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Control of red cell urea and water permeability by sulfhydryl reagents

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The binding constant for pCMBS (*p*-chloromercuribenzenesulfonate) inhibition of human red cell water transport has been determined to be 160 ± 30 μM and that for urea transport inhibition to be 0.09 ± 0.06 μM, indicating that there are separate sites for the two inhibition processes. The reaction kinetics show that both processes consist of a bimolecular association between pCMBS and the membrane site followed by a conformational change. Both processes are very slow and the on rate constant for the water inhibition process is about 10^5 times slower than usual for inhibitor binding to membrane transport proteins. pCMBS binding to the water transport inhibition site can be reversed by cysteine while that to the urea transport inhibition site can not be reversed. The specific stilbene anion exchange inhibitor, DBDS (4,4′-dibenzamidostilbene-2,2′-disulfonate) causes a significant change in the time-course of pCMBS inhibition of water transport, consistent with a linkage between anion exchange and water transport. Consideration of available sulfhydryl groups on band 3 suggests that the urea transport inhibition site is on band 3, but is not a sulfhydryl group, and that, if the water transport inhibition site is a sulfhydryl group, it is located on another protein complexed to band 3, possibly band 4.5.

Introduction

When Macey and Farmer [1] first showed that pCMBS inhibited water and urea transport in human red cells, the measurements were made after a 75 min incubation period at room temperature. Subsequently, Naccache and Sha'afi [2] measured the time-course of the inhibition and found that 1 mM pCMBS caused water permeability to decrease slowly over a 75 min period at room temperature. Sha'afi and Feinstein [3] found that another mercurial sulfhydryl reagent, pCMB, bound strongly to band 3, the anion transport protein, and Brown et al. [4] suggested that band 3 provides the channel for aqueous transport across the red cell membrane. Solomon et al. [5] subsequently proposed that the same sulfhydryl group on band 3 was also responsible for the inhibition of urea transport (see also Refs. 6–8). We found that pCMBS bound to band 3 within 2 min at 0°C, showing that binding was very fast, in contrast to the slow inhibition of water transport. As we began to characterize the inhibition processes in more detail, we found that pCMBS inhibition of urea transport was complete in 1 or 2 minutes at room temperature (Chasan, B. and Solomon, A.K., unpublished data), much faster than water transport inhibition, in agreement with Macey's results [9]. This difference in time course indicates that two different pCMBS sites are involved. We have now made a detailed study of the binding

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Abbreviations: pCMBS, *p*-chloromercuribenzenesulfonate; pCMB, *p*-chloromercuribenzoate; FMA, fluoresceinmercuric acetate; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate.

characteristics and kinetics of these inhibition processes and of their relation to band 3.

Methods and Materials

Methods

Osmotic permeability was measured using the stopped-flow light-scattering apparatus of Terwilliger and Solomon [10] to measure cell volume. Whole blood, outdated by no more than four days, was centrifuged, plasma and buffy coat aspirated, and then washed with a buffer of the following composition (in mM): NaCl, 125; KCl, 4.4; Na_2HPO_4, 6; $NaHCO_3$, 24.9; $MgCl_2$, 0.5; pH 7.4, 300 ± 5 mosM. Osmolalities were determined with a Fiske Model OS osmometer (Uxbridge, MA).

N-Ethylmaleimide treatment consisted of incubation at 37°C at 25% hematocrit for one hour at a final concentration of 12 mM *N*-ethylmaleimide in the above buffer. Cells were then washed three times with *N*-ethylmaleimide-free buffer and resuspended in buffer ± sulfhydryl reagents.

Cells at 2% hematocrit were then mixed with an equal volume of solution made hyperosmolal by the addition of NaCl to the buffer: the response of the cells to a 250–275 mosM gradient after mixing was the basis of all measurements of the hydraulic conductivity, L_p ($cm^3 \cdot dyn^{-1} \cdot s^{-1}$) which were carried out at room temperature, 20–22°C. After flow was stopped, scattered light intensity was sampled at 4 ms intervals over a period of 0.025–5 s. This process was repeated 5–10 times for each experimental data point. Control sets of data were obtained in the same manner, except that the cell suspensions were mixed with solutions isosmolal with the cell suspension. The difference between the averaged experimental and control curves was used to determine L_p.

Urea permeability was determined at room temperature, 20–22°C by the method of Sha'afi et al. [11] and was based on the response of the cells to a 350–400 mosM urea gradient. For each measurement of urea permeability at a specific time, L_p was determined at the same time with cells under identical conditions. As above, isosmolal control solutions were run against all solutions used to measure urea permeability.

In the cysteine reversal experiments, cysteine (or glutathione) was dissolved in a small amount of buffer and titrated to neutral pH immediately before addition to the blood suspensions.

Materials

N-Ethylmaleimide, cysteine, glutathione, and all mercurial sulfhydryl reagents were obtained from Sigma Chemical Co. (St. Louis, MO). 4,4′-Dinitrostilbene-2,2′-disulfonate (DNDS) was supplied by K and K Labs, Inc. (ICN Pharmaceuticals, Plainview, NY) and 4,4′-dibenzamidostilbene-2,2′-disulfonate (DBDS) by Dr. James Dix, SUNY, Binghamton. All other chemicals were of reagent grade and obtained from Fisher Scientific (Medford, MA). Outdated blood was kindly donated by the Children's Hospital, Boston, MA.

Results

Inhibition of water flux by pCMBS

Mercurial reagents such as pCMBS react specifically with cysteine residues on proteins. Chemical analysis [12,13] has shown that band 3 contains six cysteine residues of which five are located on the cytoplasmic side of the membrane. The five cytoplasmic cysteine groups react with either pCMBS or *N*-ethylmaleimide and reaction with one of these reagents blocks subsequent reaction with the other [14]. The sixth sulfhydryl group, which is in the 17-kDa membrane-bound fragment of band 3 between the trypsin and the chymotrypsin cuts [15], does not react with *N*-ethylmaleimide but does react with pCMBS [5,13,15]. The primary structure analysis of Kopito and Lodish [16] confirms six cysteine residues in murine band 3 and shows that one is located in the 17-kDa fragment. Solomon et al. [5] have called this the 'cryptic' sulfhydryl group and suggested that it is the locus of the mercurial inhibition of urea and water permeability. Consequently, most of our experiments have been carried out on *N*-ethylmaleimide-treated cells in which the only sulfhydryl residue in band 3 which remains to react with sulfhydryl reagents is the membrane-bound cysteine in the 17-kDa fragment.

In the course of our studies of the kinetics of pCMBS inhibition of water transport in *N*-ethylmaleimide-treated red cells, we found that periods

longer than 1 h were required for maximal inhibition at the lowest concentrations of pCMBS. When sufficient time was allowed, $K_{i,app} = 160 \pm 30$ μM as shown in Fig. 1 and Table I for the best experiment in this series. We had studied the effect of *N*-ethylmaleimide treatment on $K_{i,app}$ in two earlier experiments in which we had allowed no more than 1 h for completion of inhibition and found $K_{i,app} = 230 \pm 40$ μM (two expts.) in *N*-ethylmaleimide-treated cells, compared to $K_{i,app} = 160 \pm 40$ μM (three expts.) in non-treated controls; the difference is not significant ($p < 0.1$, Student's *t*-test). The absence of any appreciable effect of *N*-ethylmaleimide confirms the conclusion of Naccache and Sha'afi [2] that *N*-ethylmaleimide does not bind to the pCMBS water transport inhibition site. Haest et al. [17] showed that *N*-ethylmaleimide binds to 73% of the red cell membrane sulfhydryl groups and we have found

TABLE I

RATE AND INHIBITION CONSTANTS FOR MERCURIAL SULFHYDRYL REAGENTS

Reagent	Water		Urea	
	$K_{i,app}$ (μM)	k_1 ($M^{-1} \cdot s^{-1}$)	$K_{i,app}$ (μM)	k_1 ($M^{-1} \cdot s^{-1}$)
pCMBS [a]	160 ± 30	1.77 ± 0.03	0.09 ± 0.06	310 ± 200
pCMB [b]	61 ± 7	5.8 ± 0.5	0.6 ± 0.1	53 ± 5
FMA [c]	85 ± 10			
$HgCl_2$ [d]	60 ± 10		14 ± 7	

[a] No. of expts.; see legends to Figs. 1, 2, 5 and 6.

