J. Membrane Biol. 112, 25–37 (1989)

The Journal of
Membrane Biology

Interaction Among Anion, Cation and Glucose Transport Proteins in the Human Red Cell

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Summary. The time course of binding of the fluorescent stilbene anion exchange inhibitor, DBDS (4,4′-dibenzamido-2,2′-stilbene disulfonate), to band 3 can be measured by the stopped-flow method. We have previously used the reaction time constant, τ_{DBDS}, to obtain the kinetic constants for binding and, thus, to report on the conformational state of the band 3 binding site. To validate the method, we have now shown that the ID_{50} (0.3 ± 0.1 μM) for H_2-DIDS (4,4′-diisothiocyano-2,2′-dihydrostilbene disulfonate) inhibition of τ_{DBDS} is virtually the same as the ID_{50} (0.47 ± 0.04 μM) for H_2-DIDS inhibition of red cell Cl^- flux, thus relating τ_{DBDS} directly to band 3 anion exchange. The specific glucose transport inhibitor, cytochalasin B, causes significant changes in τ_{DBDS}, which can be reversed with intracellular, but not extracellular, D-glucose. ID_{50} for cytochalasin B modulation of τ_{DBDS} is 0.1 ± 0.2 μM in good agreement with $K_D = 0.06 \pm 0.005$ μM for cytochalasin B binding to the glucose transport protein. These experiments suggest that the glucose transport protein is either adjacent to band 3, or linked to it through a mechanism, which can transmit conformational information. Ouabain (0.1 μM), the specific inhibitor of red cell Na^+,K^+-ATPase, increases red cell Cl^- exchange flux in red cells by a factor of about two. This interaction indicates that the Na^+,K^+-ATPase, like the glucose transport protein, is either in contact with, or closely linked to, band 3. These results would be consistent with a transport protein *complex*, centered on band 3, and responsible for the entire transport process, not only the provision of metabolic energy, but also the actual carriage of the cations and anions themselves.

Key Words red cell · Na^+,K^+-ATPase · band 3 · anion exchange protein · glucose transport protein · stilbene anion exchange inhibitors · DBDS

Introduction

It is well known that the red cell anion exchange protein, band 3, is linked to the red cell spectrin/actin cytoskeleton by ankyrin (*see* Bennett, 1985). The cytosolic pole of band 3 has specific binding sites for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Kant & Steck, 1973) and aldolase (Yu & Steck, 1975), and through them to most of the enzymes in the glycolytic cycle (Fossel & Solomon, 1978). Using a partially purified preparation of band 3, Fossel and Solomon (1981) provided evidence that conformational information could be transmitted from ouabain, the specific extracellular Na^+,K^+-ATPase inhibitor, to intracellular G3P (glyceraldehyde 3-phosphate) along a chain comprising the Na^+,K^+-ATPase, band 3 and G3PDH. A similar cytoskeletal/transport protein link has been reported by Nelson and Veshnock (1987) who found binding between the Na^+,K^+-ATPase and ankyrin in canine kidney outer medulla.

The disulfonic stilbene inhibitors of anion exchange in the human red cell bind to the anion exchange protein, band 3, with high specificity (*see* Passow, 1986). Since the fluorescence of DBDS (4,4′-dibenzamido-2,2′-stilbene disulfonate), a member of this class of inhibitors, increases by more than a factor of two when bound to its site, we have been able to use spectrofluorimetric methods to characterize the DBDS/band 3 binding (Dix et al., 1979). The initial steps of the DBDS/band 3 binding reaction consist of a bimolecular association followed by a conformational change whose velocity we have measured by the stopped-flow method. H_2-DIDS (4,4′-diisothiocyano-2,2′-dihydrostilbene disulfonate) binds more tightly than DBDS, whose fluorescence enhancement it suppresses. We have shown that the ID_{50} for this suppression is virtually equal to the ID_{50} for H_2-DIDS inhibition of Cl^- exchange, also measured spectrofluorimetrically. These results strongly suggest that there is a 1 : 1 relationship between inhibition of DBDS/band 3 kinetics and inhibition of anion exchange.

Experiments with cytochalasin B, a specific inhibitor of glucose transport, suggest that the glucose transport protein is contiguous to band 3 or close enough that conformational information can be transmitted between the two transport proteins. Ouabain, the specific inhibitor of the Na^+,K^+-

ATPase, doubles Cl^- flux, providing further evidence of a linkage between the anion and cation transport proteins.

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 and SPQ (6-methoxy-N-(3-sulfopropyl)quinolinium) were obtained from Molecular Probes (Junction City, OR) and SITS (4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene), ouabain, digitoxigenin and HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) and *bis*-Tris-propane (1,3-*bis*-[tris(hydroxymethyl)-methylamino]propane) were obtained from Sigma (St. Louis, MO). All other chemicals, obtained from Fisher Scientific (Fairlawn, NJ), were reagent grade. Outdated bank blood was kindly supplied by the Children's Hospital (Boston, MA).

Methods

Bank blood, outdated by no more than one week, after aspiration of plasma and buffy coat, was washed three times with PBS buffer of the following composition, in mM: NaCl, 150; Na_2HPO_4, 5; pH 7.4, 300 ± 5 mOsm. Experiments with red cells always used outdated bank blood, except where otherwise specified. Unsealed, hemoglobin-free ghosts (white ghosts) were made essentially by the method of Steck and Kant (1974), by lysing the washed red cells 1 : 20 (vol/vol) in 5 mM Na_2HPO_4, pH 8.0, centrifuging at 10,000 × *g* for 10 min at 4°C, and washing the pellets three or four times in osmotic lysis medium, until white. These pellets were typically resuspended in PBS and incubated for 1 hr at 37°C, to promote resealing, and used to measure DBDS binding kinetics (*see* Fig. 3, Table 1). Since Verkman, Dix and Solomon (1983) had shown that the DBDS fluorescence enhancement sites are confined to the outside face of the membrane, and are not found on the inside face, DBDS binding kinetics may be presumed to be independent of the fraction of the preparation that was resealed.

One-step ghosts (pink ghosts) were made by lysing the washed red cells 1 : 20 (vol/vol) in the osmotic lysis medium 5 mM HEPES, pH 8.0, centrifuging at 10,000 × *g* for 10 min, and directly resuspending the red pellet at ≈1 : 5 (vol/vol) in PBS, pH 7.4. One-step ghosts were incubated for 1 hr at 37°C to reseal them, in part. These one-step ghosts were used to measure either DBDS binding kinetics, or Cl^- fluxes, measured by the fluorescence of the intracellular dye, SPQ, as described below. Since the preparation was washed three times to remove both the extracellular SPQ and the SPQ from unsealed ghosts, Cl^- flux in unsealed ghosts did not contribute to the measured Cl^- flux. A control experiment, designed to determine whether the hemoglobin present in one-step ghosts could contribute to the measured fluorescence enhancement, showed no fluorescence enhancement over the time range of 0–10 sec, when 3 μM DBDS and 3 μM hemoglobin were mixed in the stopped-flow apparatus.

Binding of DBDS

In the red cell experiments, the cell solution contained ≈0.5% washed red cells in PBS, pH 7.4. The red cell suspension was mixed with an equal volume of 1–10 μM DBDS, in the same buffer, in a stopped-flow apparatus (dead time 40 msec, Model SFA-11, Hi-Tech, Salisbury, Wiltshire, England), whose observation cuvette was placed in the light path of a Photon Technology spectrophotometer (Princeton, NJ). DBDS binding was measured by fluorescence enhancement, excited at 350 nm (resolution 4 nm) and monitored at 427 nm (resolution 4 nm). Experiments were performed at 20–25°C unless otherwise specified. The spectrophotometer output was interfaced on-line to our IBM clone (p-System, Infinity Computer, Worcester, MA). The time course of DBDS fluorescence was recorded at an acquisition rate of 4 msec/point. In order to maximize the signal/noise ratio, many runs (5–10 for ghosts and 20–100 for red cells) were averaged for each data point.

