difference in apparent K_1 values for the two types of samples. Control ghosts showed 94 % inhibition of transport (Figure 9A, open circles; $K_1 = 68.5 \pm 11.8 \text{ nM}$), which is consistent with DBDS binding to the primary

Figure 8 The pH dependence of proton transport into carbonic anhydrase-free resealed ghosts containing FITC-dextran derived from control and BS³-modified erythrocytes

Resealed ghosts, derived from control and BS³-modified erythrocytes containing FITC-dextran, were prepared, and proton influx was measured as described in the Experimental section. The time course of the reaction was analysed using the following equation: $(F_t - F_\infty) = (F_0 - F_\infty) \exp(-kt)$, where F_0 is the fluorescence at time $t = 0$, which was determined from the fits of the various time courses, F_∞ is the measured fluorescence at the end of the reaction, and F_t is the measured fluorescence at any time t . (A) Typical time courses for proton influx into control resealed ghosts containing FITC-dextran and measured at 8 °C; ●, pH 3.5, $k = 0.0558 \pm 0.0003 \text{ s}^{-1}$; ○, pH 5, $k = 0.0232 \pm 0.0001 \text{ s}^{-1}$; and ■, pH 6.0, $k = 0.005 \pm 0.0002 \text{ s}^{-1}$. (B) Plot of the observed rate constants for proton influx into control resealed ghosts versus the extracellular pH measured at 8 °C, with measurements made in the absence and presence of SD; ●, control ghosts; ○, 20 μM DBDS; and □, 20 μM DIDS. These data were taken from two independent series of experiments using blood drawn from two anonymous individuals, with the experiments performed on different days. The curve drawn through the control data was calculated by fitting the data using an equation similar to that given in the legend to Figure 3 ($pK = 4.77 \pm 0.17$). (C) Plot of the observed rate constants for proton influx versus the extracellular pH measured at 8 °C for BS³-modified resealed ghosts in the absence (●) and presence (○) of 20 μM DBDS. The time courses for BS³-modified resealed ghosts followed single exponential kinetics and were analysed similarly to those for control ghosts in (A). For both titration curves, $pK = 3.7 \pm 0.4$ (no DBDS) and $pK = 4.1 \pm 0.4$ (plus DBDS).

Figure 9 DBDS inhibition of proton transport into carbonic anhydrase-free, FITC-dextran resealed ghosts derived from control and BS³-modified erythrocytes measured at constant pH

The experimental procedures used here were similar to those in Figure 8, except that the experiments were performed at a constant extracellular pH of 3.6 and for various concentrations of DBDS. DBDS was added to the buffer before the addition of the ghosts. (A) Fractional activity plotted as k/k_0 versus [DBDS] for control (○) and BS³-modified (●) resealed ghosts (note the difference in the two abscissal scales given at the top and bottom of the Figure). These latter data were taken from two independent series of experiments using blood drawn from two anonymous individuals, with the experiments performed on different days. The inhibition of proton flux was analysed using the following decreasing hyperbolic function: $k/k_0 = (k_\infty/k_0) + \{(k_{\max}/k_0)(K_1/K_1 + [\text{DBDS}])\}$, where $k_{\max} = (k_0 - k_\infty)$. The fit of the data for control ghosts gave $K_1 = 68.5 \pm 11.8 \text{ nM}$, with $k_\infty/k_0 = 0.058 \pm 0.021$ and $k_{\max}/k_0 = 0.942 \pm 0.01$. The fit of the data for BS³-modified ghosts using the same equation gave $K_1 = 7.27 \pm 1.27 \text{ μM}$, with $k_\infty/k_0 = 0.32 \pm 0.03$ and $k_{\max}/k_0 = 0.68 \pm 0.02$. (B) Relative Dixon plots for control (○) and BS³-modified (●) resealed ghosts plotted using the following equation: $\{(k_0 - k_\infty)/(k - k_\infty)\} = 1 + (1/K_1)[\text{DBDS}]$. For control ghosts, we obtained $K_1 = 70.3 \pm 1.4 \text{ nM}$ using this equation to analyse the data, whereas, for BS³-modified resealed ghosts, we obtained $K_1 = 7.3 \pm 1.5 \text{ μM}$.

SD site on band 3 [25]. DBDS inhibited approx. 68% of the APCT function for BS³-modified cells (Figure 9A, closed circles; $K_1 = 7.27 \pm 1.27 \text{ μM}$). Thus there exists an approx. 100-fold difference in apparent inhibitory potency between the two classes

of DBDS-binding sites on band 3, as confirmed by the Relative Dixon plots shown in Figure 9(B).