[b] No. of expts. for water:
$K_{i,app}$: 1 expt., consistent with a 2nd in *N*-ethylmaleimide-treated cells and one in non-treated cells;
k_1: 1 expt.
No. of expts. for urea:
$K_{i,app}$: 1 expt.;
k_1: 2 expts.

[c] 1 expt. with *N*-ethylmaleimide cells, consistent with 2 expts. in non-treated cells.

[d] Water and urea; 1 expt. each.

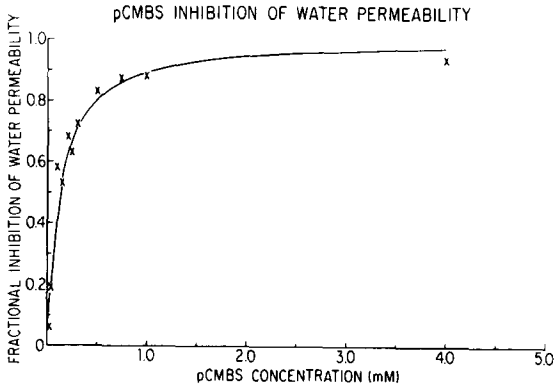

Fig. 1. Fractional inhibition of water permeability by pCMBS in *N*-ethylmaleimide-treated cells. At the lowest concentrations, periods of 1–3 h were required to complete the pCMBS binding reaction. The data were fitted to a single site binding curve by non-linear least squares with $K_{i,app} = 160 \pm 30$ μM and maximum fractional inhibition of 1.04 ± 0.07. In a second experiment, with fewer points at concentrations below 0.5 mM, $K_{i,app} = 130 \pm 30$ μM. In four similar experiments (including the two earlier ones mentioned in the text), the maximum inhibition observed at [pCMBS] between 2 and 10 mM was 0.89 ± 0.04 in agreement with the observation of Macey [9] that the maximum pCMBS inhibition of osmotic water transport is about 90% (see also Ref. 2). The maximum inhibition from the non-linear lest-squares fit to the single-site binding curves for these four experiments was 1.00 ± 0.03 and the difference between these estimates is significant ($p < 0.01$, *t*-test).

that our routine treatment of red cells with *N*-ethylmaleimide makes the cells much more fragile. However, *N*-ethylmaleimide treatment does not change either the shape or the fit of the pCMBS inhibition curve which means that the chemical group that modulates water transport is well conserved in a region of the protein resistant to a generalized attack by a powerful sulfhydryl reagent.

Kinetics of pCMBS inhibition of water flux

To determine the reaction kinetics of the pCMBS effect, we measured the rate constant of the reaction over the concentration range from 0 to 10 mM, which is near the solubility limit for pCMBS and is about sixty times the $K_{i,app}$. Fig 2(top) shows how well the individual time-courses are fitted, by non-linear least squares, to single exponentials with relaxation time, τ. If the pCMBS inhibition is produced by a coupled reaction system, as will be suggested below, the second reaction is too fast to be resolved on our time scale. When τ^{-1} is plotted against [pCMBS] as shown in Fig. 2(bottom) a straight line is obtained, consistent with a bimolecular association as shown in

the following reaction scheme

$$\text{pCMBS} + \text{band 3} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{pCMBS-band 3} \quad (1)$$

for which τ^{-1} is given by (Czerlinski [18])

$$\tau^{-1} = k_1[\text{pCMBS}] + k_{-1} \quad (2)$$

if [pCMBS] $\gg$ [band 3], as is the case in our experiments *. K_i is a dissociation constant (units of μM) and is given by k_{-1}/k_1.

Reaction kinetics can be used to show that pCMBS inhibition is not diffusion limited. If it were, the reaction would be described by

$$\text{pCMBS}_{\text{bulk}} \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} \text{pCMBS}_{\text{site}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{pCMBS-band 3} \quad (3)$$

band 3

in which $\text{pCMBS}_{\text{site}}$ denotes [pCMBS] at the reaction site. If the reaction were diffusion limited, the diffusion step would be slow compared with the reaction step and the following equation ** for τ^{-1} would apply (Czerlinski [18])

$$\tau^{-1} = k_d + k_{-d}(K_i + [\text{pCMBS}_{\text{site}}])/(K_i + [\text{pCMBS}_{\text{site}}] + [\text{band 3}]) \quad (4)$$

Since the intercept on the x-axis in Fig. 2 (bottom) is effectively zero, $k_d = 0$ and there is no measurable contribution from the diffusion term so that Eqn. 3 does not describe the system. Thus, the very slow time course of the pCMBS inhibition of water transport can not be attributed to delays caused by diffusion of pCMBS to the reaction site, unless the reaction scheme is much more complex than Eqn. 3.

We had expected that the bimolecular association would be followed by a conformation change, according to Eqn. 5, below, similar to the kinetics that characterize the binding of stilbene inhibitors of anion transport to band 3 [23].

$$\text{pCMBS} + \text{band 3} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{pCMBS-band 3} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{pCMBS-band 3}^* \quad (5)$$

in which the * denotes the conformation change. The association constants, $K_1 = k_1/k_{-1}$ and $K_2 = k_2/k_{-2}$. In this case, the apparent inhibition constant, $K_{i,\text{app}} = K_1^{-1}/(1 + K_2)$. There are simple solutions to the kinetics of Eqn. 5 under two limiting conditions [18]. If the bimolecular association is fast compared to the conformational change

$$\tau^{-1} = k_{-2} + \left(k_2[\text{pCMBS}]/\left(K_1^{-1} + [\text{pCMBS}]\right)\right) \quad (6)$$

* Solomon et al. [5] have suggested that the pCMBS binding site for inhibition of water transport is located in an aqueous pore of about 4.5 Å radius and have placed the site within about 10–15 Å of the external face of the membrane because it is external to the DIDS binding site which Rao et al. [19] have located 32 to 42 Å away from the interior membrane face. If the pCMBS concentration inside the pore differs appreciably from that in bulk solution, it is not correct to use bulk solution concentrations as the driving force for the reactions. This is formally equivalent to the unstirred layer problem which has been discussed extensively [20,21]. In qualitative terms, the unstirred layer correction may be neglected if the time required for the permeating molecule to diffuse across the unstirred layer is significantly faster than the time required for it to permeate the membrane. Rao [22] has found that the half-time for pCMBS permeation of resealed ghosts is approx. 40 min. Since diffusion is inversely proportional to distance, the time required to permeate one-third of the membrane is approx. 15 min, which is probably an overestimate since the rate-limiting step for pCMBS entrance probably is the time required to pass through the ion-exchange gate. The 15-min figure is considerably shorter than τ of 59 min when pCMBS is present at its K_i, which indicates that no unstirred layer correction is required. This conclusion is confirmed by the conclusion that pCMBS binding is not diffusion limited, as discussed following Eqn. 4.