Experiments with Fresh Blood

Fresh human blood, used in the cardiac glycoside experiments, was drawn into a tube containing 10 USP units of heparin/ml blood, washed three times in PBS buffer containing 15 mM glucose, pH 7.4 (called PBSG buffer). The packed cells were diluted in seven volumes of PBSG and incubated for 1 hr with the appropriate concentration of cardiac glycoside at 20–23°C (or 37°C). They were washed three times in PBSG, diluted 200 times in the same buffer and used in the stopped-flow apparatus as described above. For the time course of ouabain binding experiments, fresh blood was prepared as described above, incubated with 0.5 μM ouabain for the appropriate period at 20–23°C and used in the stopped-flow apparatus to measure DBDS binding or Cl^- flux. It was necessary to solubilize the digitoxigenin in ethanol. Since ethanol decreases τ_{DBDS}^{-1}, the final ethanol concentration was kept to 0.1%, and the same concentration was used in the controls.

Measurement of $^{35}SO_4$ Efflux

Fresh human red blood cells were prepared as described above and incubated at 50% hematocrit for 1 hr at 37°C plus 1 μM ethanol and 10 μCi/ml $Na_2^{35}SO_4$. One-ml aliquots of this suspension were then transferred to microfuge tubes and washed four times with ice-cold efflux buffer (10 mM Na_2SO_4, 125 mM sodium gluconate, 20 mM *bis*-Tris propane, pH 6.3, 15 mM glucose). After the fourth wash, 0.9 ml of ice-cold flux buffer was added to achieve a hematocrit of 35%. Efflux was initiated by adding 1 ml of this suspension to 14 ml of efflux buffer at 37°C. One-ml aliquots were then taken at various times, spun for 30 sec and 0.2-ml samples of supernatant (in duplicate) were counted in 5 ml Econofluor in a Tracor Model Delta 300 scintillation counter. Infinite time values were determined by adding 0.03 ml of 0.2% saponin, vortexing and then adding 0.11 ml of 50% ice-cold TCA, vortexing, spinning for 1 min (t_∞ was determined in quadruplicate) and sampling 0.2 ml of supernatant. Samples were counted to 0.5% using the SCR method for quench correction; infinite time points were corrected for dilution.

Fluorescent Method of Measuring Cl^- Exchange

Human red cells and sealed one-step ghosts were loaded with the water-soluble dye, SPQ, which accurately reports intracellular Cl^- concentration (Illsley & Verkman, 1987). Red cells were loaded with SPQ by incubation with 10 mM SPQ for 2 to 5 hr at 37°C in PBS buffer, pH 7.4. For the red cell preparation, 0.05–0.1% C_2H_5OH was added during the SPQ incubation. The dye was added to one-step ghosts just before resealing and remained during the resealing incubation. The dye leaks out of resealed one-step ghosts on a time scale measured in minutes. Extracellular SPQ was removed by three washes with PBS buffer, pH 7.4 and very low centrifugation speeds (<5000 × *g*). To minimize SPQ diffusion out of the cells or sealed ghosts, they were kept at 0°C until used. The stopped-flow experiments were carried out as described for DBDS binding. Suspensions (0.2–0.75%) of SPQ-loaded cells, or 2–6% of ghosts, were mixed with equal volumes of buffer containing in mM: 50 (or 60) $NaHCO_3$; 110 (or 92) Na gluconate, bubbled with 5% CO_2, pH 7.1–7.4. In one series of control experiments, 60 mM NO_3^- replaced 60 mM $NaHCO_3^-$. Experiments were carried out at 20–23°C unless otherwise specified. The decrease in cell Cl^- caused a rapid increase in SPQ fluorescence (excited at 350 nm, resolution 12 nm; measured at 427 nm, resolution 8 nm), which was recorded at a data acquisition rate of 4 msec/point. The rates of the Cl^- flux were calculated from the time constant, convoluted with a function describing Stern-Volmer quenching (K = 118.0 M^{-1}), by the method given by Illsley and Verkman (1987); we checked the quench constant independently (K = 116 ± 17 M^{-1}). The fluorescence lifetime (τ_o) of SPQ in the absence of quencher was measured separately for every set of experiments. The data from a large number of runs was averaged, corrected for the slow dye leakage that persists in sealed ghosts (which was measured independently for each set of measurements) and fitted to a single exponential by nonlinear least squares. Our result for the Cl^- flux rate in one-step ghosts of 20.5 mM sec^{-1} in the presence of 25 mM HCO_3^- is similar to the value of 21.1 mM sec^{-1} given by Illsley and Verkman (1987).

In order to provide further confirmation of the validity of the SPQ method, we used it to measure $Cl^-/SO_4^=$ exchange at 37°C from fresh red cells into buffer (10 mM Na_2SO_4, 125 mM Na gluconate, 15 mM glucose, 20 mM *bis*-Tris propane, pH 6.3) and determined that $\tau_{chloride}$ = 8.4 ± 0.9 min. We also measured $^{35}SO_4/SO_4$ exchange at 37°C by cells from the same blood under almost the same conditions, as described above, and determined $\tau_{sulfate}$ = 5.6 ± 0.2 min. In the SPQ experiments, we measured the loss of Cl^- from the cells; in the $^{35}SO_4^=$ experiments, we measured the loss of $^{35}SO_4^=$ from the cells so the exchange partners were not identical. Assuming the rate determining step to be primarily the $SO_4^=$ exchange at the outer face of the cell, the agreement between the two estimates of τ is satisfactory, in view of the difference in the exchange partners at the inner face.

Cytochalasin B Competition Experiments

For the experiments in which maltose or D-glucose displaced cytochalasin B from its binding site, the following mannitol [or D-glucose] buffers were used: 250 mM mannitol [or D-glucose], 25 mM NaCl, 5 mM Na phosphate, pH 7.4. Bank blood, outdated by no more than two days, was washed three times with mannitol [or D-glucose] buffer, centrifuged and resuspended in the same buffer. The cells were incubated with 2 μM cytochalasin B (freshly prepared, final ethanol concentration 0.05%) at 20–23°C for 40 min, washed twice in mannitol buffer at 5°C and resuspended 1:200 in mannitol (for t = 0 point) or D-glucose (to measure D-glucose uptake) or maltose (incubated for 8 min at 20–23°C prior to the stopped-flow measurement) buffer. For the control experiment for the $t = \infty$ measurement, red cells, washed in mannitol, were then incubated in D-glucose buffer for 90 min at 37°C. DBDS binding was measured in the stopped-flow apparatus by mixing the red cell suspension (1:200) with 6 μM DBDS (1:1) in mannitol buffer at 20–23°C.

Results

Theory

We have modeled the first steps of the DBDS binding reaction as a bimolecular association, too fast for us to measure, followed by a conformational change, according to Eq. (1), following the model of Verkman et al. (1983) in the red cell,

$$\text{DBDS} + \text{band 3} \overset{K_1}{\leftrightarrow} \text{DBDS-band 3} \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} \text{DBDS-band 3}^* \qquad (1)$$

in which * denotes the DBDS/band 3 complex following its conformation change and K_1 is the dissociation constant for the bimolecular reaction. The kinetics of the reaction are given by Eq. (2) (Czerlinski, 1966):

$$\tau_{DBDS}^{-1} = k_{-2} + (k_2[\text{DBDS}]_{total}/(K_1 + [\text{DBDS}]_{total})). \qquad (2)$$

This equation can be fitted to the data by nonlinear least squares to obtain the constants: K_1, k_2 and k_{-2}, as well as the equilibrium constant for the conformational change, $K_2 = k_2/k_{-2}$. When an equation with three coefficients is fitted to a limited number of points, as in our experiments, the statistical accuracy is less than could be desired. A great deal can be learned from direct inspection of the curves, since the intercept at [DBDS] = 0 is k_{-2} and the asymptote approaches ($k_{-2} + k_2$) as [DBDS] approaches ∞. K_1 is given by the [DBDS] at the midpoint between $\tau^{-1}_{intercept}$ and $\tau^{-1}_{asymptote}$. Since, under many conditions (Tables 1 and 2) k_{-2} is constrained to a relatively narrow range of values, the clear differences in the asymptotes, which are a distinctive feature of our results, provide visual indication of real differences in k_2, which can be more significant than those given by the errors in the least-squares fits.