THEORY

Mechanism of DBDS binding to the second site on band 3

Initial considerations

A classical mechanism that can describe the results in Figure 6(D) is:

where D represents DBDS, R_0 and T_0 represent alternate conformational states of the unliganded protein, and TD is the same T state with ligand DBDS bound. Several studies have addressed the expected behaviour of this mechanism [35–39]. When species TD is being observed, k_{obs} is given by the expression [37,38]:

$$k_{\text{obs}} = \{k_{-1}(k_{+0} + k_{-0}) + k_{+0}k_{+1}[D]\} / \{k_{+0} + k_{-0} + k_{+1}[D]\} \quad (2)$$

In this case, as $k_{+1}[D] \rightarrow \infty$, $k_{\text{obs}} \rightarrow k_{+0}$, and as $k_{+1}[D] \rightarrow 0$, then $k_{\text{obs}} \rightarrow k_{-1}$.

Model simulation results

Figure 10(A) illustrates the slow conformational transition, two-state exclusive binding mechanism, which is an extension of eqn (1), containing the isomerization step ($TD \leftrightarrow RD$). Addition of this step was necessary to accommodate the fact that we observe both a decrease in k_{obs} with increase in DBDS concentration (Figure 6D) and also an exceptionally slow rate of DBDS release in the dilution-jump experiment (Figure 5A). The mechanism in Figure 10(A) thus describes a classical, slow-transition, two-state exclusive binding mechanism [36].

Figure 10(B) shows a set of simulation results calculated at 38.45 μM DBDS using the model in Figure 10(A), with the values of the constants given in the Figure legend. The values

Figure 10 Slow-transition, two-state exclusive binding mechanism and results from theoretical simulation studies for DBDS binding to the second, proton-activated site on band 3

(A) Two-state exclusive binding mechanism. (B) Simulation of the time-dependent change in concentration of the various species in (A). The initial conditions were $[DBDS] = 38.45 \mu\text{M}$, $[band\ 3] = 1 \mu\text{M}$, with $[R_0] = 0.988 \mu\text{M}$, $[T_0] = 0.012 \mu\text{M}$ and $[TD] = [RD] = 0$. The constants used for the simulation were: $k_{+0} = 0.005 \text{ s}^{-1}$, $k_{-0} = 0.4 \text{ s}^{-1}$, $k_{+1} = 5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{-1} = 0.0275 \text{ s}^{-1}$, $k_{+2} = 0.0005 \text{ s}^{-1}$ and $k_{-2} = 0.0035 \text{ s}^{-1}$. (C) Simulations like those shown in (B) were performed at various concentrations of DBDS. The rate constant for the appearance of TD at each concentration of DBDS was determined using the equation $[TD] = [TD]_{\infty} \{1 - \exp(-kt)\}$. Semi-logarithmic plot of $\{1 - ([TD]/[TD]_{\infty})\}$ versus time for the simulated time courses calculated at 0.6 and 20 μM DBDS. The value of the rate constant at 0.6 μM DBDS was $18.2 \times 10^{-3} \text{ s}^{-1}$, whereas the value at 20 μM DBDS was $6.7 \times 10^{-3} \text{ s}^{-1}$. (D) Plot of the calculated rate constant (k_{calc} ; s^{-1} ; $\bullet$) determined at various DBDS concentrations. The following values were obtained from a fit of k_{calc} versus $[DBDS]$ using the equation given in the legend to Figure 6(D): $k^{\text{H}} = 5.3 \pm 0.4 \times 10^{-3} \text{ s}^{-1}$, $k^{\text{L}} = 14.7 \pm 0.9 \times 10^{-3} \text{ s}^{-1}$ and $K_{1/2} = 1.9 \pm 0.8 \mu\text{M}$. The solid curve was drawn on the basis of the fit using the calculated rate constants. The broken curve is a reproduction of the solid curve in Figure 6(D) using the experimentally determined rate constants.

of k_{+2} and k_{-2} were obtained by partitioning the value for the relaxation rate constant from the dilution-jump experiment of Figure 5(A) ($k = 4.4 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$), so that the value of k_{-2} was approximately equal to the observed rate constant for the release of DBDS from the second site on band 3. Figures 10(C) and 10(D) show results from the simulation calculated as a function of increase in DBDS. The broken curve in Figure 10(D) is a reproduction of the solid curve in Figure 6(D).

The overall dissociation constant for the two-state exclusive binding mechanism is:

$$K_d = K_1 \{ (1 + K_0) / [K_0(1 + K_2)] \} \quad (3)$$

The values of these constants were as follows: $K_1 = (k_{-1}/k_{+1}) = 55 \text{ nM}$, $K_0 = (k_{+0}/k_{-0}) = 0.0125$ and $K_2 = (k_{+2}/k_{-2}) = 0.1428$. We calculated that $K_d = 3.9 \mu\text{M}$. The results of Figure 5(A) suggest that the K_d value for binding of DBDS to the second site at pH 4.0 must be $\geq 3.4 \mu\text{M}$, to account for the observation that DBDS is released from the membrane with 50:50 dilution under the stated conditions of the experiment.