** In the other limit, if the diffusion step is fast compared to the reaction step, the equation for τ^{-1} is [18]

$$\tau^{-1} = k_1[\text{pCMBS}_{\text{site}}] + k_{-1} + k_1[\text{band 3}]/(1 + K_d)$$

in which $K_d = k_{-d}/k_d$. If the diffusion step is fast, $[\text{pCMBS}_{\text{site}}]$ is very nearly equal to $[\text{pCMBS}_{\text{bulk}}]$ and, since [band 3] $\ll [\text{pCMBS}_{\text{bulk}}]$, the solution reduces to that given in Eqn. 2. Thus the data in Fig. 2(bottom) conform to the condition that the diffusion step is fast compared to the reaction step. k_1 is the rate constant for the binding of pCMBS to band 3. In this sense, band 3 is construed to include its Gouy-Chapman layer, so that any effects involved in penetrating its surface potential barrier are included in k_1. Furthermore, if band 3 has to undergo a conformation change to allow pCMBS to penetrate the protein in order to bind to its reaction site, that reaction rate is subsumed into k_1.

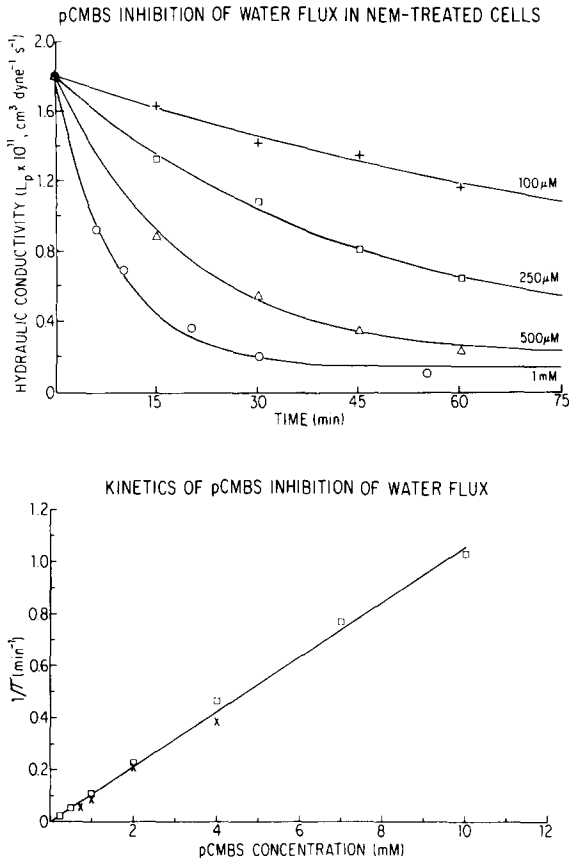

Fig. 2. (Top) Time-course of development of pCMBS inhibition of water permeability in one experiment, typical fo two, in *N*-ethylmaleimide (NEM)-treated cells. The hydraulic conductivity (L_p) data were fitted to single exponentials by non-linear least squares and the time constants were used in the bottom figure. The pCMBS concentrations are shown by each curve. (Bottom) Dependence of the rate constant (τ^{-1}) for water inhibition on pCMBS concentration in two experiments. The data were fitted to a straight line with slope of 1.77 ± 0.03 $M^{-1} \cdot s^{-1}$ and intercept of $(-0.3 \pm 2) \cdot 10^{-4}$ s^{-1}.

Under these conditions, the curve of τ^{-1} against [pCMBS] would saturate at high concentrations, but Fig. 2(bottom) shows no trace of curvature at the highest concentration which could be achieved (because of the limited solubility of pCMBS). In the other limit, if the velocity of the conformational change is fast compared to the bimolecular association, the equation is [18]

$$\tau^{-1} = k_1[\text{pCMBS}] + (k_{-1}/(1+K_2)) \quad (7)$$

In both Eqns. 2 and 7, the velocity of the forward reaction, k_1, is given by the slope of the curve which is 1.77 ± 0.03 $M^{-1} \cdot s^{-1}$. The only difference between Eqns. 2 and 7 is in the τ^{-1} intercept ($(-0.3 \pm 2) \cdot 10^{-4}$ s^{-1}) which is divided by the factor $(1 + K_2)$ in Eqn. 7. Since the intercept does not differ significantly from zero, no information can be obtained about k_{-1} or K_2.

The on-rate constant, $k_1 = 1.77$ $M^{-1} \cdot s^{-1}$ is about six orders of magnitude smaller than the values of 10^6–10^8 which characterize the association rate constants for enzyme-ligand association in free solution [24]. Binding to an intrinsic membrane protein, such as band 3, is at the low end of this range since Smith and Dix [25] give an on rate constant of $(1–3) \cdot 10^6$ $M^{-1} \cdot s^{-1}$ for the binding of the stilbene anion transport inhibitor, DBDS (4,4′-dibenzamidostilbene-2,2′-disulfonate), to band 3. The rate constant for Cl^- binding to band 3 has not been measured directly but it must be at least as fast as the Cl^- turnover number measured by Brahm [26] of $6.2 \cdot 10^3$ s^{-1} at 20°C and 150 mM Cl^- which leads to a minimum value of $4.1 \cdot 10^4$ M^{-1} s^{-1} for k_{on}. Schwarz et al. [27] report that k_{on} for tetrodotoxin binding to nerves from *Xenopus* or *Rana* at room temperature is approx. $4 \cdot 10^6$ $M^{-1} \cdot s^{-1}$, similar to the figure for the DBDS/band 3 reaction. Thus, on rates of approx. 10^6 $M^{-1} \cdot s^{-1}$ appear to be characteristic of the binding of specific transport inhibitors to their sites on integral transport membrane proteins.

It is very difficult to understand why the on rate constant of pCMBS is many orders of magnitude slower than these typical figures. Cantor and Schimmel [24] point out that binding rate constants depend sensitively upon the fit between enzyme and substrate. For example, when *S*-methylaspartate replaces aspartate as substrate for reaction with aspartate aminotransferase, k_{on} is reduced from $> 10^7$–10^8 $M^{-1} \cdot s^{-1}$ to $1.2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$. pCMBS is a specific sulfhydryl reagent, but there is no reason to expect it to be a good fit to the cysteine residue with which it is presumed to react. Even so, the on rate seems remarkably small, particularly since we have shown that it can not be attributed to the time required for pCMBS to diffuse to its site of action (Eqns. 3 and 4). These considerations indicate that binding of

pCMBS to its reaction site involves a profound rearrangement of the protein.

The off rate constant, $k_{-1} = k_1 K_{i,app}(1 + K_2)$ or $3 \cdot 10^{-4}$ $s^{-1}(1 + K_2)$. Though it cannot be evaluated without knowing K_2, it is very small compared to the values of 10^3–10^4 s^{-1} which characterize enzyme-ligand complexes [24].