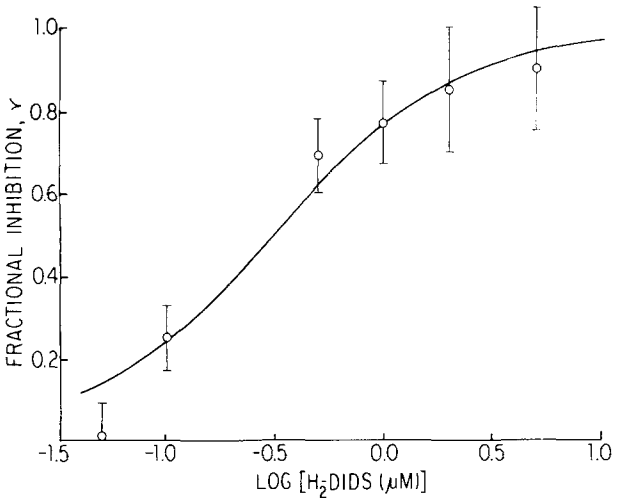

Fig. 1. Effect of H_2-DIDS on kinetics of DBDS binding (4 μM) to one-step ghosts: fractional inhibition of τ_{DBDS} (maximum: 100% inhibited [τ^{-1}_{DBDS} = 0 sec^{-1}]; minimum: 0 inhibition [τ^{-1}_{DBDS} = 6.0 sec^{-1}]). Packed pellets of resealed one-step ghosts were washed and then diluted 30 times in PBS buffer, pH 7.4. H_2-DIDS was added to the ghost suspension 5 min before measurement at 20–23°C. ID_{50} = 0.3 ± 0.1 μM. The points were obtained by fitting the time-course data by nonlinear least squares to a single exponential; the dose-response curve was fitted to the points by nonlinear least squares. Each point is the average of 5–10 measurements; average of two experiments. Errors are SD

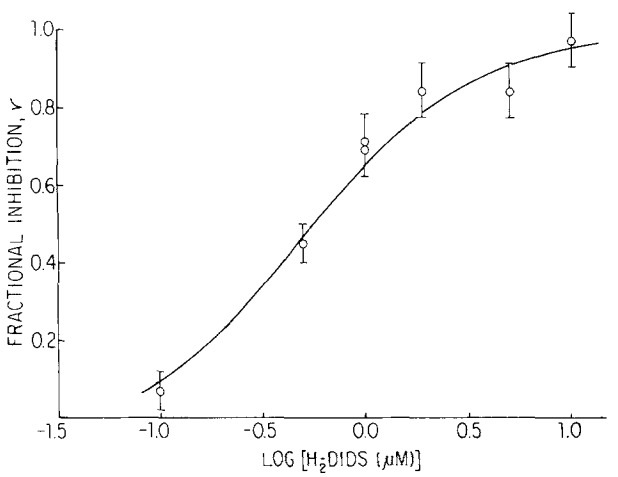

Fig. 2. Effect of H_2-DIDS on Cl^- flux in one-step ghosts. One-step ghosts were incubated with SPQ dye as described under Methods, then diluted 15 times in the same buffer. The stopped-flow experiments were performed by mixing ghosts in PBS buffer with an equal volume of buffer containing 110 mM Na gluconate, 50 mM $NaHCO_3$. τ_{Cl^-} was obtained from the time course, fitted to a single exponential by nonlinear least squares. Fractional inhibition, ν, (maximum: 100% [$\tau^{-1}_{Cl^-}$ = 0 sec^{-1}]; minimum, 0% [$\tau^{-1}_{Cl^-}$ = 6.0 sec^{-1}]) of τ_{Cl^-} was plotted against [H_2-DIDS] and the curve was fitted by nonlinear least squares. ID_{50} = 0.47 ± 0.04 μM. Every point is the average of five measurements; average of two experiments

Since the effects that we have observed often involve two of the constants in Eq. (1) and sometimes all three, it is useful to compute $K_{1,app}$, the apparent DBDS binding constant. $K_{1,app} = K_1/(1 + K_2)$ is the binding constant that would be obtained in a Scatchard plot, and contains contributions from all three coefficients. Though the constraints introduced by the propagation of errors lead to a very large error in this derived constant, $K_{1,app}$ provides a snapshot of how these three coefficients interact to determine how tightly the ligand is bound to band 3.

Relation of DBDS Binding Kinetics to Anion Exchange

The specific anion transport inhibitor, H_2-DIDS, inhibits red cell anion exchange with ID_{50} = 0.3 μM (Knauf, 1979); the fractional inhibition of Cl^- exchange increases linearly with the number of H_2-DIDS molecules bound to the cell (Lepke et al., 1976). We have previously reported that covalent binding of DIDS to white ghosts suppresses the time-dependent changes in fluorescence that characterize DBDS binding to ghosts (Verkman et al., 1983). In order to find out whether the DBDS fluorescence time course represented binding to the anion transport inhibition site, we determined the effect of reversible binding of H_2-DIDS on τ_{DBDS} in one-step ghosts, whose binding kinetics are compared to those of white ghosts and native red cells in the next section. Figure 1 shows that the average ID_{50} for H_2-DIDS inhibition of τ_{DBDS} is 0.3 ± 0.1 μM, in two experiments in one-step ghosts, in agreement with the ID_{50} for H_2-DIDS inhibition of anion exchange in red cells. In order to make sure that the DBDS binding kinetics measured specific binding to band 3, rather than nonspecific high affinity adsorption, we probed the specificity of the binding site with another stilbene inhibitor of lower affinity, SITS, which inhibits anion transport with $ID_{50} \approx 10$ μM (Cabantchik & Rothstein, 1972). We found that SITS inhibits τ_{DBDS} with ID_{50} = 2.7 ± 0.6 μM (average of two experiments), in reasonable agreement with the SITS ID_{50} for anion exchange. The observation that the stilbene effect on τ_{DBDS} depends upon the specific stilbene substituents, just like the effect of these stilbenes on Cl^- flux (Barzilay, Ship & Cabantchik, 1979), provides very strong support for the view that the time course of DBDS fluorescence enhancement is a probe of the configuration of the inhibition site on the anion exchange protein.

When it became possible to use the fluorescent dye, SPQ, to measure Cl^- fluxes (Illsley & Verkman, 1987) in our stopped-flow apparatus, we made a direct measurement of the H_2-DIDS inhibi-

tion of Cl^- flux in one-step ghosts. Figure 2 shows that ID_{50} for this inhibition is 0.47 ± 0.04 μM, in agreement[1] with the ID_{50} of 0.3 ± 0.1 μM for H_2-DIDS inhibition of τ_{DBDS}. Similar to the results for τ_{DBDS}, the ID_{50} for SITS inhibition of Cl^- exchange is 1.8 ± 0.2 μM (average of two experiments), greater than that for H_2-DIDS inhibition of Cl^- exchange, and in good agreement with the SITS ID_{50} we measured for τ_{DBDS}. These results, obtained with both H_2-DIDS and SITS, show that the DBDS kinetics probed by the fluorescent method are those of stilbene inhibition of Cl^- flux in one-step ghosts, and hence validate the use of our fluorescent probe as a reporter of band 3 conformation in one-step ghosts, whose DBDS binding kinetics are very similar to those for red cells and white ghosts, as discussed in the next section.