Mechanism of inhibition of proton transport by DBDS binding to the second site on BS³-modified band 3

Initial considerations

The two-state exclusive binding mechanism was used for simulation of the inhibition of proton transport by binding of DBDS to the second site on band 3 (Figure 11A). Our goal was

to simulate the pattern of DBDS inhibition of proton transport observed in Figure 9(A, closed circles).

DBDS binding to the second site is initiated concomitant to the decrease in extracellular pH (Figures 8 and 9). However, the relatively slow rate of DBDS binding (Figure 6) makes a rapid equilibrium assumption invalid and requires one to simulate the inhibition process to interpret the data. The model in Figure 11(A) does assume that there is both rapid and saturating proton binding when the extracellular pH is changed from 7.1 to 3.6. It is assumed, furthermore, that this pH jump and the associated proton binding reaction converts the AE functional state of band 3 into the APCT state rapidly. It was necessary to assume that R_0 and T_0 states of unprotonated BS³-modified band 3 (as shown in the model in Figure 11A) pre-exist in the AE functional state. These species are proposed to be protonated rapidly at the active site, generating the APCT functional state without changing the initial distribution of the two states. Failure to include the pre-existing T_0 state in the simulation leads to failure in simulating the inhibition of proton transport by DBDS in the type of experiments illustrated in Figures 8 and 9.

Selection of the values for the first two rate constants in the DBDS binding manifold (k_{+0} and k_{-0}) and the concentrations of the R_0 and T_0 states present initially were determined from the kinetics of binding of DBDS to the second site on BS³-modified band 3 measured at 8 °C (Figure 7B, open diamonds). DBDS binding to BS³-modified band 3 showed a biphasic kinetic time course (Figure 7B). Given the existence of two prebinding conformational states (R_0 and T_0) in the established mechanism

Figure 11 Simulation of DBDS inhibition of proton transport into BS³-modified resealed ghosts at constant pH using the slow-transition, two-state exclusive binding mechanism

(A) The slow-transition, two-state exclusive binding model for inhibition of proton transport by DBDS binding to the second site on band 3. (B) Simulation of the time-dependent change in the concentrations of species for proton transport into BS³-modified cells measured in the absence of DBDS (i.e. part of the mechanism shown by a dashed box in A). The initial conditions and values for the various rate constants used were: [band 3] = 0.55 μM, with $\{[H^+]_{in}\}_0 = 79$ nM, $[R_0] = 0.35$ μM and $[T_0] = 0.2$ μM, $[TD] = [RD] = 0$, $k_{T1} = 0.056$ s⁻¹, $k_{+0} = 0.005$ s⁻¹, $k_{-0} = 0.0087$ s⁻¹ and $k_{T2} = 0.16$ s⁻¹. We used these constants to calculate the increase in intracellular proton concentration $\Delta[H^+]_{in}$ as a function of time. The apparent rate constant was calculated using the equation $\{[H^+]_{in}\}_t = \{[H^+]_{in}\}_0 + \{\Delta[H^+]_{total}\}[1 - \exp(-kt)]$, where $\Delta[H^+]_{total} = (\{[H^+]_{in}\}_\infty - \{[H^+]_{in}\}_0)$. The value of k calculated at [DBDS] = 0 was 0.0842 ± 0.0006 s⁻¹. (C) Simulation of the time-dependent change in the concentration of species for proton transport into BS³-modified cells measured in the presence of 30 μM DBDS. This simulation is based on the entire mechanism shown in (A). The values for the initial conditions and constants were the same as those in (B) with the additional constants: $k_{+1} = 6 \times 10^4$ M⁻¹ s⁻¹, $k_{-1} = 0.01$ s⁻¹, $k_{T3} = 0.019$ s⁻¹, $k_{+2} = 0.0005$ s⁻¹, $k_{-2} = 0.0035$ s⁻¹ and $k_{T4} = 0.005$ s⁻¹. The rate constant for the appearance of intracellular protons was calculated to be 0.0438 ± 0.0006 s⁻¹ using the equation described above for (B). (D) Time courses for proton transport simulated at 0 and 20 μM DBDS concentration. These time courses followed single exponential kinetics on the basis of an analysis of residuals from a fit to the equation given in (B) where deviations were < 5% of the total signal change. The values of the simulated rate constants were, for 0 μM DBDS, $k = 0.0842 \pm 0.0006$ s⁻¹, and for 20 μM DBDS, $k = 0.0443 \pm 0.0004$ s⁻¹. (E) Plot of k/k_0 versus DBDS concentration for the simulated rate constants for proton transport into BS³-modified cells. The solid curve was drawn on the basis of a fit of the ratio of the calculated constants (●) using the equation given in the legend to Figure 9(A). The value of K_1 from this fit was 2.7 ± 0.1 μM, with $k_\infty/k_0 = 0.47 \pm 0.01$ and $k_{max}/k_0 = 0.53 \pm 0.01$. The broken curve was reproduced from the fitted curve for the experimental data for BS³-modified band 3 (Figure 9A, ●).