Reversal of pCMBS effects by cysteine

Naccache and Sha'afi [2] had shown that 10 mM cysteine completely reversed 1 mM pCMBS inhibition of water transport, thus implicating membrane sulfhydryl groups as the causative agents. We extended these studies on reversal by comparing the effects of cysteine and glutathione. Since it appeared that a chemical bond had been formed between pCMBS and band 3, washing the cells with pCMBS-free buffer was expected to have no effect, and it did not, as Fig. 3 shows. Addition of cysteine reverses the inhibition with essentially no time delay, confirming Naccache and Sha'afi's observation, but glutathione reverses it more slowly, as shown in Fig. 3. The delay in glutathione action may be ascribed to steric hindrance since its molecular mass (307 Da) is more than 2.5-times greater than that of cysteine (121 Da). This would be consistent with location

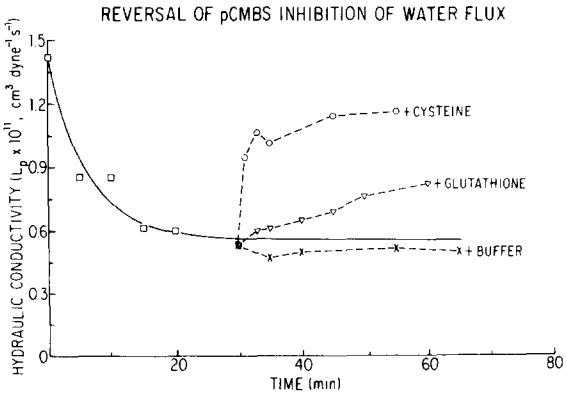

Fig. 3. Reversal of inhibition of water transport produced by 2 mM pCMBS in one of two experiments with identical results. At 30 min, concentrated solutions of cysteine and glutathione were added to produce final buffer concentrations of 5 mM. To obtain the curve marked buffer, a sample was washed twice with 100 vols. of pCMBS-free buffer and resuspended before taking subsequent data.

of the pCMBS site in a channel with restricted access, as suggested by Solomon et al. [5].

The difference in the time course of cysteine and glutathione reversal may be used to eliminate one possible mechanism of their action. Since these reagents reduce the external pCMBS concentration to zero, their mechanism of action could be reversal of the direction of the reaction by the law of mass action. In the extracellular solution, both reagents would reduce the pCMBS concentration essentially instantaneously and the time delays would be occasioned by dissociation of pCMBS from band 3 and diffusion through the channel to the extracellular space. But the dissociation and diffusion processes would be independent of the nature of the reagent, so the difference in the time-course between cysteine and glutathione action means that the rate depends upon the time of diffusion to, or reactions at, the pCMBS-band 3 site.

To study the kinetics of cysteine reversal the pCMBS concentration was set at 150 μM, near its K_i, and the reaction was reversed with 2 mM cysteine, a concentration which almost saturates the reversal reaction. As Fig. 4 shows, the time required to return to the control permeability (dashed line) increases with time of pCMBS incubation. The time required to reach the apparent new asymptote increases smoothly from 15 ± 2 min, when cysteine was added 15 min after the pCMBS reaction began, to 34 ± 4 min, after a 90 min delay.

If there had been no conformational change, the reaction scheme would be

$$\text{cysteine} + \text{pCMBS-band 3} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{cysteine-pCMBS} + \text{band 3} \qquad (8)$$

for which the kinetics are given by

$$\tau^{-1} = k_3[\text{cysteine}] \qquad (9)$$

since [cysteine] $\gg$ pCMBS-band 3 and the concentration of the reaction products is negligible (Czerlinski [18]). Since [cysteine] is essentially constant and τ^{-1} is not, Eqn. 9 is not valid which means that Eqn. 8 does not describe the system and an additional reaction step is required. Addition of a conformational change, as shown in

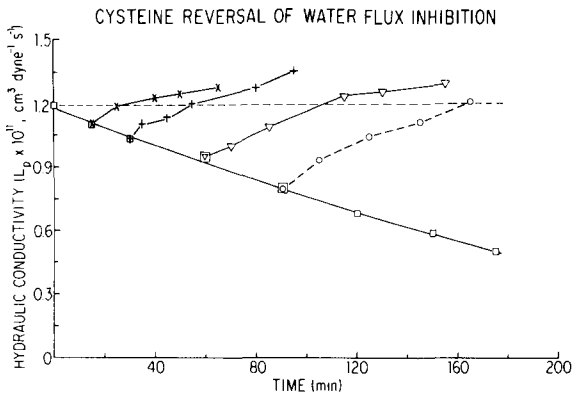

Fig. 4. Time dependence of cysteine (2 mM) reversal of inhibition produced by 0.15 mM pCMBS in one of three experiments with similar results. Cysteine was added after delays of 15, 30, 60 and 90 min. The time required for return to half of the estimated asymptotic value of the hydraulic conductivity varied from 15 ± 2 min when cysteine was added after a 15 min delay to 34 ± 4 min when cysteine was added after a 90 min delay. In this experiment, as well as two others done at the same time, the time-course of pCMBS inhibition of water transport was unusually slow, possibly because the red cells in all three experiments were unusually fragile. The cysteine reversal in these three experiments was similar, as well as in a fourth experiment of the same type, in which the pCMBS inhibition was faster, though still slower than the experiment at 100 μM pCMBS shown in Fig. 2 (top).

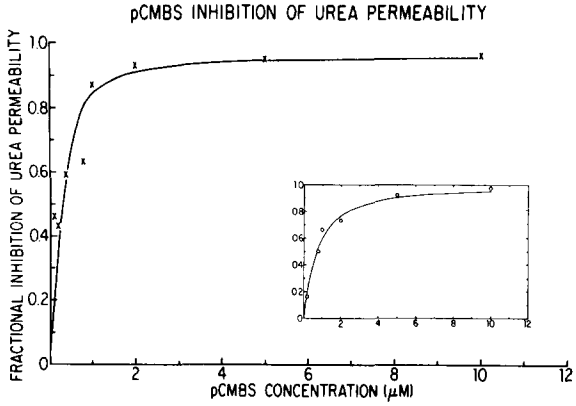

Fig. 5. pCMBS inhibition of urea permeability in *N*-ethylmaleimide-treated cells. The data have been fitted to a single-site binding curve by non-linear least squares with $K_{i,app} = 0.093 \pm 0.06$ μM and maximal inhibition $= 0.96 \pm 0.06$. In addition to the second experiment discussed in the text, five earlier experiments were carried out with consistent results. As discussed in the text, the shape of the binding curve can be used to determine whether the binding reaction is irreversible. The single-site binding curve to which the data have been fitted is derived from reversible reaction kinetics. If the reaction were irreversible, the fractional inhibition would rise linearly to saturation at which point there would be a sharp intersection with a horizontal line, and the inhibition would become independent of [pCMBS]. When $K_{i,app}$ is very small, as in Fig. 5, it is difficult to exclude the possibility that the data would also fit an irreversible reaction scheme satisfactorily. However, when $K_{i,app}$ is larger, as in the experiment with non-*N*-ethylmaleimide-treated cells shown in the inset, ($K_{i,app} = 0.52$ μM; units same as in large graph), the binding curve is typical of a reversible reaction.

Eqn. 5, appears to be the simplest reaction scheme consistent with our experiments.

Kinetics of pCMBS inhibition of urea flux

The pCMBS concentration required to inhibit urea flux is very much smaller than that required for water as Macey [9] has found. Fig. 5 shows the single site binding curve for one experiment in *N*-ethylmaleimide-treated cells in which pCMBS concentrations as low as 0.1 μM were used. The $K_{i,app}$ is 0.09 ± 0.06 μM, three orders of magnitude lower than that for water. (In an earlier experiment in which [pCMBS] was only reduced to 1 μM, $K_{i,app} = 0.2 \pm 0.2$ μM). When normal cells, not exposed to *N*-ethylmaleimide, were probed with low pCMBS concentrations (0.2 μM), $K_{i,app}$ was higher, 0.52 ± 0.07 μM, as shown in the inset to Fig. 5.

