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Interaction of Thiourea with Band 3 in Human Red Cell Membranes

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Summary. Although urea transport across the human red cell membrane has been studied extensively, there is disagreement as to whether urea and water permeate the red cell by the same channel. We have suggested that the red cell anion transport protein, band 3, is responsible for both water and urea transport. Thiourea inhibits urea transport and also modulates the normal inhibition of water transport produced by the sulfhydryl reagent, *p*CMBS. In view of these interactions, we have looked for independent evidence of interaction between thiourea and band 3. Since the fluorescent stilbene anion transport inhibitor, DBDS, increases its fluorescence by two orders of magnitude when bound to band 3 we have used this fluorescence enhancement to study thiourea/band 3 interactions. Our experiments have shown that there is a thiourea binding site on band 3 and we have determined the kinetic and equilibrium constants describing this interaction. Furthermore, *p*CMBS has been found to modulate the thiourea/band 3 interaction and we have determined the kinetic and equilibrium constants of the interaction in the presence of *p*CMBS. These experiments indicate that there is an operational complex which transmits conformational signals among the thiourea, *p*CMBS and DBDS sites. This finding is consistent with the view that a single protein or protein complex is responsible for all the red cell transport functions in which urea is involved.

Key Words red cell · band 3 · thiourea · anion transport · urea transport · *p*CMBS · stilbene inhibitor · fluorescence enhancement · stopped-flow

Introduction

Urea permeates the human red cell membrane so rapidly that extracellular concentrations as high as 0.5 M equilibrate with the cytoplasm in about 0.3 sec (Sha'afi et al., 1971). Solomon et al. (1983) have presented evidence indicating that the transport takes place through an aqueous channel in band 3. Others, particularly Macey and Farmer (1970), Wieth et al. (1974), Mayrand and Levitt (1983) and Brahm (1983) have argued that urea transport takes place by a specialized transport system whose locus has not been specified. Wieth et al. (1974) reported that thiourea inhibits urea transport; this inhibition has been shown to be specific to amides by Solomon and Chasan (1980) and Mayrand and Levitt (1983). In order to throw further light on the mechanism and location of the urea transport system, we have looked for independent evidence of an interaction between thiourea and band 3.

It has been shown that anion exchange across the human red cell membrane is mediated by band 3 and that the stilbene anion transport inhibitor, DIDS (4,4′-diisothiocyano-2,2′-disulfonic stilbene), binds to an external site on band 3 with 1 : 1 stoichiometry and specifically inhibits anion exchange (*see* Knauf, 1979). A fluorescent DIDS analogue, DBDS (4,4′-dibenzamido-2,2′-disulfonic stilbene) binds reversibly and specifically to the DIDS-reactive site where it also inhibits anion exchange (Cabantchik & Rothstein, 1972; Rao et al., 1979); the fluorescence intensity of DBDS increases by two orders of magnitude when bound to the red cell membrane. We have previously measured DBDS fluorescence enhancement by stopped-flow and temperature-jump methods to characterize the reaction kinetics of DBDS binding (Verkman et al., 1983) and have shown that the first steps of the process take place according to the reaction scheme:

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

$$\text{DBDS}$$

in which the first step is a bimolecular association (Eq. constant, K_1), too fast to measure on our apparatus, and the second is a conformation change to produce band 3*-DBDS with rate constants k_2 and k_{-2}. We have now found that thiourea alters the time course of DBDS fluorescence and has significant effects on the entire reaction scheme.

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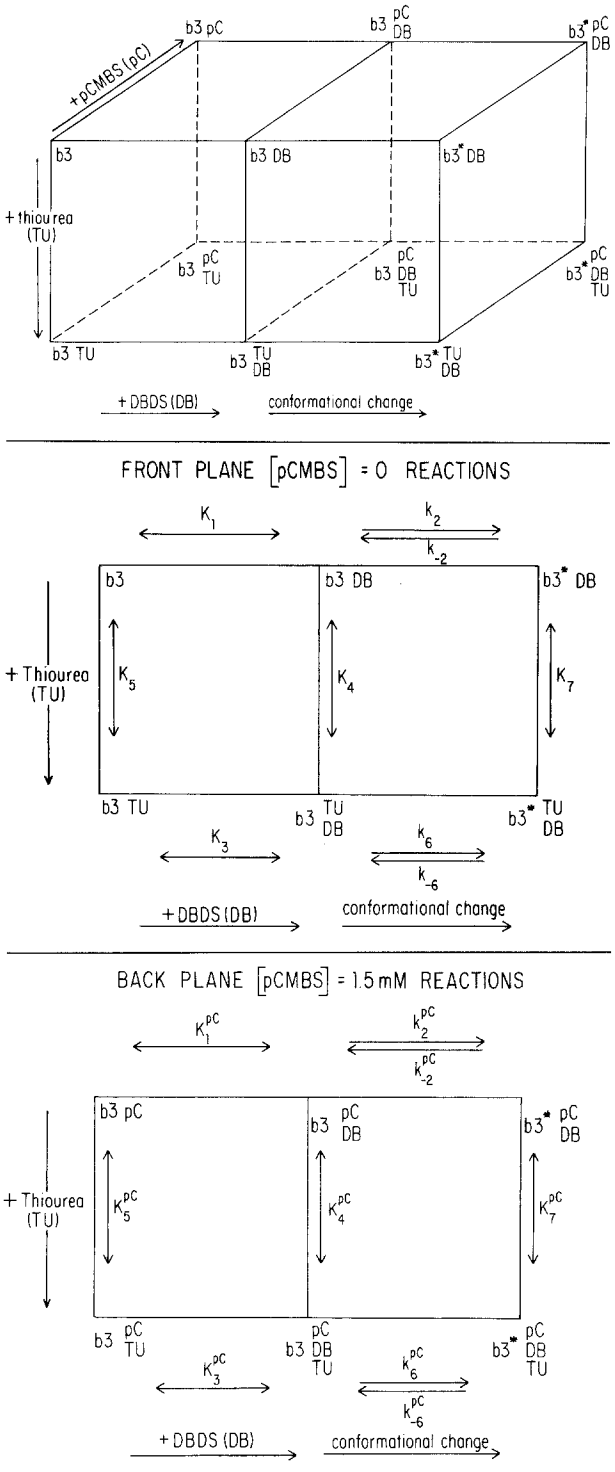


Fig. 1. (Top) Generalized reaction scheme for the band 3/DBDS/*p*CMBS/thiourea system. (Middle) Front plane showing reactions in the absence of *p*CMBS. (Bottom) Back plane showing reactions in the presence of *p*CMBS