(Figures 10A and 10D), we interpreted the observation of a 'burst phase' by proposing that the equilibrium between states ($R_0 \leftrightarrow T_0$) has shifted towards the T_0 state for membrane-bound, BS³-modified band 3. As established above, the T_0 state binds DBDS exclusively, whereas the R_0 state must be converted slowly into the T_0 state for ligand binding to occur. This implies that the kinetics will show a 'burst phase' when the fraction of T_0 state has increased initially. We assign the fast phase to the second-order binding of DBDS to the T_0 state and the slow phase to the $R_0 \rightarrow T_0$ transition. Thus, for simulating DBDS inhibition of

proton transport, we assigned a value of 0.005 s⁻¹ to k_{+0} (Figure 11). The initial concentrations of the R_0 and T_0 states were determined using the fraction of the fast and slow phases seen for DBDS binding to BS³-modified band 3 at 8 °C (Figure 7B, open diamonds) as a measure of the fraction of T_0 (fast phase) and R_0 (slow phase) states present initially.

It was necessary to impose the rather stringent requirement that any mechanism selected and all of the constants used for the simulation must yield monophasic, single exponential kinetic time courses for the intracellular acidification process. This

requirement followed from our experimental observations with control (Figure 8A) and BS³-modified (results not shown) cells. We found that a proton transport step had to be assigned to all of the molecular species in the ligand-binding manifold to generate exponential time courses and simulate the experimental data (Figure 11).

Model simulation results

Figure 11(B) presents results from the simulation without DBDS binding steps. Figure 11(C) shows the time courses for all of the species in the reaction sequence when DBDS binding steps were included ($[DBDS] = 30 \mu\text{M}$). Figures 11(D) and 11(E) show simulation results for the effect of increasing the DBDS concentration on the kinetics of proton transport into BS³-modified cells. Single exponential kinetic time courses were generated using the mechanistic constants given in the legend to that Figure. Increasing the concentration of DBDS caused the transport rate to decrease. Figure 11(E, closed circles) shows a plot of the values of (k/k_0) versus the concentration of DBDS using the calculated rate constants. The solid line in Figure 11(E) was calculated using these simulated (k/k_0) values and the equation given in the legend to Figure 9(A). This calculation gave a value of $K_1 = 2.7 \pm 0.1 \mu\text{M}$, which differed by only a factor of approx. 2 from the value observed experimentally in Figure 9(A, closed circles) ($K_1 = 7.27 \pm 1.27 \mu\text{M}$). The broken curve in Figure 11(E) is a reproduction of the solid curve from Figure 9(A, closed circles) that was determined using the experimental data.

We have used the mechanistic constants from the above simulation (Figure 11) to calculate the overall equilibrium DBDS dissociation constant (K_d) for BS³-modified band 3. The overall dissociation constant was defined by eqn (3) above. The values used for this calculation were as follows: $K_1 = (k_{-1}/k_{+1}) = 0.17 \mu\text{M}$, $K_0 = (k_{+0}/k_{-0}) = 0.5747$ and $K_2 = (k_{+2}/k_{-2}) = 0.1429$. Using these values, we obtained a calculated value for K_d of $0.41 \mu\text{M}$ at 8°C. This value is approx. 7-fold smaller than the calculated value for K_1 ($2.7 \pm 0.1 \mu\text{M}$; Figure 11E, closed circles) and approx. 18-fold smaller than the observed value of K_1 ($7.27 \pm 1.27 \mu\text{M}$; Figure 9A, closed circles) measured at the same temperature. We suggest that these differences between the calculated K_d value and the calculated and observed K_1 values are a direct result of the failure of the rapid equilibrium assumption to be applicable in the proton transport inhibition experiments involving binding of DBDS to the second binding site on band 3. When the rapid equilibrium assumption fails to be applicable, $K_d \neq K_1$ and one must simulate the mechanism to determine the actual value of K_d .

The K_d value calculated from the transport studies at 8°C is smaller for BS³-modified band 3 ($0.41 \mu\text{M}$) compared with the value calculated for DIDS-modified band 3 measured in direct kinetic binding experiments at 25°C ($3.9 \mu\text{M}$). Lowering the temperature over this range did not affect the 'on' kinetic constants significantly (Figure 7B). Such a difference in K_d values is due to the shift in the initial equilibrium from R_0 to T_0 observed for BS³-modified band 3 [compare the slow monophasic binding kinetics for DIDS-band 3 in Figure 6C, which implies that $R_0 \gg T_0$, with the biphasic kinetics for BS³-modified band 3 in Figure 7B, where significantly more T_0 state (fast phase) was present initially].