Comparison of Properties of Red Cells, White Ghosts and One-Step Ghosts

Ojcius et al. (1988) had found that the permeability of well-washed white ghosts differs from that of the normal red cell in two important ways. First, white ghosts do not retain the normal high urea permeability characteristic of the native red cell and second, water flux is no longer inhibitable by *p*-chloromercuribenzene sulfonate (*p*CMBS), as it is in the normal red cell. However, one-step ghosts, prepared in HEPES buffer without washing, retain normal urea and pCMBS-inhibitable water permeability; Ojcius et al. (1988) suggested that the loss of these permeability properties in white ghosts might be due to cytoskeletal effects. Since some 15% of the band 3 in the membrane is linked to the spectrin tetramer by ankyrin (Low, 1986), we thought that cytoskeletal effects might also lead to significant changes in DBDS binding kinetics and, therefore, compared these preparations. As Fig. 3 shows, the asymptotic value of τ^{-1}_{DBDS} at high [DBDS] clearly depends upon the state of the cell membrane while the intercept changes hardly at all. The data in Table 1 confirm this interpretation, showing that there are significant differences in k_2 while k_{-2} is independent of the state of the membrane. Though the resting configuration of band 3 is independent of the membrane state, as shown by K_1, the equilibrium constant for the conformational change, K_2, is shifted to the left, away from the activated state, as the red cell undergoes additional washes. These data show that osmotic lysis and subsequent washing of the cell are inimical to the ability of band 3 to make the conformational change, which locks DBDS into its inhibitory site.

Effect of Glucose Transport Inhibitor on DBDS Binding Kinetics

Red cell glucose transport is specifically inhibited by cytochalasin B (Lin, 1978) with a $K_I \approx 0.3$–0.6 μM. In addition, there is a second class of high affinity cytochalasin B sites in the red cell, which interact with the spectrin-actin cytoskeletal complex with $K_D = 6 \times 10^{-2}$ μM (Lin & Lin, 1978). Cytochalasin E, which binds more tightly than cytochalasin B to the higher affinity cytoskeletal site, displaces cytochalasin B from this site, but does not affect cytochalasin B binding to the glucose transport protein (Jung & Rampal, 1977). In order to study the cytochalasin B effect on the glucose transport protein, we have pretreated the red cells with cytochalasin E to block the cytoskeletal sites, similar to the method used by Cushman and Wardzala (1980). As the dose response curve in Fig. 4 shows, cytochalasin B has a significant effect on the reaction, slowing down the rate by about 40% from $\tau^{-1}_{DBDS} = 8.0$ to 5.0 sec^{-1} at 4 μM DBDS (a similar decrease was observed in the signal amplitude). The ID_{50} for this

[1] We determine ID_{50} in our experiments, rather than K_I, which is the correct parameter to use in relating inhibition processes in different experiments. The relation between K_I and ID_{50} is given by Segel (1975) who derives equations for (ID_{50}/K_I) as a function of the three ratios, $[S]/K_S$, $[I]/K_I$ and $[J]/K_J$ in which S stands for substrate, I for the inhibitor and J for a second inhibitor; the K's are dissociation constants. These equations are eq VIII-16 on p. 477 and eq III-4 on p. 105, which are valid for competitive inhibitors only but apply to our experiments because the stilbene inhibitors and Cl^- are all mutually competitive. In our experiments on the effect of H_2-DIDS on DBDS, DBDS is the substrate because it is the kinetics of DBDS binding that we measure, and H_2-DIDS is the inhibitor. Since these experiments were carried out with 150 mM Cl^- on both sides of the membrane, Cl^- also competes for the binding site and replaces J in the equation. We take 65 mM to be K_{Cl} (Brazy & Gunn, 1976), but we have to use the ID_{50} value for DBDS, 1.3 μM (Barzilay et al., 1979), to replace K_S in eq VIII-16, since we can find no determination of the K_I. With these values $(ID_{50}/K_I)_{DBDS\ expt} = 1.8$.

In the case of the Cl^- flux measurements by the SPQ method, Cl^- is the substrate and there is no second inhibitor. In these experiments, a Cl^- gradient was necessary to provide the driving force, so the extracellular Cl^- was set at 75 mM in eq III-4, which leads to $(ID_{50}/K_I)_{SPQ\ expt} = 2.2$. On the basis of these calculations, $(ID_{50})_{DBDS\ expt}$ should be 1.2 times greater than $(ID_{50})_{SPQ\ expt}$, which agrees very well with the factor of about 1.5 that we observe, but the uncertainties are such that agreement within a factor of two or three is satisfactory.

Knauf et al. (1980) have pointed out that ID_{50} for stilbene inhibitor binding depends upon the ratio of $(Cl^-)_{out}/(Cl^-)_{in}$ and that a fivefold increase in this ratio increases the ID_{50} by a factor of about two. In our case the $(Cl^-)_{out}/(Cl^-)_{in}$ ratio is 0.5 for the SPQ experiments and 1 for the DBDS experiments, which would provide a further increase in the predicted value of $ID_{50,DBDS\ expt}$ as compared to $ID_{50,SPQ\ expt}$.

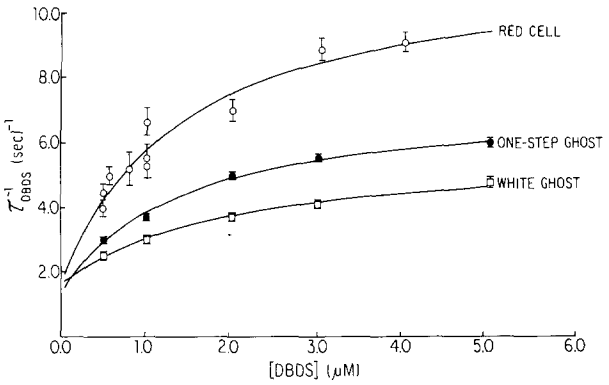

Fig. 3. Kinetics of DBDS binding in red cell preparations. White and one-step ghosts were diluted 25 and 50 times from packed pellets in PBS buffer, pH 7.4; each point is the average of 5–10 measurements. Average of two experiments. Red cells (bank blood) were diluted 600 times from packed cells in the same buffer; each point is the average of 50–80 measurements. The data were fitted to Eq. (2) by nonlinear least squares and the coefficients are given in Table 1. Average of four experiments

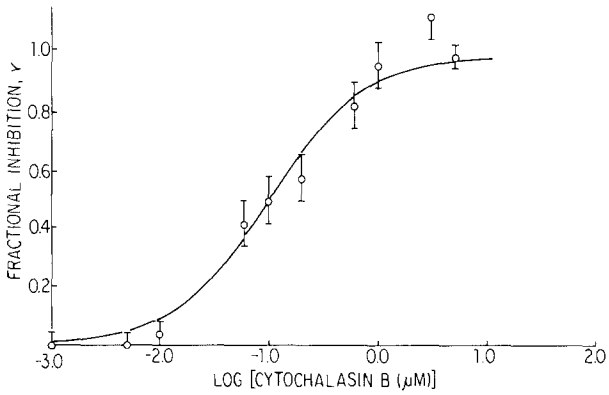

Fig. 4. Fractional inhibition of τ^{-1}_{DBDS} by cytochalasin B in red cells at 4 μM [DBDS] in one of two experiments. Red cells (bank blood) were diluted 350 times from packed cells in PBS, treated with 2 μM cytochalasin E and incubated for 60 min at 20–23°C and then incubated again at 20–23°C with different concentrations of cytochalasin B for 25 min, prior to the stopped-flow measurements in PBS. To solubilize the cytochalasins, C_2H_5OH (<1 μM) was added to experimental and control suspensions. The dose-response curve was obtained as in Fig. 1. ν was set at 1.0 at $\tau^{-1}_{DBDS} = 5.0$ sec^{-1} (maximum inhibition) and 0 at $\tau^{-1}_{DBDS} = 8.0$ sec^{-1} (0 inhibition). $ID_{50} = 0.1 \pm 0.2$ μM. Every point is the average of 30–45 measurements

Table 1. Kinetics of DBDS binding to red cell preparations[a]