Fig. 6 shows that the relationship between τ^{-1} and [pCMBS] is linear over an extended concentration range from 1 to 500 μM, which is 5000-times greater than the K_i. At 500 μM pCMBS, τ is 12 s which is at the limit of our resolution time. There is no trace of curvature at these high concentrations so the reaction can be described either by Eqn. 2 or 4 with an average $k_1 = 310 \pm 200$ $M^{-1} \cdot s^{-1}$, as given in Table I. The τ^{-1} intercept at the [pCMBS] ordinate is not significantly different from zero, $(0.3 \pm 5) \cdot 10^{-3}$ s^{-1} in Fig. 6. Though the value of k_1 is two orders of magnitude greater than that for pCMBS inhibition of water transport, it is still about four orders of magnitude smaller than other values for inhibitor binding to transport proteins.

We used a very low [pCMBS] (2.5 μM) to study the reversibility of the pCMBS/band 3 reaction and added 2 mM cysteine, an excess of almost three orders of magnitude. As Fig. 7 shows, we

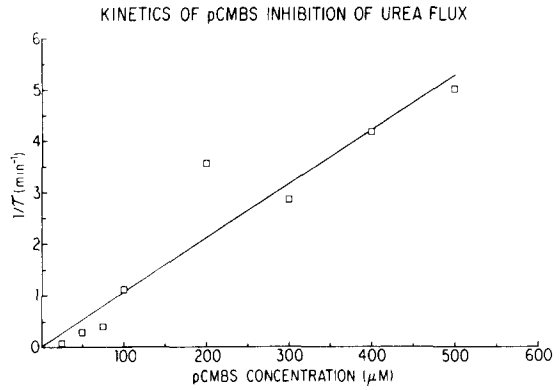

Fig. 6. Dependence of the rate constant (τ^{-1}) for urea flux inhibition in *N*-ethylmaleimide-treated cells on pCMBS concentration in one of four experiments. The data were fitted to a straight line with slope 180 ± 20 $M^{-1} \cdot s^{-1}$ and intercept $(0.3 \pm 5) \cdot 10^{-4}$ s^{-1} and the averages for the four experiments were slope $= 310 \pm 200$ $M^{-1} \cdot s^{-1}$ and intercept $(0.8 \pm 1.5) \cdot 10^{-3}$ s^{-1}. There were eight earlier experiments under somewhat different conditions which gave similar results. As stated in the text, the resolution time of these experiments kept us from using higher [pCMBS]. Each point in Fig. 6 is the characteristic time of an exponential time-course similar to those shown in Fig. 2 (top). Each point in the exponential time course is a single value for the urea permeability coefficient determined by non-linear least-squares fits to the average time course of five runs over the entire time course of a stopped-flow experiment. Our resolution time is determined by the time required to flush the machine and change solutions. At the highest [pCMBS], τ was 12 s.

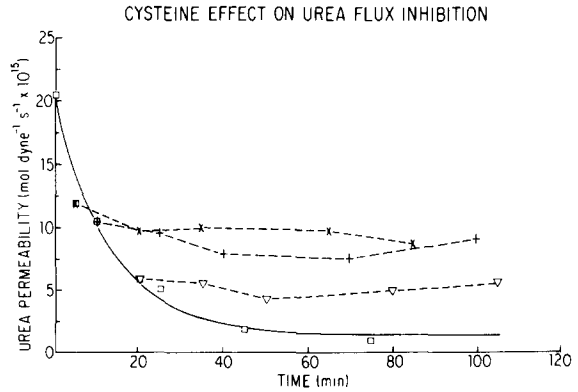

Fig. 7. Time dependence of cysteine (2 mM) effect on 2.5 μM pCMBS inhibition of urea flux in *N*-ethylmaleimide-treated cells. Cysteine was added after delays of 5, 10 and 20 min. The basic inhibition curve was fitted to a single exponential by non-linear least squares with $\tau = 14 \pm 3$ min. Two additional experiments were carried out with similar results.

were unable to obtain any sign of reversal, even as early as 10 min after beginning the pCMBS reaction. The inability of cysteine to reverse the reaction on our time scale is not consistent with the reaction scheme in Eqn. 5, even if the equilibrium is displaced far to the right. As in the case of pCMBS inhibition of water flux, k_{-1} is a function of the unknown K_2, $k_{-2} = 2.8 \cdot 10^{-5}(1 + K_2)s^{-1}$. Even if K_2 were of the order of 10^3 so that there were 1000-times as much pCMBS-band 3* as pCMBS-band 3, k_{-1} would be approx. 0.03 s^{-1}. Though this is orders of magnitude slower than the off rate constants of 10^3–10^4 s^{-1} which characterize most of the enzyme-ligand interactions discussed by Cantor and Schimmel [24], it is orders of magnitude faster than the data in Fig. 7, which would require that k_{-1} be measured in hours rather than seconds. Although we have been unable to reverse the inhibition with cysteine, the reaction is not irreversible, as shown by the fit of the urea transport inhibition data to the single site binding curve shown in Fig. 5 (and its inset) and discussed in its legend.

The question of whether the reaction of a mercurial sulfhydryl reagent is reversible by cysteine is a question of the relative affinity of the mercurial to the protein as compared to its affinity to cysteine. Thus it is possible for the neighboring groups in the protein to increase the relative affinity of a sulfhydryl group to pCMBS so that cysteine no longer can remove the mercurial. If this were the case, the reaction should be reversible by the law of mass action, when cysteine reduces the pCMBS concentration to zero. However, the arguments in the paragraph above exclude this possibility. Thus, it seems likely that pCMBS binding to the urea inhibition site causes the protein to change its conformation so that cysteine can no longer reach the pCMBS site as, for example, by moving the site to a lipid environment.

Effects of other mercurial reagents

In order to determine the effect of lipid permeability, other mecurial reagents were studied, particularly pCMB which Sha'afi and Feinstein [3] had shown to inhibit water permeability. pCMB is a weak acid which makes it much more lipid soluble than pCMBS with its sulfonate group, so that pCMB can readily permeate the cell mem-

brane, while pCMBS can not. Ashley and Goldstein [28] had found that there was a fast component ($k \sim 0.2$ min^{-1}) in the time-course of pCMB inhibition of red cell water transport. Our first point was taken at five minutes after the reaction began, by which time the fast component would no longer have been evident, and our curves showed no sign of more than a single exponential term. The results are given in Table I.

In the course of these studies, we also measured the $K_{i,app}$ values of two other mercurial sulfhydryl reagents that had previously been found to affect red cell water transport [3,29,30,31], fluorescein-mercuric acetate (FMA) and $HgCl_2$. Benga et al. [29] had shown that FMA inhibits diffusional permeability of water with a K_i of 50–70 μM at 37°C, and our value of $K_{i,app}$, 85 ± 10 μM for osmotic permeability in *N*-ethylmaleimide-treated cells at 20–22°C, as given in Table I, is in reasonable agreement; they also found that FMA inhibition of diffusional water flux could not be reversed even by a 10^3-fold excess of cysteine. Benga et al. [30] found that $HgCl_2$ inhibited diffusional permeability of water with a K_i of around 20 μM at 37°C, and our $K_{i,app}$ of 60 ± 10 μM for osmotic permeability in *N*-ethylmaleimide-treated cells at 20–22°C is in reasonable agreement. $HgCl_2$ also inhibits urea permeability with a $K_{i,app}$ only about a quarter of that for the water inhibition site.