DISCUSSION

Interpretation of the results presented in this study requires the understanding that most experiments were performed with the primary SD-binding site occupied either by saturating concentrations of reversibly bound DBDS or by covalently bound

DIDS. Carrier-type transport mechanisms, such as the ping-pong [41] or the ordered sequential [42] mechanisms, involve transport site(s) alternation between inward-facing and outward-facing forms. Binding of SD molecules to the primary allosteric SD inhibitory site is known to 'recruit' the transport site to the outward-facing form [6]. Thus we can assume that the properties we have observed are those belonging to the carrier 'locked' in the outward-facing form. Furthermore, we suggest that lowering the pH with SD molecules bound to the primary site leads to formation of the unloaded, outward-facing form of the carrier. The results in Figure 3 show that chloride does not compete with DBDS for binding at the second site. We suggest that this apparent lack of competition is related directly to the fact that chloride does not bind to the transport site at low pH. The loss of chloride-induced acceleration of DBDS release on lowering the pH (Figure 4, open and closed circles) occurred independently of whether chloride was premixed with ghosts or mixed simultaneously on initiation of the reaction (Figure 4, open versus closed circles). There was no effect of lowering the pH in the absence of chloride (Figure 4, open squares), and the rate constants were indistinguishable at pH 4 irrespective of chloride concentration. These findings support the hypothesis that the decrease in rate is related to loss of chloride binding to the transport site. Finally, there is clear evidence in the literature that the chloride-induced acceleration in SD release from band 3 is related to binding to the transport site [28], and ³⁵Cl-NMR line broadening studies show decrease in chloride binding over the same pH range [17,19].

The discovery that the rate constant for DBDS binding to the second site decreased with increase in DBDS concentration (Figure 6D) is the key to understanding how this second site may be related to the band 3 transport site. The kinetic behaviour in Figure 6(D) is diagnostic for a mechanism where a slow, initial conformational isomerization step occurs before the faster ligand-binding step (eqn 1). However, if the carrier is 'locked' in the outward-facing, unloaded form at low pH, with an SD molecule bound to the primary site, one must ask exactly what type of initial conformational change is being probed in the kinetic studies of Figure 6. We suggest that the $R_0 \leftrightarrow T_0$ conformational change (Figure 10A) is a slow, localized 'gating-like' conformational change within the outward-facing, unloaded form of the carrier, which becomes observable at low pH. In this context, we may assign the R_0 species to the 'closed form' of the access channel, with the T_0 species representing an 'open form'. Such a 'gating-like' conformational change may be part of a 'tunnelling' mechanism [43–45] for the conductance of anions through the outward-facing, unloaded form of the carrier. Tunnelling-like conformational changes do not require re-orientation of the carrier [43], which is consistent with our experimental situation. Indeed, Jennings [43] has stated that such a tunnelling phenomenon is evidence that the transport pathway has some characteristics of a channel.

DBDS binding within the access channel of band 3 would need to occur, despite having an SD molecule bound to the primary SD-binding site. It is conceivable that a localized 'gating-like' conformational change could occur within the outward-facing, unloaded form of the protein, since the entrance to the channel and the primary SD site are most probably well separated, as suggested by ³⁵Cl-NMR studies [9]. Recent studies using single cysteine mutants in an otherwise cysteineless background [46] seem to support this view. In addition, fluorescence energy transfer measurements support the view that the transport and SD sites are separated by a considerable distance [47].

The results showing that DBDS binding to the second binding site inhibited proton transport (Figure 8C and Figures 9A and 9B,

closed circles), taken together with the gating-like characteristics of the DBDS binding kinetics, offer support for the hypothesis that the second site may be located within a gated access channel leading to the transport site. Further support comes from our ability to simulate the inhibition pattern in Figure 9(A, closed circles) using the slow-transition, two-state exclusive binding mechanism (Figure 11). Finally, if we accept the view that the primary SD-binding site is not coincident with the transport site [8,27,28,46,47] nor does it block access to the transport site [9], the discovery of a second DBDS binding site that inhibits APCT may prove to be an important finding in our attempt to map the structure of band 3 and relate such structural insights to function.