	Red cell	One-step ghost	White ghost
K_1 (μM)	1.4 ± 0.3	1.4 ± 0.6	1.9 ± 0.6
k_2 (sec^{-1})	10.0 ± 0.9	6.0 ± 0.4	4.2 ± 0.2
k_{-2} (sec^{-1})	1.6 ± 0.4	1.3 ± 0.6	1.6 ± 0.3
K_2	6.3 ± 1.6	4.6 ± 2.1	2.6 ± 0.5
$K_{1,app}$ (μM)	0.19 ± 0.09	0.25 ± 0.22	0.53 ± 0.27

[a] The experiments were done on four sets of the three preparations, each set from a single blood, except for white ghosts in which two of the bloods provided samples for two sets each.

effect in one of two experiments was 0.1 ± 0.2 μM (in the other, $ID_{50} = 0.2 \pm 0.1$ μM). Carruthers (1986) found an apparent K_D of 0.06 ± 0.005 μM for cytochalasin B binding to a single class of sites on stripped red cell ghost membranes. Carruthers (1986) identified the site as the glucose transport protein and reported that the number of sites agreed well with the number of copies of band 4.5 in the preparation. Our ID_{50} is slightly lower than the K_I range found by Lin (1978) to inhibit glucose transport and slightly higher than the apparent K_D of Carruthers (1986) for binding to stripped ghosts. This agreement supports the view that the effects we observe on τ_{DBDS} are consistent with cytochalasin B binding to the glucose transport protein.

In order to study the cytochalasin effect on the three kinetic constants, we have used 10 μM concentrations to saturate the system and obtained the results shown in Fig. 5 (left) and Table 2. As the curves show, and the Table confirms, the cytochalasins cause a large shift in K_2, as a result of a decrease in k_2, and an even larger decrease in k_{-2}. It is clear that cytochalasin B, with or without cytochalasin E, has a significant effect on k_2 and k_{-2}. Although cytochalasin E does not cause a statistically significant change in k_2, we find that the difference between the asymptotes, together with the small error bars in Fig. 5, are very persuasive. When there is no difference between two treatments, as in the comparison between cytochalasin B alone and cytochalasins B and E together, as in the bottom curve, the experimental points lie virtually on top of one another. The cumulative effect of the cytochalasin treatments is to drive the conformational change reaction over to the right, stabilizing the activated form and locking the ligand even tighter to band 3, as indicated by the decrease in $K_{1,app}$.

Helgerson and Carruthers (1987) have studied competitive effects of a number of sugars on cytochalasin B binding to the sugar transporter in human red cell membranes.[2] They report that extra-

[2] Helgerson and Carruthers (1987) used 10 μM cytochalasin D in almost all of their experiments because it blocks cytochalasin B binding to the cytoskeletal sites and has no effect on cytochalasin B binding to the glucose transport protein. We used 10 μM cytochalasin E for the same purpose and found, as shown in Fig. 5 (left), that treatment with cytochalasin E does not alter the effect of 10 μM cytochalasin B on the kinetics of DBDS binding. Consequently, our control experiments with maltose and D-glucose were carried out with no cytochalasin E treatment.

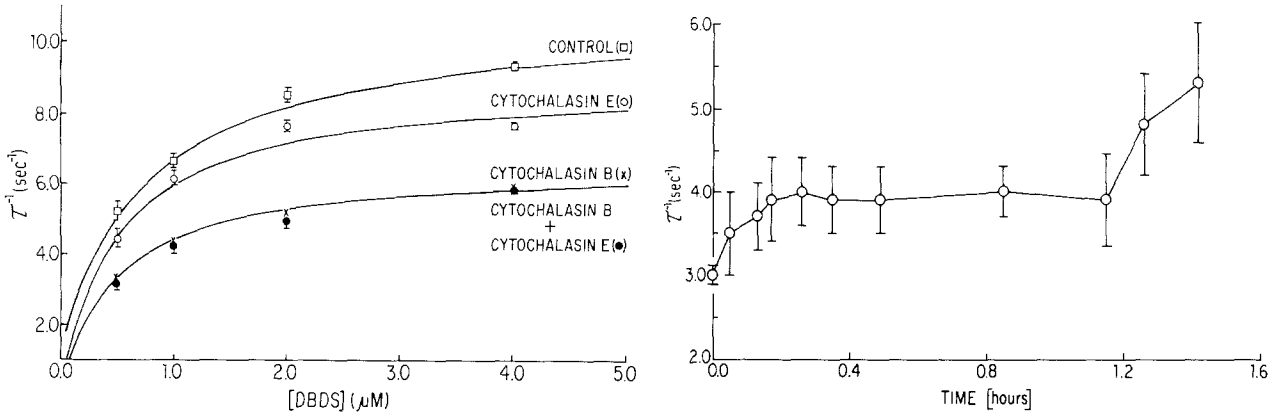

Fig. 5. (Left): Effect of cytochalasins B and E on DBDS binding kinetics to red cells. Packed red cells (bank blood) were diluted 500 times in PBS buffer, pH 7.4, prior to stopped-flow measurements with DBDS (4 μM) in PBS. Incubation conditions—Cytochalasin E (CE): 10 μM added, incubated for 30 min at 20–23°C; cytochalasin B (CB): 10 μM, 20 min at 20–23°C; CE + CB: 10 μM CE, 20 min at 20–23°C, then 10 μM CB added for further 20 min at 20–23°C. Every point is the average of 30–40 measurements; average of two experiments. The data have been fitted to Eq. (2) by nonlinear least squares and the coefficients are given in Table 2. (Right): Effect of D-glucose on cytochalasin B modulation of DBDS binding kinetics. Red cells (bank blood) were washed in mannitol buffer and incubated with 2 μM cytochalasin B (solubilized in ethanol, final ethanol concentration 0.05%) for 40 min at 20–23°C, washed twice at 5°C and resuspended in 200 volumes of D-glucose (or mannitol) buffer. Stopped-flow measurements were made by mixing equal volumes of the red cell suspension with mannitol buffer containing 6 μM DBDS. For the zero time point, the red cell suspension in mannitol buffer was mixed (1 : 1) with mannitol buffer in the stopped-flow apparatus. Every point is the average from two separate bloods (20–60 duplicates for each value). Similar results were obtained in a third experiment under similar conditions (100 mM NaCl, 100 mM D-glucose, 5 mM PO_4, pH 7.4)

Table 2. Effect of cytochalasins on DBDS binding kinetics in red cells[a]

	Control	Cytochalasin E	Cytochalasin B	Cytochalasin B + cytochalasin E
K_1 (μM)	0.8 ± 0.5	0.5 ± 0.7	0.5 ± 0.3	0.6 ± 0.3
k_2 (sec^{-1})	10.0 ± 1	8.9 ± 0.7	6.6 ± 0.7	6.5 ± 0.6
k_{-2} (sec^{-1})	1.2 ± 0.5	0.04 ± 0.4	0.05 ± 0.4	0.1 ± 0.5
K_2	8 ± 3	200 ± 100	130 ± 70	60 ± 30
$K_{1,app}$ (nM)	83 ± 89	2.2 ± 4.7	3.8 ± 4.3	10 ± 10

[a] The concentrations of the cytochalasins were each 10 μM; the incubation conditions are given in the legend to Fig. 5. Data is from two experiments.

cellular addition of 100 mM maltose, a reactive but nontransportable sugar, to whole red cells causes an immediate release of cytochalasin B from its intracellular binding site. We have carried out a similar experiment and obtained a similar maltose effect on cytochalasin B displacement, as measured by τ^{-1}_{DBDS}. The control τ^{-1}_{DBDS} for red cells is 4.3 ± 0.3 sec^{-1} in mannitol [or D-glucose] buffer (250 mM mannitol [or D-glucose], 25 mM NaCl, 5 mM PO_4, pH 7.4). When these cells were incubated with 2 μM cytochalasin B, as described under Methods, τ^{-1}_{DBDS} was changed to 2.8 ± 0.2 sec^{-1}. Substitution of 250 mM maltose for the mannitol shifted τ^{-1}_{DBDS} back to 3.8 ± 0.3 sec^{-1}, in good agreement with the original control. This experiment shows that the displacement of cytochalasin B from its binding site on the glucose transport protein is accompanied by a change in τ^{-1}_{DBDS}, providing further evidence of a linkage between the glucose transport protein and band 3.