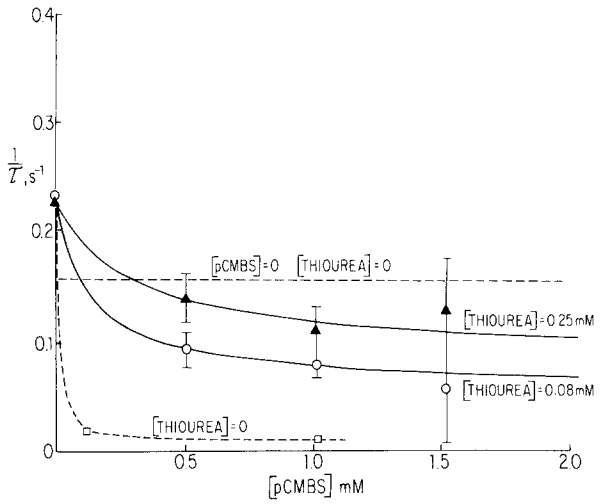


Fig. 2. Suppression of fluorescence enhancement rate, τ^{-1}, at two different concentrations of thiourea (TU), 0.08 mM (○) and 0.25 mM (▲) (five runs; errors are SEM). The upper dashed line indicates the base rate, at [TU] = 0. $[DBDS]_{tot}$ = 0.11 μM, $[band\ 3]_{tot}$ = constant = 0.02 to 0.04 μM, 28.5 mM sodium citrate, pH 7.4, 21°C. The lower dashed line with points (□) intercepts the upper dashed line on the $1/\tau$ axis and shows the effect of *p*CMBS at [TU] = 0 calculated from the binding constants of Lukacovic et al. (1984, Table 1)

Macey and Farmer (1970) showed that *p*CMBS (*p*-chloromercuribenzene sulfonate) inhibited the flux of water across the red cell membrane. We have suggested that red cell water transport utilizes the aqueous channel in band 3 and that the *p*CMBS site is located on band 3 (Solomon et al., 1983). Recently, Chasan et al. (1984) have shown that thiourea suppresses the *p*CMBS inhibition of water transport. The present paper presents further evidence showing that *p*CMBS modulates the specific reactions between thiourea and band 3.

Materials and Methods

Ghost Preparation

Red cell ghosts were prepared from human whole blood outdated less than 3 days using a hemolysis procedure similar to that of Dodge et al. (1963). Whole blood was washed three times with PBS (phosphate-buffered saline containing 150 mM NaCl and 5 mM Na_2HPO_4), pH 8.0 at 4°C, and was lysed in 5 mM Na_2HPO_4 under the same conditions with a buffer/cell volume ratio of 40 : 1. This lysing procedure was repeated three additional times, giving finally a white, hemoglobin-free pellet of red cell ghosts. Total protein concentration in the final pellet was determined by the method of Lowry et al. (1951).

NEM (N-Ethylmaleimide) Treatment

Band 3 contains six sulfhydryl groups of which five are located on the cytoplasmic side of the membrane and react with NEM (Steck et al., 1978; Rao, 1979; Ramjeesingh et al., 1980); two of these are in the membrane bound C-terminal 35 kiloDalton (kD) fragment (Ramjeesingh et al., 1983). The sixth, which is in the 17

kD membrane-bound fragment of band 3 (between the trypsin and the chymotrypsin cuts) does not react with NEM but does react with *p*CMBS (Rao & Reithmeier, 1979; Rothstein & Ramjeesingh, 1982; Solomon et al., 1983). Rao (1978) has shown that reaction of the cytoplasmic sulfhydryl groups with NEM blocks subsequent reaction with *p*CMBS. She found that NEM uptake in red cell ghosts saturated at 0.2 to 0.5 mM NEM for 1-hr incubation in 5 mM Na_2HPO_4, pH 7.0 at 37°C. All of our experiments have been done with NEM-treated ghosts that have been incubated with one volume of 2 mM NEM (Sigma Chemical Co., St. Louis, Mo.) in PBS, pH 7.4 for one hour at ambient temperature (21 to 23°C) and subsequently washed three times with NEM-free PBS at pH 7.4. Since we have used a higher NEM concentration, a lower temperature and a slightly different pH than Rao, we have carried out control experiments to see whether longer times of incubation or higher temperatures had any effect in our system. Neither extending the incubation time to 2 hr at 23°C or raising the temperature for a 1-hr incubation to 37°C caused any change in the effect of *p*CMBS on DBDS fluorescence enhancement. This makes it likely that the *p*CMBS effects reported in this paper are not to be attributed to the cytoplasmic SH groups of band 3.

Incubation with Thiourea and *p*CMBS

A stock solution of 100 mM thiourea, pH 7.4, in 28.5 mM Na citrate was made up on the day of the experiment and was diluted with the citrate buffer to desired concentrations. For studies of thiourea effects on DBDS binding, two 15-ml samples were made up at each thiourea concentration; typically 10 to 20 μl of stock (770 μM) DBDS solution was added to one tube and 100 μl of the NEM-treated ghost pellet was added to the other. For studies of *p*CMBS effects, the ghosts were preincubated with 1.5 mM *p*CMBS for 30 min. Because thiourea reacts with ghosts practically instantaneously, no incubation time was required.

Fluorescence Studies of Anion Transport Inhibitor Binding

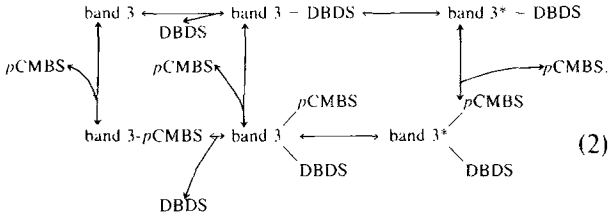
Kinetic fluorescence studies of DBDS binding to four differently treated ghost preparations (NEM, thiourea-NEM, *p*CMBS-NEM and thiourea-*p*CMBS-NEM), were performed with the stopped-flow apparatus designed and built in our laboratory and described previously (Verkman et al., 1981). The device has a dead time of 60 msec and records optical signals on a Biomation (Cupertino, Calif.) 805 waveform recorder; the data is then transferred on-line to a PDP 11/34 computer for storage and analysis. A typical trace is shown in Lukacovic et al. (1984, Fig. 2). Experiments were carried out at room temperature (21 to 23°C).

DBDS was synthesized by the method of Kotaki et al. (1971) and checked for purity by thin-layer chromatography on Silica Gel G in pyrydine/acetic acid/water (10 : 1 : 40). The concentration of DBDS was checked by its absorbance at 336 nm, and binding to ghost membranes was monitored by the accompanying fluorescence enhancement (excitation, 356 nm; emission > 410 nm).

Results

Reaction Scheme

We have previously shown that *p*CMBS binding affects the rate of the DBDS fluorescence change and have put forward the following reaction scheme to describe these interactions (Lukacovic et al., 1982, 1984)



(2)

Verkman et al. (1983) have shown that there is a second bimolecular association step in which a second DBDS molecule binds to a site on the second monomer in the band 3 dimer. The equilibrium constant for this association is difficult to resolve experimentally and Lukacovic et al. (1984) did not need to use this step in order to describe the band 3/DBDS/*p*CMBS interaction kinetics that they observed. We also will not take account of this second association step which, in any case, cannot be resolved when Cl^- is present in the medium. Our studies are concerned with interactions of thiourea with the band 3-DBDS system, both in the presence and absence of *p*CMBS, so that the reaction scheme is much more complex than shown in Eq. (2). We have used the symbolism of the generalized scheme in Fig. 1 (top) to describe the kinetics. The reactions of thiourea with band 3 in the absence of *p*CMBS are shown in the front plane (Fig. 1, middle) and those of thiourea with the band 3/DBDS/*p*CMBS system are shown in the back plane (Fig. 1, bottom).