Our original motivation for initiating the present study was to see if neutralization of Glu-681 by lowering the pH could reproduce the effects seen with modification by WRKB treatment [21]. We recall that neutralization of the negative charge on Glu-681 by treatment with the WRKB enhanced the fluorescence quantum yield of DBDS bound to the primary site on one band 3 subunit when chloride was added, and chloride apparently lowered the affinity of DBDS at that subunit by greatly accelerating the rate of DBDS release [21]. In contrast, we observed no change in the fluorescence quantum yield on lowering the pH with DBDS bound to the primary SD site (Figures 3C and 3D, closed circles). The only change observed was a marked decrease in the rate of DBDS release as discussed above (Figure 4). This implies that lowering the pH most probably increased DBDS affinity for the primary site by inhibiting chloride binding to the transport site and decreasing the allosteric site-site interactions between the primary SD site and the transport site [8]. This interpretation is supported by comparing the K_1 value for control band 3 (primary site) at low pH in 150 mM chloride buffer ($K_1 = 68.5 \pm 11.8$ nM; Figure 9A, open circles) with equilibrium binding K_d values for binding of DBDS to the primary site measured in the absence and presence of chloride [180 nM in 23 mM sodium citrate 5P(7.4) versus 770 nM in 150 mM sodium chloride 5P(7.4) at 25 °C] [25]. Since DBDS binds very rapidly to the primary site [25], we assume that the rapid equilibrium assumption is valid for DBDS inhibition of proton transport when using control ghosts and therefore that K_1 and K_d values are approximately equal. This analysis suggests that lowering the pH in chloride buffer causes the DBDS affinity to increase by approx. 10-fold, resulting in a K_d value comparable with that measured in citrate buffer at physiological pH.

At present, we are not able to explain why lowering the pH does not produce the same fluorescence spectral change in DBDS bound to the primary site of native band 3 as was seen for WRKB-modified band 3 at neutral pH in the presence of chloride [21]. It may be that the neutralization concept is oversimplified. It is worth noting that WRKB conversion of the carboxyl side chain of Glu-681 into an alcohol [14] decreases the size of that R-group. This alone could alter the steric environment at the conformationally active transport site of band 3 and can have very significant and even widespread consequences over the entire protein structure.

Knauf and co-workers [48] studied the inhibition of anion transport by DNDS (4,4'-dinitro-2,2'-stilbenedisulphonate) between pH 7.2 and pH 11.2. Their analysis suggested the presence of both competitive and non-competitive binding sites for this SD molecule. Their analysis assumed that DNDS binding to the competitive and non-competitive binding sites was mutually exclusive. Mutual exclusivity in binding would be consistent with our observations where no evidence for binding to the second site was found at neutral pH when DBDS or DIDS was bound to the primary site. Our results show that DBDS binds to both the sites simultaneously at low pH.

In summary, we have identified and characterized a second DBDS-binding site on band 3 that has binding kinetics charac-

teristic of ligand entrance into a gated access channel leading to a transport site. Transport studies support this view by showing that DBDS binding to the second site inhibits the APCT function of band 3.

We thank Dr L. M. Schopfer (Eppler Cancer Institute, University of Nebraska Medical Center) for discussions and a critical reading of this paper and Professors P. A. Knauf (Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY, U.S.A.), H. Passow (Institut für Biochemie und Biophysik, Friedrich Schiller Universität Jena, Jena, Germany) and T. L. Steck (Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, U.S.A.) for discussions. This work was supported by a Merit Review Grant from the Medical Research Service of the Veterans Administration.