Although extracellular D-glucose does not displace cytochalasin B, intracellular D-glucose does, as illustrated by an experiment in which Helgerson and Carruthers (1987) measured the time course of cytochalasin B release over a 3-hr period. They found that the ratio of free/bound cytochalasin B gradually increased, as glucose permeated the cell and released cytochalasin B, reaching the maximum effect in about 1 hr. We made analogous observations in a series of experiments. The results of two

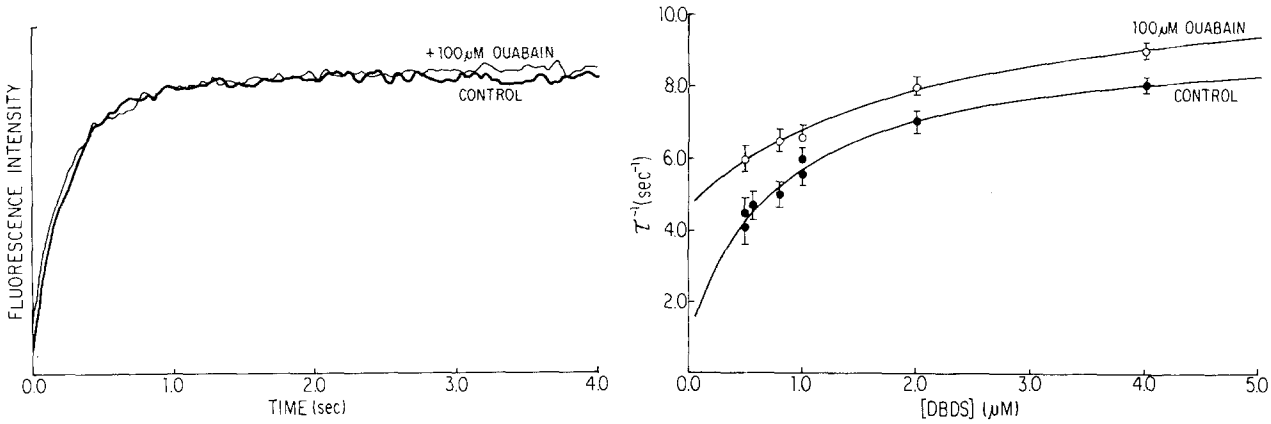

Fig. 6. (Left): Effect of 100 μM ouabain on time course of DBDS (1 μM) fluorescence intensity in white ghosts, measured by stopped flow. White ghosts in PBS, pH 7.4, were incubated with 100 μM ouabain for 5–10 min at 20–23°C. Five measurements in one experiment, typical of two. (Right): Effect of 100 μM ouabain on dependence of τ^{-1}_{DBDS} on [DBDS] in red cells. Red cells were diluted 1:400 then incubated with 100 μM ouabain for 5–10 min at 20–23°C before the experiment. Each point is an average of 40 measurements; average of four experiments. The data have been fitted to Eq. (2) by nonlinear least squares and the coefficients are given in Table 3

of these experiments are given in Fig. 5 (right), which shows the average increase in τ^{-1}_{DBDS} as D-glucose permeates the cell. The initial value of $\tau^{-1}_{DBDS} = 3.0 \pm 0.1$ sec^{-1} in the presence of 2 μM cytochalasin B; 250 mM glucose increases τ^{-1}_{DBDS} to 5.3 ± 0.7 sec^{-1} after 85 min incubation at room temperature. In order to confirm our interpretation of the data, two additional control measurements were made. To show that the properties of the red cell remained constant over the course of the experiment, we incubated a sample with mannitol rather than D-glucose, under the same conditions, and found that $\tau^{-1}_{DBDS} = 3.0 \pm 0.5$ sec^{-1} at the end of this incubation, unchanged from its initial value. To show that D-glucose displaced all of the cytochalasin B, we incubated red cells in D-glucose buffer for 1.5 hr at 37°C in the absence of cytochalasin B and found that τ^{-1}_{DBDS} (measured at 20–24°C) $= 5.6 \pm 0.4$ sec^{-1}, the same as the final value obtained after 85 min incubation with cytochalasin B and D-glucose. These experiments clearly demonstrate the relationship between τ^{-1}_{DBDS} and glucose transport and, taken together with the maltose experiment and the results in Fig. 5 (left) and Table 2, show that there is a linkage by which information can be transported between the glucose transport protein and band 3.

Effect of Ouabain on Anion Transport in Red Cells

In view of Fossel and Solomon's (1981) NMR (nuclear magnetic resonance) evidence indicating a linkage between red cell Na^+,K^+-ATPase and band 3, we had previously looked for a ouabain effect on DBDS kinetics in white ghosts, but failed to find any effect. We have now confirmed this finding in white ghosts, using the very high ouabain concentration of 100 μM, as shown in Fig. 6 (left). In the native red cell, however, 100 μM ouabain does cause an effect on τ^{-1}_{DBDS}, as shown in Fig. 6 (right) and Table 3. Ouabain is distinctly hostile to DBDS/band 3 binding. Not only does K_1 increase by a factor of more than two, showing that the DBDS fit to its binding site has been worsened, but also k_{-2} increases by a large factor, shifting the equilibrium away from the activated conformation. The net effect is an increase in the apparent dissociation constant, $K_{1,app}$ by almost an order of magnitude. Perhaps the reason that ouabain affects K_1, while the cytochalasins do not, is related to the fact that ouabain binding and DBDS binding are both on the outer face of the membrane, while the cytochalasins bind inside the cell. It is, at first sight, surprising that ouabain, which destabilizes both the binding (K_1 increases) and the conformation change (K_2 decreases), causes the reaction to go faster (τ^{-1}_{DBDS} increases), in accordance with Eq (2). However, both of these processes decrease the depth of the energy wells, so we would expect the activation energy to be lower, thus accounting for the increased reaction rate.

Since these observations had been made at very high ouabain concentrations, it was necessary to see whether low ouabain concentrations, similar to those which inhibit red cell cation transport, were effective, so the dose-response curve for τ_{DBDS} shown in Fig. 7 was obtained on fresh blood. The

Table 3. Effect of ouabain on DBDS binding kinetics in red cells[a]

	Control	Ouabain
K_1 (μM)	0.8 ± 0.2	2.2 ± 0.5
k_2 (sec^{-1})	8.3 ± 0.6	6.8 ± 0.8
k_{-2} (sec^{-1})	1.1 ± 0.5	4.7 ± 0.6
K_2	7.6 ± 3.5	1.5 ± 0.3
$K_{1,app}$ (μM)	0.093 ± 0.066	0.88 ± 0.38

[a] Four experiments with 100 μM ouabain, as shown in Fig. 6 (right).