The time constant τ of the fluorescence enhancement following rapid mixing of ghosts and DBDS, according to the reaction scheme of Eq. (1), is given by

$$\frac{1}{\tau} = k_2 \frac{[DB]}{[DB] + K_I} + k_{-2} \qquad (3)$$

in which [DB] represents the concentration of free DBDS. Equation (3) depends upon limiting conditions which are satisfied at the concentrations used in our experiments; [DBDS] $\gg$ [band 3] (Verkman et al., 1983). Figure 2 shows the competitive action of *p*CMBS and thiourea on the rate of increase of the fluorescence, τ^{-1}: *p*CMBS suppresses τ^{-1} with a K_I about 0.2 mM, whereas thiourea diminishes the *p*CMBS effect. The level of τ^{-1} at [*p*CMBS] = 0 is also elevated by thiourea, as discussed in the next section.

There are six SH groups in band 3; five are in the cytoplasm of the cell and react with NEM and

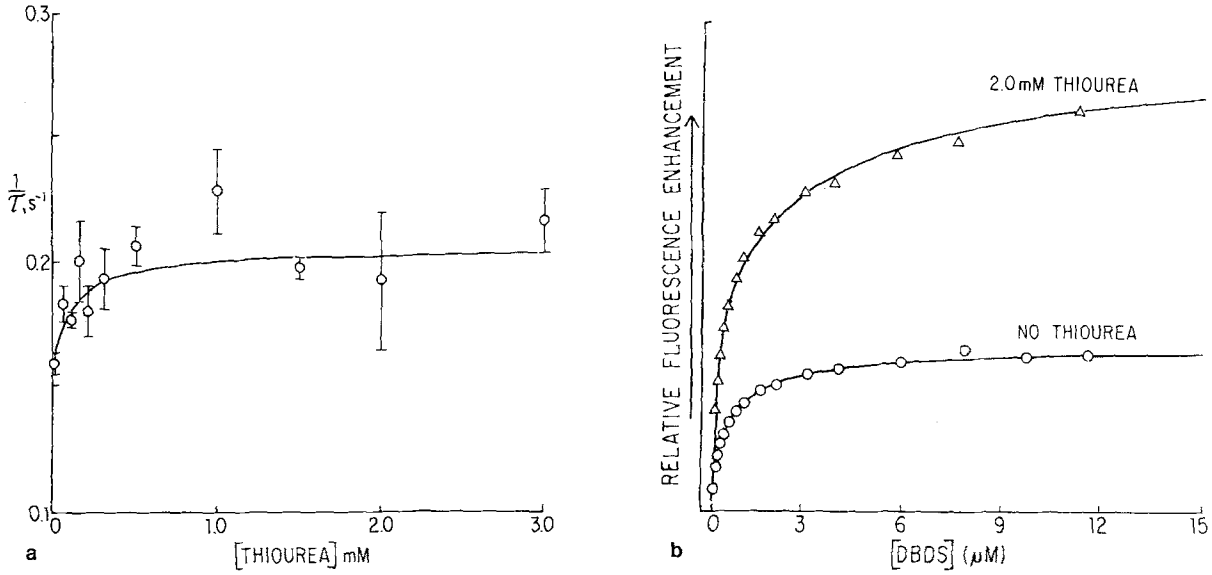


Fig. 3. *a)* Variation of fluorescence enhancement rate with total TU concentration. *b)* Dependence of equilibrium fluorescence on [DBDS] at 2.0 mM TU (△) compared with baseline values (○). Means and errors of five runs

*p*CMBS (Rao, 1979; Rao & Reithmeier, 1979). Reaction with NEM protects the cytoplasmic SH groups from reaction with *p*CMBS and vice versa. The sixth SH group is in the 17 kD membrane-bound fragment of band 3 that also contains a covalent binding site[1] for the stilbene anion transport inhibitor, DIDS (Steck et al., 1978; Ramjeesingh et al., 1980). This sixth SH group, which controls the inhibition of water transport, does not react with NEM (Solomon et al., 1983). Since we are interested in the properties of this sixth SH group, the experiments reported in this paper have been carried out on NEM-treated ghosts in which the *p*CMBS reaction with band 3 is confined to this single SH group.

For the analysis of thiourea interactions with the band 3/*p*CMBS/DBDS system, it is practical to consider only two extreme cases, [*p*CMBS] = 0 and [*p*CMBS] $\gg$ 0.2 mM, the latter being roughly the K_D for binding of *p*CMBS to the protein. It is assumed that the interaction of thiourea with band 3 is rapidly equilibrated in comparison with the fluorescence kinetics; this is corroborated by the finding, described below, that the observed kinetics can be modeled faithfully with only first-order rate constants.

Fluorescence Enhancement in the Absence of *p*CMBS (Front Plane, Fig. 1, Middle)

The dead time of our stopped-flow apparatus is approximately 60 msec, too slow to permit visualization of the fast bimolecular association of DBDS and band 3. The measurable relaxation fits well to a single exponential by least-squares analysis. Figure 3 shows that both the rate ($1/\tau$) of rise of the fluorescence and the amplitude (equilibrium fluorescence) of the slow component are increased by thiourea in one experiment, typical of three. The front plane reaction system of Fig. 1 (middle) predicts that there will be only one resolvable relaxation process, even at intermediate thiourea concentrations, because the two postulated slow steps, reactions 2 and 6, are coupled via reactions with thiourea: only one slow normal-mode relaxation is predicted. As derived in the Appendix, when [DBDS] $\gg$ [band 3] the reciprocal value of the corresponding time constant τ is given by

[1] As Jennings and Passow (1979) have shown, the other end of the difunctional DIDS molecule binds to a lysine on the 35 kD C-terminal fragment; Jennings (1984) has summarized the evidence that this second lysine is probably associated with the anion transport pathway, whereas the lysine in the 17 kD fragment is not. Although the second lysine is at least 72 residues from the first lysine, the two residues cannot be more than 12.5 Å apart since the 12.5-Å-long DIDS molecule can bind to both sites simultaneously. The evidence that DBDS and DIDS bind to the same site has been given by Rao et al. (1979).

$$\frac{1}{\tau} = \frac{(k_2 + k_6[TU]/K_4)[DB]}{(1 + [TU]/K_4)[DB] + (1 + [TU]/K_5)K_1} + \frac{k_{-2} + k_{-6}[TU]/K_7}{1 + [TU]/K_7} \quad (4)$$

in which the concentration of free thiourea is denoted by [TU]. The rate constants, k_2 and k_{-2}, and the equilibrium constant K_1 have been reported previously by Verkman et al. (1983). We repeated their experiments as our control study and obtained numerical values for these parameters similar to those originally reported (*see* Table).