Effects of stilbene inhibitors on water flux

Brahm [31] showed that the stilbene anion transport inhibitor, DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate) has no effect on the rate of water diffusion across normal red cell membranes. If DIDS is bound to a site within the aqueous channel, the large stilbene molecule would be expected to occlude the channel. In order to explain their findings on stilbene interactions with band 3, Verkman et al. [23] proposed that stilbene binding caused a conformation change which internalized the stilbene within the protein. Jennings and Passow [32] also presented evidence that one of the isothiocyano moieties of the stilbene became internalized. Macara et al. [33] used energy-transfer measurements to show that a conformation change followed stilbene binding, but the most convincing evidence was provided by Pimplikar and Reithmeier [34] who developed a stilbene affinity column to purify band 3. Before the conformation change, band 3 could be readily eluted from the SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate) bonded to the affinity column resin; after the conformation change band 3 could no longer be eluted. Solomon et al. [5] suggested that, after the conformation change internalized the stilbene, the channel became unencumbered so that water could easily traverse the channel. Even though there is no direct stilbene effect on water transport, we thought that stilbenes might have an indirect effect on the kinetics of pCMBS modulation of transport, which must also depend upon the conformation of band 3.

We have therefore made a search for evidence of an interaction between the stilbenes and the kinetics of the pCMBS effect and found that two reversible stilbene (see Knauf [35]) inhibitors, DBDS ($K_i = 1.3$ μM) and DNDS (4,4′-dinitrostilbene-2,2′-disulfonate; $K_i = 2$ μM), have a significant effect on the kinetics of 0.4 mM pCMBS inhibition of water transport at pH 7.4. Fig. 8 shows the results for 20 μM DBDS in one experiment, characteristic of two. The ratio of the hydraulic conductivities (+DBDS/control) is 0.80 ± 0.03 and, though the difference between the points is small, it is significant at better than the 99.9% level ($p < 0.0005$ in each experiment, *t*-test). A

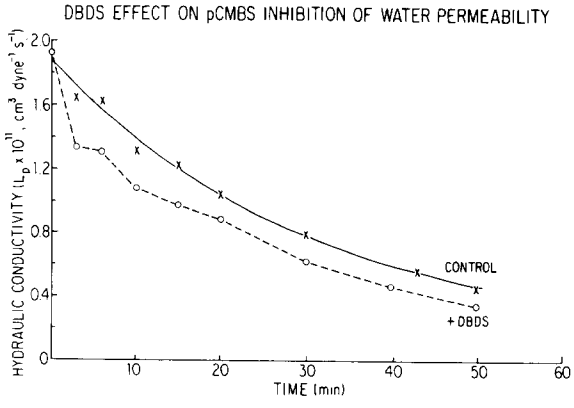

Fig. 8. The effect of 20 μM DBDS on the pCMBS (0.4 mM) inhibition of water permeability in one of three experiments with similar results. The control inhibition curve has been fitted by non-linear least-squares to a single exponential with $\tau = 30 \pm 5$ min.

similar result was obtained with 30 μM DNDS ($p < 0.0005$). Control experiments with DBDS and DNDS, in the absence of pCMBS, showed no change in the hydraulic conductivity. The time-course in Fig. 8 indicates that the stilbene inhibitor acts at the very beginning of the experiment, as if it were causing a conformational change in the pCMBS binding site that modulated the mechanism of pCMBS inhibition of water transport. This observation of a direct effect of site specific stilbene inhibitors on water transport inhibition by pCMBS provides further evidence which supports our view (see Refs. 5–8) that the anion transport site and the water transport channel are contained in the same protein or protein complex.

Discussion

Do water, urea and anions enter the cell through a common aqueous channel?

Permeation through an aqueous channel depends on at least two components, the channel which solute and solvent traverse and the modulation process which controls the flux rate. In general, when a single inhibitor is found to modulate the flux of more than a single permeating species, it follows that the fluxes of the two species are linked, but only to the extent that they are modulated by allosteric action on a single protein or a protein complex. It does not follow that the two species permeate through the same channel, which can only be established by further, very specific, experiments.

There are a number of observations that show that the fluxes of water, urea and anions are linked in the modulation sense. Thus, Reithmeier [36] found that mM concentrations of the non-mercurial sulfhydryl reagent, DTNB (5,5′-dithiobis(2-nitrobenzoic acid)), inhibited red cell anion transport by non-covalent binding at the stilbene site, acting in this instance as a molecule with steric and charge characteristics similar to the stilbene inhibitors, rather than as a sulfhydryl reagent. Toon et al. [7] confirmed Reithmeier's findings in *N*-ethylmaleimide-treated red cells and found that DTNB also inhibited water and methyl urea fluxes. K_i for sulfate efflux was 1.7 ± 0.3 mM, essentially equal to the $K_{i,app}$ of 2.5 ± 0.3 mM for water flux inhibition. Sulfate flux is inhibited by 76% and water flux by a smaller fraction, 29%. DTNB also inhibited methyl urea flux by 48%. The concentration dependence of the inhibition of water and methyl urea fitted the same single site binding curve, which is strong evidence that entrance of solute and solvent into the red cell is modulated by the same site. Furthermore, pretreatment with DIDS reduces the DTNB induced water flux inhibition from 29% to 12% and completely abolishes the DTNB inhibition of methyl urea flux. These experiments indicate that the fluxes of water, anions and methyl urea ar elinked in the modulation sense.

Yoon, Toon and Solomon [6] synthesized a molecule called DCMBT (4,4′-dichloromercuric 2,2,2′,2′-bistilbenetetrasulfonic acid) which combines a moiety similar to the stilbene anion transport inhibitor with a moiety similar to pCMBS. They found that this molecule strongly inhibited sulfate flux in *N*-ethylmaleimide-treated red cells. It also inhibited water transport by up to 20% and the water inhibition could be reversed by pretreatment with DIDS. They concluded that the DIDS binding site and the pCMBS water inhibition site were located within 12 Å of one another, which is the maximum distance between one Hg and the furthest stilbene, thus adding further evidence that the two sites are located in the same protein, or protein complex.

The primary argument that urea and water do not enter the cell through the same aqueous channel is an indirect one, based on a comparative physiological argument made by Brahm [37] and Macey [9], who have pointed out that red cells in other species, such as the nucleated red cell in the chicken, have a high anion permeability but a low permeability to urea and water. They use this example to argue that anions, urea and water can not share a common pathway in the human red cell membrane. This conclusion is dependent upon the premise that all the membrane bound segments of band 3 in the nucleated chicken red cell membrane are identical with those found in the non-nucleated human red cell membrane. Although these two proteins exchange anions at similar rates and appear to be similar by partial proteolysis, Jay [38] has found that they differ in isoelectric point and proteolytic patterns. Jay also

points out that antisera raised against human and chicken band 3 do not cross react, which suggests that the two sera do not recognize any common antigenic determinants.

The most direct approach to the question of whether urea and water go through the same channel is to determine the reflection coefficient, σ, for urea permeation through the red cell membrane. In general, values of $\sigma < 1.0$ provide thermodynamic proof that coupling exists between solute and solvent flow in a single channel (see Katchalsky and Curran [39]). Chasan and Solomon [40] have recently made a new determination of σ_{urea} in the human red cell and obtained a value of 0.7 ± 0.02. Treatment with 1 mM pCMBS increased σ to 0.93 ± 0.02. Under these specific conditions, the criterion for coupled flow, as Chasan and Solomon point out, is $\sigma_{urea} < 0.95$. Levitt and Mlekoday [41] have also recently determined σ_{urea} for the human red cell and obtained a value of 0.95; they point out that their experimental curve can also be fit by $\sigma = 0.75$, which agrees with the Chasan and Solomon figure. Aside from this experiment, all the experimental data in the literature give values of σ_{urea} less than 0.95. Thus, the measurements of σ_{urea} prove that urea and water share the same aqueous pathway through the membrane, not necessarily that all of the urea molecules do, but that there is a significant degree of coupling between urea and water flow in the same channel.