REFERENCES

- Casey, J. R. and Reithmeier, R. A. F. (1991) Analysis of the oligomeric state of band 3, the anion transport protein of the erythrocyte membrane, by size exclusion chromatography: oligomeric stability and origin of heterogeneity. *J. Biol. Chem.* **266**, 15726–15737
- Salhany, J. M., Cordes, K. A. and Sloan, R. L. (1997) Gel filtration chromatographic studies of the isolated membrane domain of band 3. *Mol. Membr. Biol.* **14**, 71–79
- Schopfer, L. M. and Salhany, J. M. (1992) Factors determining the conformation and quaternary structure of isolated human erythrocyte band 3 in detergent solution. *Biochemistry* **31**, 12610–12617
- Vince, J. W., Sarabia, V. E. and Reithmeier, R. A. F. (1997) Self-association of band 3, the human erythrocyte anion exchanger, in detergent solution. *Biochim. Biophys. Acta* **1326**, 295–306
- Lemieux, M. J., Reithmeier, R. A. F. and Wang, D. N. (2002) Importance of detergent and phospholipid in the crystallization of the human erythrocyte anion-exchanger membrane domain. *J. Struct. Biol.* **137**, 322–332
- Knauf, P. A. and Pal, P. (2003) Band 3 mediated transport. In *Red Cell Membrane Transport in Health and Disease* (Bernhardt, I. and Ellory, J. C., eds.), pp. 253–301, Springer-Verlag, Berlin
- Cabantchik, Z. I. and Greger, R. (1992) Chemical probes for anion transporters of mammalian cell membranes. *Am. J. Physiol.* **262**, C803–C827
- Salhany, J. M. (1996) Allosteric effects in stilbenedisulfonate binding to band 3 protein (AE1). *Cell. Mol. Biol.* **77**, 543–549
- Salhany, J. M. (2001) Mechanistic basis for site-site interactions in inhibitor and substrate binding to band 3 (AE1): evidence distinguishing allosteric from electrostatic effects. *Blood Cells Mol. Dis.* **27**, 901–912
- Jennings, M. L. (1976) Proton fluxes associated with erythrocyte membrane anion exchange. *J. Membr. Biol.* **28**, 187–205
- Jennings, M. L. (1978) Characteristics of CO₂-independent pH equilibration in human red blood cells. *J. Membr. Biol.* **40**, 365–391
- Milanick, M. A. and Gunn, R. B. (1982) Proton-sulfate cotransport: mechanism of H⁺ and sulfate addition to the chloride transporter of human red cells. *J. Gen. Physiol.* **79**, 87–113
- Milanick, M. A. and Gunn, R. B. (1986) Proton inhibition of chloride exchange: asynchrony of band 3 proton and anion transport sites. *Am. J. Physiol.* **250**, C955–C969
- Jennings, M. L. and Al-Raiyel, S. (1988) Modification of a carboxyl group that appears to cross the permeability barrier in the red blood cell anion transporter. *J. Gen. Physiol.* **92**, 161–178
- Jennings, M. L. and Smith, J. S. (1992) Anion-proton cotransport through the human red blood cell band 3 protein. Role for glutamate 681. *J. Biol. Chem.* **267**, 13964–13971
- Jennings, M. L. (1995) Rapid electrogenic sulfate-chloride exchange mediated by chemically modified band 3 in human erythrocytes. *J. Gen. Physiol.* **105**, 21–47
- Müller-Berger, S., Karbach, D., Kang, D., Aranibar, N., Wood, P. G., Rütterjans, H. and Passow, H. (1995) Roles of histidine 752 and glutamate 699 in the pH dependence of mouse band 3 protein-mediated anion transport. *Biochemistry* **34**, 9325–9332
- Müller-Berger, S., Karbach, D., König, J., Lepke, S., Wood, P. G., Appelhans, H. and Passow, H. (1995) Inhibition of mouse erythroid band 3-mediated chloride transport by site-directed mutagenesis of histidine residues and its reversal by second site mutation of lys 558, the locus of covalent H₂DIDS binding. *Biochemistry* **34**, 9315–9324
- Lepke, S., Heberle, J. and Passow, H. (2003) The band 3 protein: anion exchanger and anion-proton cotransporter. In *Red Cell Membrane Transport in Health and Disease* (Bernhardt, I. and Ellory, J. C., eds.), pp. 221–252, Springer-Verlag, Berlin
- Milanick, M. A. and Gunn, R. B. (1984) Proton-sulfate cotransport: external proton activation of sulfate influx into human red blood cells. *Am. J. Physiol.* **247**, C247–C259
- Salhany, J. M., Sloan, R. L. and Cordes, K. A. (2003) The carboxyl side chain of glutamate 681 interacts with a chloride binding modifier site that allosterically modulates the dimeric conformational state of band 3 (AE1). Implications for the mechanism of anion/proton cotransport. *Biochemistry* **42**, 1589–1602