The data shown in Fig. 3*a* are typical of three studies suggesting that, when [TU] $\gg$ 0.5 mM, the effect of thiourea on DBDS binding kinetics is saturated. Since τ^{-1} saturates at high [TU], the relations $k_2 \ll k_6[TU]/K_4$ and $k_{-2} \ll k_{-6}[TU]/K_7$ must hold when [TU] $\gg$ 0.5 mM. Therefore, when one observes the kinetics at [TU] $\gg$ 0.5 mM, Eq. (4) can be approximated by the simpler expression

$$\frac{1}{\tau} = \frac{k_6[DB]}{[DB] + K_3} + k_{-6}. \quad (5)$$

This approximation is justified because [DBDS] $\ll K_1$(K_1 = 3 μM, as compared to [DBDS] $\leq$ 0.5 μM in all our experiments). The approximation also depends on the observation from data such as those in Fig. 3*a*, showing that all thiourea binding equilibria have a K_D on the order of 0.1 mM. Hence, $[TU]/K_4$, $[TU]/K_5$, and $[TU]/K_7 \gg 1$ if [TU] $\gg$ 0.1 mM. Additionally, Eq. (5) incorporates the relation

$$K_3 = K_1K_4/K_5 \quad (6)$$

which is the thermodynamic requirement of detailed dynamic balance.

When [TU] $\gg$ 0.1 mM, all band 3 is complexed with thiourea, and Eq. (5) can be used to extract the equilibrium and rate parameters from variation of τ with varying [DBDS]. Figure 4 shows the plot of reciprocal time constants against [DBDS], under conditions in which thiourea effects are saturated ([TU] = 2.0 mM; one experiment typical of three). The linear relation in Fig. 4 would be predicted if $K_3 \gg$ [DB] in Eq. (5) when [DB] $\leq$ 0.5 μM. The intercept of the fitted linear regression line with the τ^{-1} axis gives the value

$$k_{-6} = 0.10 \pm 0.04 \text{ sec}^{-1}$$

and the slope gives the parameter ratio

$$k_6/K_3 = 0.50 \pm 0.13 \text{ sec}^{-1}\ \mu\text{M}^{-1}.$$

Table. Effects of *p*CMBS on kinetic and equilibrium binding parameters for DBDS and thiourea binding to band 3

A. DBDS binding reaction scheme for the conditions [band 3] $\ll$ [DBDS] < 0.5 μM:

$$\text{DBDS} + \text{band 3} \overset{K_i}{\leftrightarrow} \text{band 3} \cdot \text{DBDS} \underset{k_{-j}}{\overset{k_j}{\rightleftharpoons}} \text{band 3}^* \cdot \text{DBDS}$$

Reaction conditions	Parameter values	Units	Fig. 2 symbol
[Thiourea] = 0	$K_i = 3.0 \pm 1.5$	μM	K_1
[*p*CMBS] = 0[a]	$k_j = 1.7 \pm 0.5$	sec^{-1}	k_2
	$k_{-j} = 0.09 \pm 0.03$	sec^{-1}	k_{-2}
[Thiourea] = 2.0 mM	$K_j = 4.2 \pm 1.7$	μM	K_3
[*p*CMBS] = 0	$k_j = 2.1 \pm 0.3$	sec^{-1}	k_6
	$k_{-j} = 0.10 \pm 0.04$	sec^{-1}	k_{-6}
[Thiourea] = 0	$K_i = 28 \pm 5$	μM	K_1^{pC}
[*p*CMBS] = 1.5 mM	$k_j = 4.0 \pm 0.5$	sec^{-1}	k_2^{pC}
	$k_{-j} = 0.10 \pm 0.05$	sec^{-1}	k_{-2}^{pC}
[Thiourea] = 20.0 mM	$K_i = 0.6 \pm 0.3$	μM	K_3^{pC}
[*p*CMBS] = 1.5 mM	$k_j = 0.2 \pm 0.2$	sec^{-1}	k_6^{pC}
	$k_{-j} = 0.20 \pm 0.03$	sec^{-1}	k_{-6}^{pC}

B. Thiourea (TU) binding reactions with band 3 (*b*3):

$$\text{TU} + b3 \overset{K_l}{\rightleftharpoons} b3\text{TU} \qquad \text{TU} + \text{DB}b3 \overset{K_m}{\rightleftharpoons} \text{DB}b3\text{TU} \qquad \text{TU} + \text{DB}b3^* \overset{K_n}{\rightleftharpoons} \text{DB}b3^*\text{TU}$$

[*p*CMBS] = 0	$K_l \approx 0.23$	mM	K_5
	$K_m \approx 0.23$	mM	K_4
	$K_n \approx 0.19$	mM	K_7
[*p*CMBS] = 1.5 mM	$K_l \approx 0.42$	mM	K_5^{pC}
	$K_m = 9 \pm 3$	μM	K_4^{pC}
	$K_n \approx 0.36$	mM	K_7^{pC}

[a] Values for DBDS binding to band 3 have been reported previously by Verkman et al. (1983) as $K_1 = 3.0 \pm 1.4$ μM, $k_2 = 4.0 \pm 1.5$ sec^{-1} and $k_{-2} = 0.09 \pm 0.02$ sec^{-1}. The DBDS used in their experiments came from an earlier synthesis of DBDS.

With these estimates, k_6 and K_3 can be calculated from the relation

$$k_6^{-1} = (\tau^{-1} - k_{-6})^{-1} - \{(k_6/K_3)[DB]\}^{-1}. \quad (7)$$

This approach is, however, highly inaccurate when [DB] $\ll K_3$, and k_6 can be more accurately estimated from the curvature of the τ^{-1}-[DB] plot when [DB] approaches K_3. Nonlinear curve-fitting gives

$$k_6 = 2.1 \pm 0.3 \text{ sec}^{-1},$$

from which

$$K_3 = 4.2 \pm 1.7 \ \mu\text{M}$$

is calculated. Although it is still difficult to pinpoint the reaction step responsible for the kinetic en-

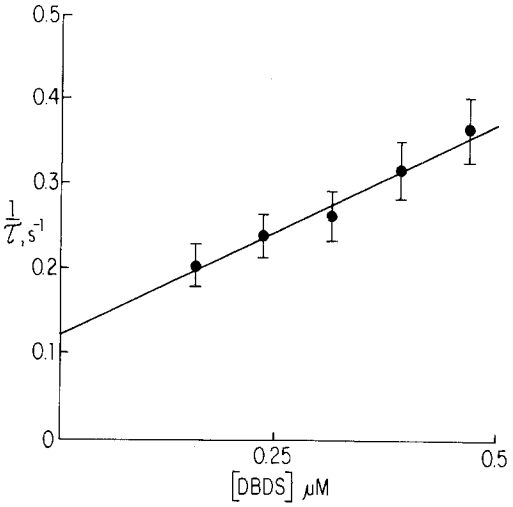


Fig. 4. Rate of fluorescence increase plotted against total DBDS concentration when [TU] = 2.0 mM, fitted by least-squares linear regression. Means and errors of five runs