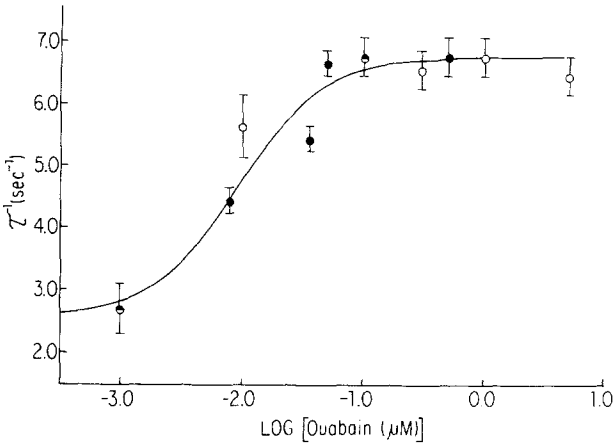

Fig. 7. Effect of ouabain on τ^{-1}_{DBDS} in red cells. Red cells from freshly drawn blood were washed three times in PBSG, then diluted 200 times in the same buffer. The cells were incubated with ouabain for 60 min at either 20–23°C (filled circles) or at 37°C (open circles) and then washed three times with PBSG; experiments at 2.0 μM DBDS were carried out at 20–23°C by the stopped-flow method. The dose-response curve was fitted as in Fig. 1. ID_{50} = 0.010 ± 0.003 μM. Every point is the average of 60–80 measurements at 20–23°C; average data from two experiments

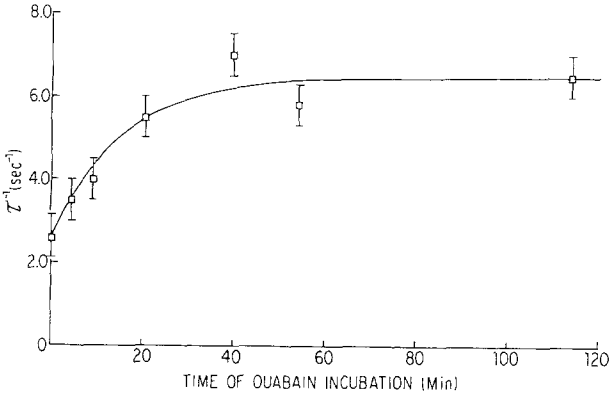

Fig. 8. Time course of ouabain effect on τ^{-1}_{DBDS}. Experimental conditions as in Fig. 7 except that ouabain concentration was set at 0.5 μM; the abscissa gives the time of ouabain incubation at 20–23°C and the ordinate gives τ^{-1}_{DBDS}. Points were fit to a single exponential for τ^{-1}_{DBDS}; the half time = 11 ± 3 min. Each point is the average of 30 measurements; one experiment

ID_{50} for ouabain inhibition of τ^{-1}_{DBDS} is 0.010 ± 0.003 μM, in good agreement with the K_I of 0.017 μM given by Solomon, Gill and Gold (1956) for inhibition of K^+ flux. Figure 8 shows that the half time of the effect of 0.5 μM ouabain on τ^{-1}_{DBDS} is 11 ± 2.5 min which, after taking account of the difference in concentration, is consistent with the half time for 0.17 mM ouabain uptake by the red cell of ≈30 min (Gardner & Conlon, 1972; *see also* Hoffman, 1969). The time course in Fig. 8 shows that the ouabain effect on τ^{-1}_{DBDS} cannot be attributed to nonspecific adsorption, which is essentially instantaneous on this time scale.

In order to confirm that the cardiac glycoside effect on τ_{DBDS} was a consequence of binding to the Na^+,K^+-ATPase, we carried out an analogous series of experiments with digitoxigenin, whose K_I for inhibition of K^+ flux is 0.24 μM (Solomon et al., 1956), about an order of magnitude less than that of ouabain. Figure 9 shows that the ID_{50} for digitoxigenin inhibition of τ^{-1}_{DBDS} is 0.14 ± 0.03 μM, in very good agreement with the value for K^+ flux inhibition. Taken together, these cardiac glycoside experiments show that the ouabain effect on τ^{-1}_{DBDS} is to be attributed to actions at the cation transport inhibition site on the membrane Na^+,K^+-ATPase.

The ouabain effect has been confirmed by direct measurement of the red cell Cl^- flux by the SPQ method. The top two curves in Fig. 10 show that 10 μM ouabain increases $\tau^{-1}_{Cl^-}$ significantly ($P < 0.02$, t test) from 1.4 ± 0.1 sec^{-1} to 2.5 ± 0.3 sec^{-1} in one of four similar experiments. It is interesting that the cation transport *inhibitor* increases the velocity of the anion transport system (increases $\tau^{-1}_{Cl^-}$) just as it increases τ^{-1}_{DBDS}. The bottom time course in Fig. 10 shows that 10 μM H_2-DIDS abolishes all but 7.5% of the Cl^- flux.

In order to make sure that these results could not be ascribed to a change in cell pH as HCO_3^- exchanged for cell Cl^-, we carried out an additional set of experiments in which NO_3^- replaced HCO_3^- as exchange partner. The results of the control experiments, in the absence of ouabain, in the top section of Table 4 show that τ_{Cl^-} is independent of the exchange partner. The effects of 0.1–10 μM ouabain on Cl^-/NO_3^- exchange are considerably greater than those on Cl^-/HCO_3^- exchange, as shown in the bottom two sections of Table 4. The experiments with NO_3^- show that the effect of ouabain on band 3 is dependent upon the identity of the exchange partner, which means that the linkage between the oua-

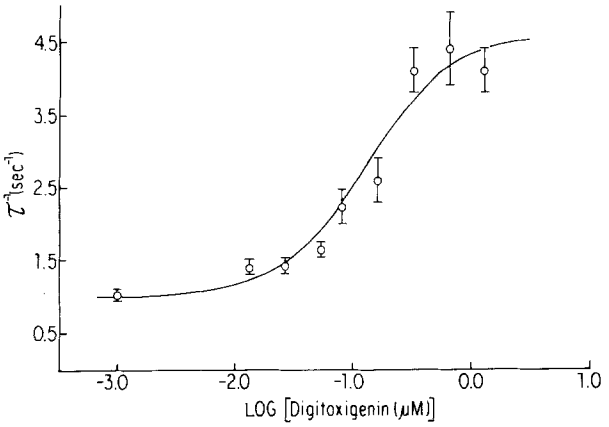

Fig. 9. Effect of digitoxigenin on τ^{-1}_{DBDS} in red cells. Red cells from freshly drawn blood were treated as in Fig. 7. Experiments at 2.0 μM DBDS were carried out at 20–23°C by stopped-flow. Digitoxigenin was solubilized with ethanol (final ethanol concentration, 0.1%) and the cells were incubated with digitoxigenin for 60 min at 20–23°C and then washed three times with PBSG. ID_{50} = 0.14 ± 0.03 μM. Every point is the average of 60–80 measurements; one experiment

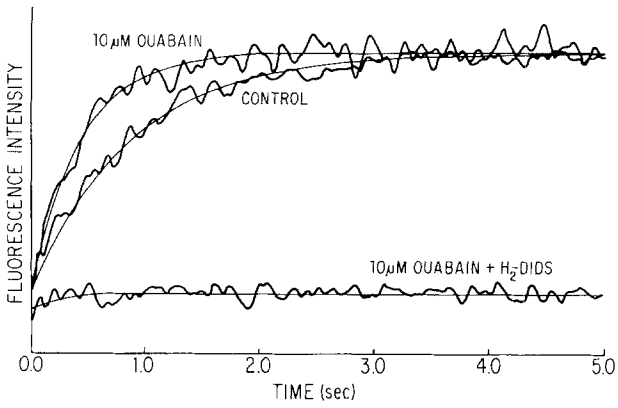

Fig. 10. Effect of 10 μM ouabain on time course of red cell Cl^- flux. Red cells were incubated with SPQ dye (with 15 mM D-glucose), washed three times as described under Methods, and diluted 150 times with PBS buffer, pH 7.4. The stopped-flow experiments were performed by mixing the red cell suspension in PBS with an equal volume of 92 mM Na gluconate + 60 mM $NaHCO_3$, bubbled with 5% CO_2. After correction for the slow leak of SPQ dye from the cells, the time course was fitted to a single exponential by nonlinear least squares. In the control, $\tau^{-1}_{Cl^-}$ = 1.4 ± 0.1 sec^{-1}; after 10 min incubation with 10 μM ouabain at 20–23°C, $\tau^{-1}_{Cl^-}$ = 2.5 ± 0.3 sec^{-1}. The bottom curve, in which [H_2-DIDS] = 10 μM, shows that 7.5% of the Cl^- flux is not H_2-DIDS inhibitable. Each time course is the average of 30 runs. One experiment at 20–23°C, typical of two additional experiments on different bloods and one additional experiment with 110 mM Na gluconate and 50 mM $NaHCO_3$

Table 4. Comparison of ouabain effect on Cl^-/NO_3^- and Cl^-/HCO_3^- exchange kinetics

Ouabain concentration	τ_{Cl^-}, (sec)	Amplitude, (fluorescence units)
0		
Cl^-/NO_3^-	1.0 ± 0.1	102 ± 3
Cl^-/HCO_3^-	1.2 ± 0.1	104 ± 4
0.1 μM		
Cl^-/NO_3^-	0.25 ± 0.02	76 ± 3
Cl^-/HCO_3^-	0.62 ± 0.03	67 ± 2
10 μM		
Cl^-/NO_3^-	0.29 ± 0.06	59 ± 3
Cl^-/HCO_3^-	0.54 ± 0.04	74 ± 3

bain receptor and band 3 is intimate enough to modulate the basic ion exchange property of band 3. We may also conclude that the effect of ouabain on τ_{DBDS} is not the result of a cell pH shift, following replacement of cell Cl^- with HCO_3^-.