Taken together, these various lines of evidence implicate a single protein, or protein complex, as a common factor in the transport of anions, urea and water across the human red cell membrane. In this context, our observation that the site for pCMBS inhibition of water transport is different from that for urea does not mean that water and urea use different channels. Instead it means only that at least one rate controlling step is different but has no bearing on whether solute and solvent traverse the same channel. The only evidence which bears on this point is that of σ which shows that urea and water do indeed, at least in part, share a common channel as they cross the membrane.

What is the locus of the sulfhydryl reagent action?

Water inhibition site

Localisation of the water transport inhibition site depends upon experiments with specific stilbene anion transport inhibitors, particularly DBDS which fluoresces when bound to band 3. We have seen (Fig. 8) that DBDS can also modulate the kinetics of pCMBS inhibition of water transport. Lukacovic et al. [42] have previously used DBDS fluorescence to study the kinetics of interactions in the pCMBS/DBDS/band 3 system. In the course of this study they made an independent determination of the equilibrium constant for pCMBS binding to band 3 (their K_i), which is equivalent to $K_{i,app}$ in our notation. Their constant has a value of approx. 110 μM which agrees very well with the present value of 160 ± 30 μM for pCMBS binding to the water inhibition site. This agreement reinforces the view that the pCMBS water inhibition site and the DBDS binding site are located in the same protein or protein complex.

The characteristics of the water transport inhibition site are most unusual. It reacts specifically with lipid soluble mercurial reagents. As we have seen, it does not react with DTNB nor *N*-ethylmaleimide and we have probed the site with a variety of lipophilic maleimides but never found any evidence of reaction with a maleimide which would protect the water transport inhibition site from its characteristic reaction with pCMBS. Sha'afi and Feinstein [3] showed that a wide variety of sizes and shapes of mercurial reagents inhibited water transport.

Although the water inhibition site requires a mercurial reagent, the further properties of the ligand are not important since Table I shows that $K_{i,app}$ is low, being in the range of 10^{-4}–10^{-5} M, and varies only by a small factor between the very small $HgCl_2$ molecule and the much larger FMA. The site does discriminate against the charge on the sulfonate, but only by a factor of less than three between the charged pCMBS and the less polar pCMB. The changes in k_1 are much more striking since FMA requires 20 min of incubation at 37°C in order to produce inhibition whereas $HgCl_2$ inhibition is complete within 5–15 min at room temperature. This suggests that a profound rearrangement of the protein is needed to accommodate the bulky molecules that contain aromatic rings. The very long reaction times indicate that the path between the native and final configura-

tion is long and tortuous for these aromatic molecules, but it is very much shorter for $HgCl_2$. The fact that k_1 for pCMBS is slower by a factor of three than k_1 for pCMB suggests that the path leads through a hydrophobic region. Kopito and Lodish [16] suggest that there are twelve α-helical segments in band 3 that span the membrane and that polar faces on five or more of these helices act cooperatively to form a channel (see also Solomon et al. [5]). Such an ordered structure might have great difficulty in accommodating a pCMBS molecule.

Urea flux inhibition site

Sha'afi and Feinstein [3] used polyacrylamide gel electrophoresis to study the locus of ^{14}C-pCMB binding in red cells that had been treated with *N*-ethylmaleimide, iodoacetamide and mersalyl to block many of the membrane SH groups. After 30 min incubation with 1 mM pCMB at room temperature, the predominant ^{14}C peak was found at the band 3 position. Solomon et al. [5] obtained similar results for ^{203}Hg-pCMBS binding to *N*-ethylmaleimide-treated ghosts that had been treated with 0.1 mM pCMBS for 2 min at 0°C. They found a predominant peak at the band 3 location, but also reported a peak at band 4.5 that contained up to 30% of the number of band 3 sites. At the time these experiments were done, we did not know the kinetics of the pCMBS reaction and thought that this peak represented binding to the water inhibition site. The data in Table I for a pCMBS concentration of 0.1 mM show that τ for the water inhibition site is 94 min at room temperature and that for urea is 0.5 min. Thus there would have been no pCMBS reaction with the water site and the urea site would be less than half filled, depending upon the temperature coefficient for the binding reaction. Toon et al. [7] computed that 10^5–10^6 urea channels are required to accommodate the observed red cell urea flux and pointed out that bands 3 and 4.5 are the only integral red cell membrane proteins which contain a sulfhydryl group and are present in sufficient number.

Fig. 9 shows a Scatchard plot of our ^{203}Hg-pCMBS binding data (Lukacovic, unpublished data) fitted by non-linear least squares to a two site model. The high-affinity site has $K_1 = (1.3 \pm 7.6) \cdot 10^{-6}$ M and the number of sites, $N_1 = (0.8 \pm 0.2) \cdot 10^6$/cell. In view of the very large errors, these data are consistent with the characteristics of the urea transport inhibition site, as given in Table I. There should have been essentially no binding to the water transport inhibition site in this short time, so that Fig. 9 contains no information about the locus of this site and we can assign the primary gel electrophoresis peaks at band 3 to the urea inhibition site.

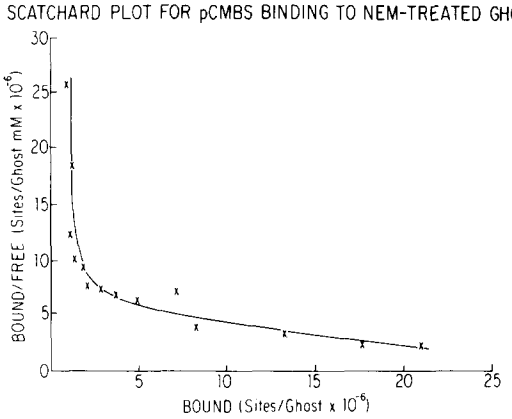

Fig. 9. Scatchard plot for ^{203}Hg-pCMBS binding to *N*-ethylmaleimide-treated resealed ghosts. Ghosts were treated with 2 mM *N*-ethylmaleimide (NEM) in phosphate-buffered saline (0.15 M NaCl, 0.005 M Na_2HPO_4, pH 7.5) for 1 h at 25°C and then incubated with 0.1 mM ^{203}Hg-pCMBS for 2 min at 0°C. Ghosts were washed thrice in phosphate-buffered saline containing 2 mM *N*-ethylmaleimide (20 vols.). The data were fitted by non-linear least squares to a two site Scatchard equation with the number of sites/cell of the high affinity site, $N_1 = (0.8 \pm 0.2) \cdot 10^6$ and affinity, $K_1 = 1.3 \pm 7.6$ μM. For the low affinity site, $N_2 = (30 \pm 10) \cdot 10^6$ and $K_2 = 5 \pm 3$ mM. Although the fit appears to be quite good by eye the errors are very large, probably because the high-affinity site binding curve is so steep.

The urea transport inhibition site binds pCMB and pCMBS very tightly with $K_{i,app}$ of approx. 10^{-7} M and discriminates in favor of the charged pCMBS by a factor of six. The aromatic component clearly favors binding since $K_{i,app}$ for $HgCl_2$ is larger by two or more orders of magnitude than that of the aromatic mercurials. These requirements are reminiscent of those for binding of the stilbene anion transport inhibitors to band 3 which are favored by charged sulfonates and aromatic rings. The binding constants are just about as tight, since $K_{i,app}$ for DBDS binding to band 3,

given by Smith and Dix [25] is 1.3 μM, within an order of magnitude of that for pCMBS binding to the urea transport inhibition site.