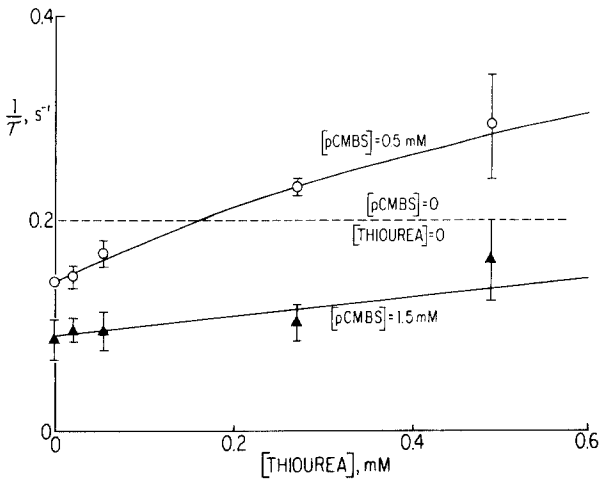


Fig. 5. Variation of fluorescence enhancement rate with thiourea concentration. (○) [*p*CMBS] = 0.5 mM; (▲), [*p*CMBS] = 1.5 mM. The dashed line shows the baseline value ([*p*CMBS] = 0; [TU] = 0). $[DBDS]_{tot}$ = 0.11 μM, $[band\ 3]_{tot}$ = constant = 0.02 to 0.04 μM. Means and errors of five runs

hancement shown in Fig. 3*a*, these calculations suggest that the 25% increase in τ^{-1} probably results from k_6 being larger than k_2. Interestingly, the overall equilibrium constants for DBDS binding in the absence and presence of thiourea are roughly equivalent:

$$K_1^* = K_1/(1 + k_2/k_{-2}) = 0.17\ \mu M, \quad (8)$$

$$K_3^* = K_3/(1 + k_6/k_{-6}) = 0.14\ \mu M. \quad (9)$$

This suggests that the enhancement of equilibrium fluorescence shown in Fig. 3*b* is a result of either the appearance of additional DBDS binding sites with identical affinities, or of an increased fluorescence of bound DBDS in the presence of thiourea. Of these two choices, the second is more likely, and our finding that raising [TU] from 0 to 10 mM does not influence the fluorescence of ghost-free DBDS solutions suggests that the thiourea-mediated fluorescence enhancement is a membrane effect. Hence, thiourea appears to influence either the quantum yield or the excited-state lifetime of bound DBDS.

With DBDS binding parameters determined, it is possible to estimate the three equilibrium constants that describe thiourea interactions, K_4, K_5 and K_7 in Fig. 1. From detailed dynamic balance requirements,

$$K_7 = K_3K_6K_5/(K_1K_2). \quad (10)$$

This relation, along with Eq. (6), leaves only one independent parameter to describe thiourea binding. The above data analysis allows the approximation

$$K_3 \approx K_1 \quad (11)$$

and from Eq. (6),

$$K_5 \approx K_4. \quad (12)$$

With $K_6 = k_{-6}/k_6 = 0.048$ and $K_2 = k_{-2}/k_2 = 0.059$, Eq. (10) gives

$$K_7 \approx 0.81\ K_5 \approx 0.81\ K_4. \quad (13)$$

When [DB] is constant (0.13 μM), a simplified approximation for Eq. (4) may be obtained by setting $K_7 = K_5 = K_4$ and using the numerical value of [DB]. Under these conditions, Eq. (4) can be reduced to a function of K_4 only:

$$\frac{1}{\tau} = \frac{0.50 + 1.1[TU]/K_4 + 0.59([TU]/K_4)^2}{3.1 + 6.2[TU]/K_4 + 3.1([TU]/K_4)^2}. \quad (14)$$

For purposes of analysis, it is possible to determine K_4 with an expression equivalent to Eq. (14) within experimental errors;

$$\frac{1}{\tau} = (0.16)\left[\frac{1 + 1.15X}{1 + X}\right] \quad (15)$$

in which the new variable X is defined as

$$X = 2[\mathrm{TU}]/K_4 + ([\mathrm{TU}]/K_4)^2. \quad (16)$$

The numerical values of Eqs. (14) and (15) differ generally by less than 5%. The advantage in using Eq. (15) is that the half-maximum increase in τ^{-1} must occur at $X = 1$. The data shown in Fig. 3*a* indicate that $X = 1$ when [TU] $\cong$ 0.1 mM, and therefore that K_4 is approximately the solution of Eq. (16) with $X = 1$ and [TU] = 0.1 mM. This yields the value

$K_4 = 0.23$ mM.

Equations (12) and (13) then give

$K_5 \approx 0.23$ mM

$K_7 \approx 0.19$ mM.

The uncertainties in these values are large, 100 to 200%, but these calculations do provide quantitative estimates for the affinity of the various band 3 forms for thiourea. All the parameter values calculated for the front plane in Fig. 1 (middle) are summarized in the Table.

Thiourea Effects in the Presence of *p*CMBS (Back Plane, Fig. 1, Bottom)

Both the amplitude and rate of rise of fluorescence following binding of DBDS to band 3 are greatly reduced if the ghosts are prereacted with *p*CMBS. However, if the *p*CMBS-ghost reaction takes place in the presence of thiourea, the repressive influence of *p*CMBS on DBDS binding is greatly reduced. Figure 5 shows both the inhibitory influence of *p*CMBS on the rate of DBDS fluorescence enhancement and the reduction of the *p*CMBS effect due to thiourea. Figure 5 shows a residual *p*CMBS effect, even at high thiourea concentrations, suggesting that thiourea does not displace all the *p*CMBS from the protein entirely. Considered within the framework of schematic models, the data argue against direct site-to-site competition, and favor a shunt model in which *p*CMBS and thiourea influence the DBDS binding site collectively, as shown in Fig. 1 (bottom).

Figure 6 illustrates the effect of thiourea on equilibrium fluorescence; the [TU] = 1.0 mM data point shows the asymptotic values of the recovery from *p*CMBS treatment in one experiment, typical of five. The K_I value of the thiourea inhibition of the *p*CMBS effect is estimated to be about 0.5 mM.

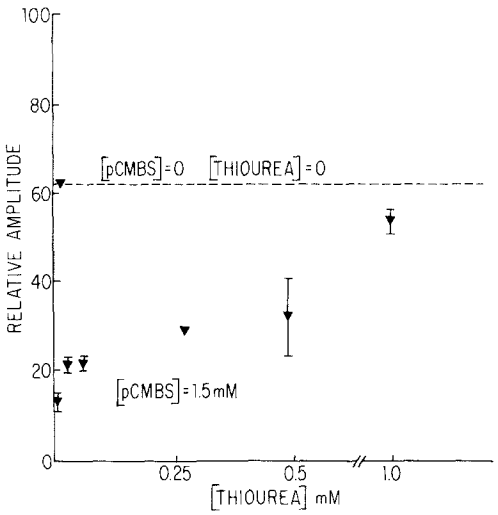


Fig. 6. Equilibrium fluorescence after DBDS complexing with band 3 protein in 1.5 mM *p*CMBS at various concentrations of thiourea. Dashed line marks [*p*CMBS] = 0 value. The K_I for inhibition of the *p*CMBS effect is about 0.5 mM. $[\mathrm{DBDS}]_{tot} = 0.11$ μM, $[\text{band 3}]_{tot}$ = constant = 0.02 to 0.04 μM. Five runs at all thiourea concentrations except for the single experiment at [TU] = 0.25 mM

Therefore, if [TU] $\gg$ 0.5 mM, DBDS binding can be modeled with only the lower rail of the back plane model (Fig. 1, bottom). The three parameters describing DBDS binding under these conditions, K_3^{pC}, k_6^{pC} and k_{-6}^{pC}, can be determined as in the *p*CMBS-free case.