These experiments show that the specific extracellular cation transport inhibitor, ouabain, has a direct effect on red cell anion flux and very strongly suggest that the cation and anion transport proteins are contiguous to one another in the red cell membrane or connected by a link through which conformational information can flow. It is generally agreed that the number of ouabain sites/cell, and hence of copies of membrane Na^+,K^+-ATPase is ≈250 (Hoffmann, 1969), though Gardner and Conlon (1972) report that ouabain binds to a single class of specific sites with a maximum binding affinity of 1200 sites/cell. The disproportion between the relatively few ouabain binding sites on the red cell membrane and the 10^6 copies of band 3 means that some sort of an amplification mechanism is necessary to link these two processes. Assuming that the amplification factor can be approximated by the ratio of copies/cell, it would be in the range of 1,000–10,000.

The NO_3^- experiment with 0.1 μM ouabain (Table 4) speaks directly to the question of the amplification factor. In these experiments 1 ml of packed red cells was diluted to 7 ml, with a final ouabain concentration of 0.1 μM. One ml of packed red cells contains 1.2 × 10^{16} molecules of band 3. Six ml of 0.1 μM ouabain contains 4.2 × 10^{14} molecules of ouabain, which means that there is only one ouabain molecule in the system for every 30 band 3 molecules. Since the cells were washed after the ouabain reaction was completed, this figure is a maximum, which shows that a single ouabain molecule must have interacted with at least 30 band 3 molecules, and probably many more. In the case of the DBDS data shown in Fig. 7, 10^{-8} M ouabain produced a significant change in τ^{-1}_{DBDS}. A similar calculation on the molecular ratio of molecules

shows that the change in τ^{-1}_{DBDS} was produced by one ouabain molecule for each 200 band 3 molecules. These considerations show that it is not possible to ascribe our observed shifts in $\tau^{-1}_{Cl^-}$ and τ^{-1}_{DBDS} to a 1 : 1 interaction between ouabain and band 3 and provide firm support for our conclusion that an amplification factor is required.

Fossel and Solomon (1977) found that extracellular ouabain caused changes in the NMR spectrum of intracellular 2,3-DPG (2,3-diphosphoglycerate) in the native red cell. Since the intracellular 2,3-DPG concentration is ≈4.5 mmol/liter of cells, Solomon (1978) concluded that an amplification factor of the order of 1.5×10^3 was required in order to account for the measured resonance shifts. It is interesting that this factor, obtained from an entirely different type of experiment, is in reasonable agreement with our present estimate. Band 3, which is known to bind to the cytoskeleton, has binding sites for at least some, and possibly all, the glycolytic enzymes (*see* Fossel & Solomon, 1978; Low, 1986) and 2,3-DPG is a cofactor for the glycolytic enzyme, monophosphoglycerate mutase. If, as is not unlikely, Na^+,K^+-ATPase also binds to the cytoskeleton, transmission of conformational information along the cytoskeletal network could provide the mechanism for these amplification factors.

Discussion

Maintenance of Native Red Cell Integrity Is Essential

One of the striking features of these experiments is the observation that both of the linkages we have observed are apparent in native red cells, but are not found in white ghosts. These observations appear to be related to Ojcius et al. (1988) findings that the permeability properties of white ghosts differ from those of the native red cell, discussed above. Beth et al. (1986) have reported that red cell lysis causes a marked change in the rotational mobility of band 3, indicating a significant disruption of interactions of band 3 with fixed membrane elements. These elements were initially thought to be the cytoskeleton, but subsequently the other membrane proteins that remain after extraction of bands 1, 2 and 5 have been implicated (Beth et al., 1987). This finding is consistent with our observation of close linkages among band 3, the glucose transport protein and the Na^+,K^+-ATPase.

Salhany, Rauenbuehler and Sloan (1987) have found that preparation of white ghosts exposes new binding sites on the membrane-bound fragment of band 3, with which the anion transport inhibitor, pyridoxal 5'-phosphate can react. Since these sites are nonreactive in the native red cell, the results of Salhany et al. (1987) show that preparation of white ghosts causes a substantive conformational change in the membrane bound fragment of band 3. Bennett (1985) reports that tropomyosin is released from its binding to actin during the preparation of white ghosts in phosphate buffered solutions. Thus, it seems clear that band 3 in white ghosts no longer retains its native configuration, and it is probable that the cytoskeleton/band 3 linkage has also been modified. Our studies provide evidence that these changes affect the anion transport system, as shown by the altered kinetic constants for DBDS binding in Table 1. Thus, we may conclude that the loss of native membrane integrity goes hand-in-hand with the loss of transport protein interconnections and configurational changes in band 3.

Evidence for a Membrane/Cytoskeletal Enzyme Transport Complex

The common thread that links these diverse observations together is cation transport. Glucose provides the fuel to drive the cation pump. If there is a requirement for delivery of exogenous phosphate to a specific locale to maintain the ADP-ATP balance, band 3 would provide the route. The cytoskeletal pole of band 3 provides the binding sites for the glycolytic enzymes. Proverbio and Hoffman (1977) have shown that membrane-bound phosphoglycerate kinase (PGK) generates ATP in a compartmentalized pool, which is used to drive the cation pump. Mercer and Dunham (1981) have confirmed this finding and have shown that membrane-bound G3PDH, with PGK, forms a membrane-bound complex to provide the membrane-associated ATP.

Low (1986) has pointed out that the cytoplasmic domain of band 3 is poised "under physiological conditions in a sensitive metabolic equilibrium among three conformations" and suggested among other possibilities, that interactions with peripheral proteins, such as G3PDH, could alter this sensitive equilibrium. Kant and Steck (1973) have shown that the binding of G3PDH to band 3 is ionic strength dependent. Fossel and Solomon (1981) found that DIDS modulated the effect of ouabain on resonance shifts in intracellular 2,3-DPG. They also used the ^{31}P resonance of glyceraldehyde 3-phosphate (G3P) to report on the conformation of G3PDH in inside-out red cell vesicles and found that G3P resonance shifts were dependent upon the Na/K concentration ratio on both sides of the membrane (Fossel & Solo-

mon, 1979). These studies had been carried out to show the relationship of G3PDH conformation to Glynn and Lew's (1970) finding that Na and K gradients could be used to drive the red cell cation transport pump backwards and produce ATP. All of these observations, from diverse sources, suggest that band 3 is well placed to serve as a centerpiece for the assembly of a complex of membrane transport proteins that combine their functions to produce an orderly movement of ions across the membrane, and that G3PDH may occupy a privileged position to serve as regulator for the concerted process.

Just as the assembly of the glycolytic enzymes into a single megadalton complex in the cytoplasm expedites the transfer of intermediate products in the glycolytic cycle from enzyme to enzyme, so would an enzyme transport complex, centered upon band 3, discharge the same function for cation transport and provide, as well, a ready explanation for all of our observations in this communication.

This work was supported in part by a National Institutes of Health grant GM 34099, by the American Cancer Society, Massachusetts Division, and by the Council for Tobacco Research-U.S.A. A.J. is a Fellow of the American Heart Association, Massachusetts affiliate.

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Received 2 November 1988; revised 18 May 1989