- 22 Salhany, J. M. (2004) Slow transitions between two conformational states of band 3 (AE1) modulate divalent anion transport and DBDS binding to a second site on band 3 which is activated by lowering the pH (pK ~ 5.0). *Blood Cells Mol. Dis.* **32**, 372–378
- 23 Schopfer, L. M. and Salhany, J. M. (1995) Characterization of the stilbenedisulphonate binding site on band 3. *Biochemistry* **34**, 8320–8329
- 24 Salhany, J. M., Sloan, R. L. and Cordes, K. A. (1991) Evidence for the development of an intermonomeric asymmetry in the covalent binding of 4,4'-diisothiocyanatostilbene-2,2'-disulphonate to human erythrocyte band 3. *Biochemistry* **30**, 4097–4104
- 25 Salhany, J. M., Sloan, R. L., Cordes, K. A. and Schopfer, L. M. (1995) Quantitative analysis of the kinetics of stilbenedisulphonate binding to band 3. *Int. J. Biochem. Cell Biol.* **27**, 953–964
- 26 Salhany, J. M., Cordes, K. A. and Schopfer, L. M. (1993) Kinetics of conformational changes associated with inhibitor binding to the purified band 3 transporter. Direct observation of allosteric subunit interactions. *Biochemistry* **32**, 7413–7420
- 27 Salhany, J. M., Sloan, R. L., Cordes, K. A. and Schopfer, L. M. (1994) Kinetic evidence for ternary complex formation and allosteric interactions in chloride and stilbenedisulphonate binding to band 3. *Biochemistry* **33**, 11909–11916
- 28 Salhany, J. M. (1999) Anion binding characteristics of the band 3/4,4'-dibenzamidostilbene-2,2'-disulphonate binary complex: evidence for both steric and allosteric interactions. *Biochem. Cell Biol.* **77**, 543–549
- 29 Salhany, J. M. and Schopfer, L. M. (2001) Kinetic mechanism of DIDS binding to band 3 (AE1) in human erythrocyte membranes. *Blood Cells Mol. Dis.* **27**, 844–849
- 30 Staros, J. V. (1982) N-hydroxysulfosuccinimide active esters: bis(N-hydroxysulfosuccinimide) esters of two dicarboxylic acids are hydrophilic, membrane-impermeant, protein cross-linkers. *Biochemistry* **21**, 3950–3955
- 31 Staros, J. V. and Kakkad, B. P. (1983) Crosslinking and chymotryptic digestion of the extracytoplasmic domain of the anion exchange channel in intact human erythrocytes. *J. Membr. Biol.* **74**, 247–254
- 32 Jennings, M. L. and Nicknisch, J. S. (1985) Localization of a site of intermolecular crosslinking in human erythrocyte band 3 protein. *J. Biol. Chem.* **260**, 5472–5479
- 33 Jennings, M. L., Monaghan, R., Douglas, S. M. and Nicknisch, J. S. (1985) Functions of extracellular lysine residues in the human erythrocyte anion transport protein. *J. Gen. Physiol.* **86**, 653–669
- 34 Pool, R. C. and Halestrap, A. P. (1991) Reversible and irreversible inhibition by stilbenedisulphonates of lactate transport into rat erythrocytes. Identification of some new high-affinity inhibitors. *Biochem. J.* **275**, 307–312
- 35 Kirschner, K., Eigen, M., Bittman, R. and Voight, B. (1966) Binding of nicotinamide-adenine dinucleotide to yeast D-glyceraldehyde-3-phosphate dehydrogenase: temperature-jump relaxation studies on the mechanism of an allosteric enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1661–1667
- 36 Halford, S. E. (1972) *Escherichia coli* alkaline phosphatase. Relaxation spectra of ligand binding. *Biochem. J.* **126**, 727–738
- 37 Rhee, S. G. and Chock, P. B. (1976) Mechanistic studies of glutamine synthetase from *Escherichia coli*: kinetics of ADP and orthophosphate binding to the unadenylated enzyme. *Biochemistry* **15**, 1755–1760
- 38 Garland, D. L. (1978) Kinetics and mechanism of colchicine binding to tubulin: evidence for ligand-induced conformational change. *Biochemistry* **17**, 4266–4272
- 39 Schopfer, L. M., Massey, V., Ghisla, S. and Thorpe, C. (1988) Oxidation-reduction of general acyl-CoA dehydrogenase by the butyryl-CoA/crotonyl-CoA couple. A new investigation of the rapid reaction kinetics. *Biochemistry* **27**, 6599–6611
- 40 Appleman, J. R. and Lienhard, G. E. (1985) Rapid kinetics of glucose transporter from human erythrocytes. Detection and measurement of a half-turnover of the purified transporter. *J. Biol. Chem.* **260**, 4575–4578
- 41 Gunn, R. B. and Fröhlich, O. (1979) Asymmetry in the mechanism for anion exchange in human red blood cell membranes. Evidence for reciprocating sites that react with one transported anion at a time. *J. Gen. Physiol.* **74**, 351–374
- 42 Salhany, J. M. and Rauenbuehler, P. B. (1983) Kinetics and mechanism of erythrocyte anion exchange. *J. Biol. Chem.* **258**, 245–249
- 43 Jennings, M. L. (1992) Anion transport proteins. In *The Kidney: Physiology and Pathophysiology* (Seldin, D. W. and Giebisch, G., eds.), pp. 113–145, Raven Press, New York
- 44 Fröhlich, O. (1988) The 'tunneling' mode of biological carrier-mediated transport. *J. Membr. Biol.* **101**, 189–198
- 45 Knauf, P. A., Law, F.-Y. and Marchant, P. (1983) Relationship of net chloride flow across the human erythrocyte membrane to anion exchange. *J. Gen. Physiol.* **81**, 95–126
- 46 Zhu, Q. and Casey, J. R. (2004) The substrate anion selectivity filter in the human erythrocyte Cl⁻/HCO₃⁻ exchanger protein, AE1. *J. Biol. Chem.* **279**, 23565–23573
- 47 Knauf, P. A., Law, F.-Y., Leung, T.-W. V. and Atherton, S. J. (2004) Relocation of the disulfonic stilbene sites of AE1 (Band 3) on the basis of fluorescence energy transfer measurements. *Biochemistry* **43**, 11917–11931
- 48 Liu, S. Q., Ries, E. and Knauf, P. A. (1996) Effects of external pH on binding of sulfate, 4,4'-dinitro-stilbene-2,2'-disulphonate (DNDS), and chloride to the band 3 anion exchange protein. *J. Gen. Physiol.* **107**, 293–306

Received 19 July 2004/2 December 2004; accepted 13 January 2005

Published as BJ Immediate Publication 13 January 2005, DOI 10.1042/BJ20041211