The dependence of τ^{-1} on DBDS concentration when [*p*CMBS] = 1.5 mM and [TU] = 20.0 mM is shown in Fig. 7. Unlike the linear trend seen in Fig. 4, τ^{-1} begins to saturate at relatively low DBDS concentrations (one experiment, typical of two), suggesting that $K_3^{pC} \ll K_3$. A second *p*CMBS effect is that τ^{-1} saturates at a much lower value of DBDS; $\tau^{-1} \approx 0.4$ sec^{-1} when [DBDS] ≥ 0.8 μM (not shown in Fig. 7). Under these conditions, τ^{-1} is given by an expression analogous to Eq. (5), and the asymptotic value of τ^{-1} leads to the relation

$$k_6^{pC} + k_{-6}^{pC} \approx 0.4 \text{ sec}^{-1}. \quad (17)$$

The nonlinear least-squares fit of Eq. (5) (*p*CMBS analogue) to the data shown in Fig. 7 gives the values

$k_6^{pC} = 0.2 \pm 0.2$ sec^{-1},

$k_{-6}^{pC} = 0.20 \pm 0.03$ sec^{-1},

$K_3^{pC} = 0.6 \pm 0.3$ μM.

From comparison of these results with those given in the previous section, it is seen that the effect of

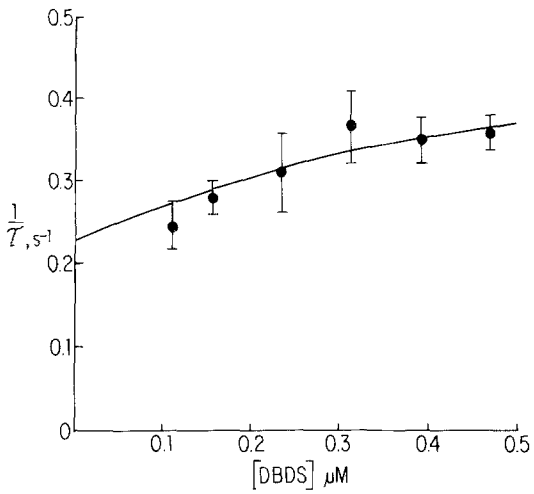


Fig. 7. Rate of fluorescence increase after DBDS binding as a function of [DBDS] when $[p\text{CMBS}]_{tot}$ = 1.5 mM and [TU] = 20 mM. The curve is a nonlinear least-squares fit of the analogue of Eq. (5) to the data. $[\text{band 3}]_{tot}$ = constant = 0.02 to 0.04 μM. Means and errors of five runs

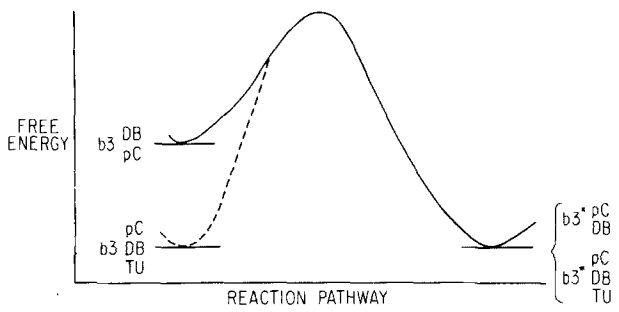


Fig. 8. Energy diagram illustrating effect of thiourea on the DBDS-band 3 complex in the presence of *p*CMBS. Depression of the free energy of the low-fluorescence complex can account for the different kinetics and amplitudes in the presence of thiourea

*p*CMBS is to reduce K_3 and k_6 by about one order of magnitude. The relation $k_6^{pC} \approx k_{-6}^{pC}$ indicates that the low and high fluorescence states (band 3-DBDS-*p*CMBS-thiourea and band 3*-DBDS-*p*CMBS-thiourea) are about equally stable. This is in marked contrast with the *p*CMBS-free case, in which (band 3*-DBDS-thiourea)/(band 3-DBDS-thiourea) ≈ 20 at equilibrium. The free energy distribution among the DBDS states is illustrated schematically in Fig. 8, in which the free energy of the two high fluorescence species, band 3*-DBDS-*p*CMBS and band 3*-DBDS-*p*CMBS-thiourea, have been taken as equivalent for demonstration purposes. Figure 8 also illustrates that the main effect of thiourea on DBDS binding to *p*CMBS-complexed band 3 is to stabilize the rapid, bimolecular step; the driving force for the conformational change is thereby significantly reduced.

Thiourea binding parameters can be determined with an expression corresponding to Eq. (14). Using the mean values of k_6^{pC} and k_{-6}^{pC} calculated above and thermodynamic constraints (as in Eqs. 6 and 10), the number of independent thiourea binding parameters is reduced to one, K_4^{pC}

$$\frac{1}{\tau} = \frac{4.4 + 0.35[\text{TU}]/K_4^{pC} + 0.0064\,([\text{TU}]/K_4^{pC})^2}{28.4 + 1.58[\text{TU}]/K_4^{pC} + 0.021([\text{TU}]/K_4^{pC})^2}. \quad (18)$$

In order to avoid complicated statistical procedures, Eq. (18) can be rearranged to the standard quadratic form;

$$ay^2 + by + c = 0, \quad (19)$$

in which $y = [\text{TU}]/K_4^{pC}$ and the three coefficients of the polynomial are determined by the value of the experimental parameter τ^{-1}: $a = 0.021\,\tau^{-1} - 0.0064$, $b = 1.58\,\tau^{-1} - 0.35$ and $c = 28.4\,\tau^{-1} - 4.4$. The solutions of Eq. (19) are determined by τ^{-1}, and a plot of these y_i against the corresponding values of [TU] must be linear, with slope $(K_4^{pC})^{-1}$. In other words, τ^{-1} varies with [TU], and this is reflected in the calculated y_i. The plot of the y_i against [TU] is shown in Fig. 9, and gives

$$K_4^{pC} = 9 \pm 3\ \mu\text{M}.$$

As previously, the thermodynamic constraints allow K_5^{pC} and K_7^{pC} to be estimated;

$$K_5^{pC} = (K_1^{pC}/K_3^{pC})K_4^{pC} \approx (28/0.6)\,(9) = 0.42\ m\text{M},$$
$$K_7^{pC} = (K_3^{pC}K_6^{pC}K_5^{pC})/(K_1^{pC}K_2^{pC})$$
$$\approx (0.0006)\,(1.0)\,(0.42)/[(.028)(.025)] = 0.36\ \text{mM}.$$

All these parameters are also summarized in the Table.

Studies with Substituted Thioureas and Urea

The hydrogen bonding of thiourea is weaker than that of urea because the —C=S group does not normally form hydrogen bonds whereas —C=O does. This difference is reflected in the olive-oil/water partition coefficient (Collander & Barlund, 1933) which is 0.0012 for thiourea, almost an order of magnitude greater than that for urea, 0.00015. In order to explore the influence of hydrogen bonding on the thiourea/band 3 system, we reduced thiourea hydrogen bonding ability further by introducing methyl groups into the $—NH_2$ residues. From Fig.

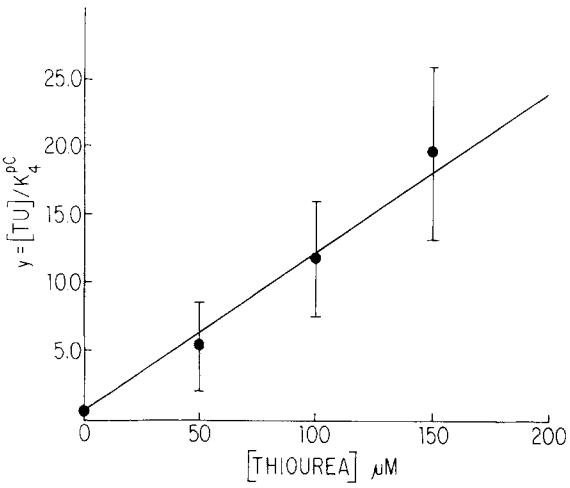


Fig. 9. Plot of solutions, $y = [TU]/K_4^{pC}$, of Eq. (19): the slope of the fitted regression line gives $K_4^{pC} = 9 \pm 3$ μM. $[DBDS]_{tot} = 0.39$ μM, $[band\ 3]_{tot}$ = constant = 0.02 to 0.04 μM. Means and errors of five runs

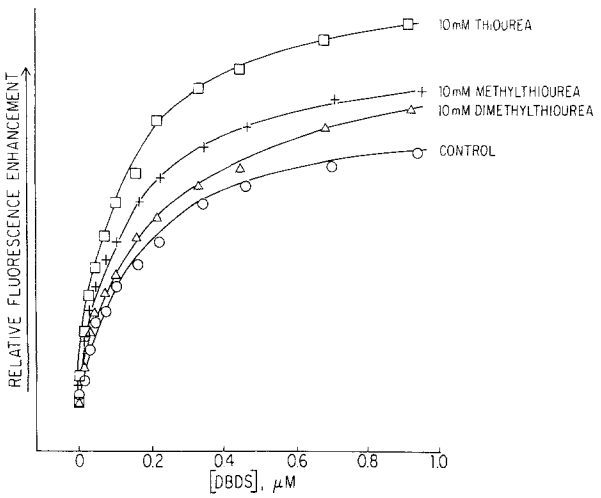


Fig. 10. Equilibrium DBDS binding curves comparing fluorescence enhancement in 10 mM thiourea [*p*CMBS = 0] with that in methyl-substituted analogues

10, which compares the equilibrium fluorescence enhancement of thiourea to that of two methyl-substituted compounds, it is seen that equilibrium fluorescence enhancement decreases as methyl groups are introduced. This correlation is consistent with the view that thiourea effects are related to solute hydrogen bonding, but it is not clear whether the relationship results from direct effects on band 3 or from indirect interactions mediated by membrane lipids.

In the course of these experiments, we also studied the effect of urea on the rate constant for the DBDS fluorescence enhancement, $1/\tau$. Concentrations of urea in the mM range did not have any effect on red cells treated with 1 mM *p*CMBS, but urea concentrations above 0.1 M decreased $1/\tau$ from its control value of 0.24 sec^{-1}, to 0.17 sec^{-1} at 0.1 M, 0.14 sec^{-1} at 0.25 M, and 0.09 sec^{-1} at 0.5 M. This effect may be relevant for the urea permeation process, which is known to saturate above 1 M urea, with a half-concentration of 0.33 M (Brahm, 1983). The change in $1/\tau$ induced by urea in the 0.1 to 0.5 M range indicates that urea can alter the conformation of band 3; either directly, as a function of urea binding, or indirectly, as a result of urea's structure-breaking ability.

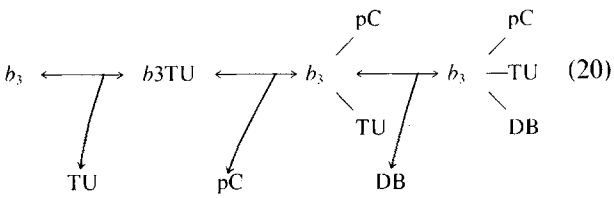
Discussion

Our experiments demonstrate that there are one or more thiourea binding sites on band 3 which affect the interaction of the protein with both the specific anion transport inhibitor, DBDS, and the water transport inhibitor, *p*CMBS. One aspect of our findings relates to the structural integrity of band 3 itself; the effects of thiourea are much greater after treatment with *p*CMBS, as the data in the Table show. This is illustrated by the striking effect of *p*CMBS on the equilibrium constant, K_4, which decreases from 0.23 mM in the absence of *p*CMBS to 9 μM in the presence of *p*CMBS (K_4^{pC}). This difference may indicate that the SH group with which *p*CMBS interacts is important to the structural integrity of band 3. In the absence of *p*CMBS, band 3 retains essentially its native configuration. When *p*CMBS reacts with the SH group, a restraint appears to be removed from the protein, which then exhibits a much larger conformational change in response to thiourea binding.

Our experiments show that there is a thiourea site on red cell membranes that modulates DBDS fluorescence. Since DBDS binds primarily to band 3 (Rao et al., 1979; Verkman et al., 1983) and exhibits no fluorescence enhancement when the stilbene site has been blocked by covalent reaction with DIDS, the thiourea site we have characterized is most probably on, or connected to, band 3. The observation that *p*CMBS modulates the thiourea/DBDS interaction adds further support to our previous findings showing a DBDS/*p*CMBS interaction (Lukacovic et al., 1984). The present results also provide evidence of an operational complex which transmits conformational signals among the thiourea, *p*CMBS and DBDS sites.

As pointed out earlier, thiourea also interacts with both the water and urea transport system in the red cell. If we make the assumption, for the moment, that these transport functions are on band 3,

we can illustrate the several thiourea functions schematically, as follows,



reaction 1	reaction 2	reaction 3
inhibits	interacts with	interacts with
urea transport	water transport system	fluorescence enhancement
$K_{I(1)} \approx$ 12–35 mM	$K_{I(2)} \approx$ 3 mM	$K_{I(3)} \approx$ 0.2 mM.

As Mayrand and Levitt (1983) showed, the inhibition site for urea transport (reaction 1 above) is not very specific; they found almost 50 urea and thiourea analogues which produce some degree of inhibition. This inhibition was favored by introduction of hydrophobic side chains, and by substitution of —C=S for —C=O. Thus, many analogues are able to fit into the site and we would expect that the high-affinity analogues studied by Mayrand and Levitt would also cause changes in DBDS fluorescence kinetics. Once the site is occupied the changes in transport properties are quite specific. Chasan and Solomon (1980) and Mayrand and Levitt (1983) reported that thiourea inhibited amide permeation, but had no effect on alcohol permeation. The K_I for urea flux inhibition is 12 mM on the outside of the cell and 35 mM on the inside.

We reported previously that thiourea blocks the *p*CMBS inhibition of red cell water transport (Chasan et al., 1984). This process, which is reaction 2 in Eq. (20), is characterized by K_I = 3 mM at 2 mM *p*CMBS and takes place in the absence of DBDS. The association constant for the reaction of thiourea with band 3/*p*CMBS/DBDS, reaction 3 in the scheme above, is given in the Table as ≈0.2 mM.

The question that should be addressed is whether these differences in association constants, which extend over more than two orders of magnitude, indicate that there is more than a single binding site or that there is more than a single protein. As the reaction scheme in Eq. (20) shows, the protein has been modified by *p*CMBS treatment for the studies of reaction 2 and modified again by DBDS treatment for the studies of reaction 3. Not only does *p*CMBS modify the protein conformation sufficiently to inhibit water transport, but it also seems to affect the general stability of the conformation, as discussed above. Verkman et al. (1983) have presented evidence that DBDS binding causes a conformational change in band 3; Macara et al. (1983) have also provided evidence that DBDS causes a band 3 conformational change and Bjerrum et al. (1983) have shown that a related stilbene anion transport inhibitor, DNDS (4,4′-dinitro-2,2′-stilbene disulfonate) causes a significant change in the reactivity of a band 3 arginine residue that is important to anion transport. Passow et al. (1980) have shown that stilbene inhibitors affect the rate of dinitrophenylation of a lysine residue in band 3 which is also believed to be important in anion transport. These findings show that the conformation of the protein was different when each of the reactions 1, 2 and 3 were studied. Thus, it is not necessarily to be expected that the conformation of the thiourea binding site remain invariant under these conditions.

The data we have presented give evidence of a defined reaction between thiourea and band 3, both in the presence and absence of *p*CMBS. The kinetic and thermodynamic constants have been presented in the Table. It is important to point out that the means and standard errors of the 16 constants listed in the Table refer to experiments on a single ghost preparation, and that these values differ slightly for different ghost samples. The proposed reaction model is, however, consistent for all experiments. Furthermore, these data are entirely consistent with the view that all the red cell transport functions in which thiourea is involved are carried out by a single protein or protein complex.

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Appendix

Derivation of Equation (4)

The derivation below is based on the assumption that all bimolecular steps are rapidly equilibrated and that the optical properties of band 3*-DBDS are the same as those of band 3*-DBDS-TU. Verkman et al. (1983) have assumed that the bimolecular association of DBDS to band 3 is faster than the conformational change and found that the data fit kinetics based on this assumption. In the presence of excess thiourea, the bottom limb of Eq. (4) is predominant. If thiourea affects the fluorescence of bound DBDS directly, increased fluorescence would follow the addition of further thiourea after saturation of the bottom limb. Since we have observed no such additional fluorescence the assumption given above is justified.

Because the three thiourea binding steps are assumed to be rapid, the observed (slow) relaxation can be described as the interaction of three "pools":

$$\mathrm{DB} + \left\{\begin{matrix} b_3 \\ + \\ b_3\mathrm{TU} \end{matrix}\right\} \underset{\text{fast}}{\rightleftharpoons} \left\{\begin{matrix} \mathrm{DB}b_3 \\ + \\ \mathrm{DB}b_3\mathrm{TU} \end{matrix}\right\} \underset{\text{slow}}{\rightleftharpoons} \left\{\begin{matrix} \mathrm{DB}b_3^* \\ + \\ \mathrm{DB}b_3^*\mathrm{TU} \end{matrix}\right\}$$

which can be rewritten in the simpler form

$$DB + b_3' \overset{K_1'}{\leftrightarrow} DBb_3' \underset{k'_{-2}}{\overset{k_2'}{\rightleftharpoons}} DBb_3^{*\prime} \tag{A1}$$

with the definitions

$$K_1' = \frac{[DB][b_3']}{[DBb_3']} = \frac{[DB][b_3]}{[DBb_3]} \cdot \frac{\left(1 + \frac{[TU]}{K_5}\right)}{\left(1 + \frac{[TU]}{K_4}\right)}$$

$$= K_1 \frac{\left(1 + \frac{[TU]}{K_5}\right)}{\left(1 + \frac{[TU]}{K_4}\right)} \tag{A2}$$

in which $K_4 = \frac{[DBb_3][TU]}{[DBb_3TU]}$ and $K_5 = \frac{[b_3][TU]}{[b_3TU]}$.

Analogously

$$k'_{-2}/k_2' = K_2' = \frac{[DBb_3']}{[DBb_3^{*\prime}]} = \frac{[DBb_3]}{[DBb_3^*]} \cdot \frac{\left(1 + \frac{[TU]}{K_4}\right)}{\left(1 + \frac{[TU]}{K_7}\right)}$$

$$= K_2 \frac{\left(1 + \frac{[TU]}{K_4}\right)}{\left(1 + \frac{[TU]}{K_7}\right)}. \tag{A3}$$

On the basis of Eq. (A1), the relaxation signal reflects the rate of formation of $DBb_3^{*\prime}$; the rate equation is (*see* Fig. 1, middle):

$$\frac{d[DBb_3^{*\prime}]}{dt} = \frac{d[DBb_3^*]}{dt} + \frac{d[DBb_3^*TU]}{dt} = k_2[DBb_3]$$
$$+ k_6[DBb_3TU] - k_{-2}[DBb_3^*] - k_{-6}[DBb_3^*TU]. \tag{A4}$$

Using the definition of equilibrium constants, this can be simplified to read,

$$\frac{d[DBb_3^{*\prime}]}{dt} = k_2'[DBb_3'] - k'_{-2}[DBb_3^{*\prime}], \tag{A5}$$

in which

$$k_2' = \frac{k_2 + k_6 \frac{[TU]}{K_4}}{1 + \frac{[TU]}{K_4}}; \quad k'_{-2} = \frac{k_{-2} + k_{-6} \frac{[TU]}{K_7}}{1 + \frac{[TU]}{K_7}}. \tag{A6}$$

The slow relaxation time constant for Eq. (A1) has the form

$$\frac{1}{\tau} = k_2' \frac{[DB]}{[DB] + K_1'} + k'_{-2} \tag{A7}$$

when $[DB] \gg [b_3]$ and the definitions in Eqs. (A2) and (A6) apply. Written in terms of the parameters in Fig. 1, middle,

$$\frac{1}{\tau} = \frac{\left(k_2 + k_6 \frac{[TU]}{K_4}\right)[DB]}{\left(1 + \frac{[TU]}{K_4}\right)[DB] + \left(1 + \frac{[TU]}{K_5}\right)K_1} + \frac{k_{-2} + k_{-6} \frac{[TU]}{K_7}}{1 + \frac{[TU]}{K_7}}. \tag{A8}$$