The rearrangements required to accommodate the mercurial sulfhydryl reagents can be achieved more readily by this site than the water transport inhibition site since k_1 is faster by one to two orders of magnitude. The pathway probably leads through a hydrophilic environment since pCMBS reacts much more rapidly than pCMB. These observations would be consistent with a binding site in a less constrained portion of the protein, possibly in apposition to the external face of the membrane.

Are the pCMBS transport inhibition binding sites on band 3?

As has been discussed, our experiments have been carried out under conditions in which the *N*-ethylmaleimide reaction is complete, so that the sixth cryptic cysteine is the single sulfhydryl available on band 3 to accommodate the two distinct binding sites described above. This remaining cysteine, at residue 498, has been placed by Jay and Cantley [43] just inside the membrane on the extracellular face.

The most likely candidate to occupy this place is DTNB, rather than either pCMBS (water transport or urea transport inhibition site). The gel electrophoresis pattern of Brown et al. [4] shows a single prominent peak for ^{14}C-DTNB at band 3 and no other peak in *N*-ethylmaleimide- and iodoacetamide-treated red cell ghosts. Toon et al. [7] assayed DTNB binding by spectrophotometric determination of the colored reaction product and found that there was covalent DTNB binding to band 3 at a ratio of 1.1 ± 0.2 DTNB sites to band 3 sites and that the site was saturated when the DTNB content equalled the band 3 content, which is normally in the μmolar concentration range. Presence of the reaction product proves that reaction took place with a sulfhydryl group. Although it is possible that the agreement between the number of binding sites and the number of copies of band 3 is fortuitous, the observation of Toon et al. [7] that DTNB binding affects the kinetics of the DBDS/band 3 reaction makes the argument that DTNB binds to a cysteine on band 3 extremely strong.

DTNB occupation of the covalent site appears to have no effect on the pCMBS inhibitable transport processes. Toon et al. [7] found no inhibition of water transport in the μmolar concentration range, confirming the findings of Benga [30], Macey [9], Brahm [31] and others. Furthermore, Toon et al. [7] showed that covalent binding of DTNB did not interfere with the normal pCMBS inhibition of water transport and we have found (data not shown) that the same is true for the urea inhibition site. This means that there is a DTNB site, located on band 3 by gel electrophoresis, that is different from either the water or urea transport inhibition sites.

This argument leaves no sulfhydryl groups available for the pCMBS binding to the water and urea transport inhibition sites. It follows that: either the two pCMBS binding sites are not sulfhydryl groups or they are not on band 3. Although organic mercurial compounds are generally considered to be specific reagents for sulfhydryl groups in proteins, this is not necessarily the only reaction site (Liu [44]). A good example is the specific binding of pCMBS to crystalline sperm whale myoglobin, which contains no sulfhydryl groups (Watson et al. [45]).

The gel electrophoresis pattern of Solomon et al. [5] provides a strong argument for placing the pCMBS urea transport inhibition site on band 3, a finding which agrees with the conclusions from the gel electrophoresis data of Sha'afi and Feinstein [3] which showed the pCMB peak to be located on band 3. This conclusion is supported by the close relationships between the pCMBS/urea-transport-inhibition site and the stilbene-inhibitor/band 3 system, which shows that the two sites are closely related in space.

Logic would dictate that the urea inhibition site is on band 3, but is not a sulfhydryl group. The binding reaction would appear to be a chemical reaction, since binding is relatively tight and there is a reasonable sensitivity to the chemical nature of the mercurial ligand. As we have discussed above, the fact that pCMBS binding to the urea inhibition site can not be reversed by cysteine is not a proof that a sulfhydryl group is not involved, but rather a reflection of a conformation change that moves the mercurial into an environment that can not easily be reached by cysteine.

Localization of the water transport inhibition site presents a more difficult problem because there are no gels which demonstrate pCMBS binding to such a site on band 3, just as there are no sulfhydryl groups remaining to which the pCMBS could bind. The evidence that the water transport inhibition site is coupled, in the modulation sense, to the anion and urea transport processes is very strong and has been given above. The only experiments which localize the site spatially are those with DCMBT which place the water inhibition site within about 12 Å of the anion transport inhibition site. Our evidence [5] also indicates that the water inhibition site is in a cleft or channel in the membrane and that the site is external to the anion transport site since anion transport inhibitors which inhibit entrance of pCMBS into the cell do not inhibit pCMBS inhibition of water transport. The observation that cysteine reverses binding to this site strongly suggests that the water transport inhibition is governed by a sulfhydryl reaction.

If this is the case, it follows that the probable locus for the water trnasport inhibition site is on another protein, complexed with band 3. Solomon et al. [5] have computed that $2.7 \cdot 10^5$ aqueous pores are required to account for the red cell membrane water flux – a figure that agrees reasonably well with the $6 \cdot 10^5$ copies of the band 3 dimer. As previously discussed, there are only two integral membrane proteins present in comparable amounts: glycophorin, which has been sequenced and found to contain no sulfhydryl groups [46] and band 4.5, the glucose transport protein, which Shelton and Langdon [47] report to be present in approx. $3 \cdot 10^5$ copies/cell. In view of the uncertainty in the estimates of the band 4.5 content (see Refs. 48 and 49) and the possibility that band 3 may be present as a tetramer rather than a dimer [50,51] no convincing argument can be based on the stoichiometry. Band 4.5 has been sequenced by Mueckler et al. [52], whose preliminary model shows two of the six cysteine residues to be located at or near the outside surface. Deziel et al. [53] have found that *N*-ethylmaleimide reacts with five sulfhydryl groups on band 4.5. The finding that only one of these can also react with pCMBS in situ is used to localize the pCMBS-reactive group to the outside of the cell. This may be presumed to be the extracellular pCMBS site, cryptic to *N*-ethylmaleimide, which Batt et al. [54] have found to inhibit some fraction of the net glucose efflux. Furthermore, Carruthers and Melchior [55,56] have shown that reconstitution of band 4.5 into unilamellar lipid vesicles provides a significant increase in water permeability. These considerations mean that band 4.5 is a viable candidate for the water transport inhibition site, but resolution of this question must await further experiments in reconstituted band 3 and band 4.5 vesicles.

Acknowledgement

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References

1 Macey, R.I. and Farmer, R.E.L. (1970) Biochim. Biophys. Acta 211, 104–106
2 Naccache, P. and Sha'afi, R.I. (1974) J. Cell. Physiol. 84, 449–456
3 Sha'afi, R.I. and Feinstein, M.B. (1977) Membrane water channels and SH-groups. in Adv. Exp. Med. Biol. Vol. 84 (Miller, M.W., Shamoo, A.E. and Brand, J.S., eds.), pp. 67–80, Plenum Press, New York
4 Brown, P.A., Feinstein, M.B. and Sha'afi, R.I. (1975) Nature 254, 523–525
5 Solomon, A.K., Chasan, B., Dix, J.A., Lukacovic, M.F., Toon, M.R. and Verkman, A.S. (1983) Ann. N.Y. Acad. Sci. 414, 97–124
6 Yoon, S.C., Toon, M.R. and Solomon, A.K. (1984) Biochim. Biophys. Acta 778, 385–389
7 Toon, M.R., Dorogi, P.L., Lukacovic, M.F. and Solomon, A.K. (1985) Biochim. Biophys. Acta 818, 158–170
8 Toon, M.R. and Solomon, A.K. (1985) Biochim. Biophys. Acta 821, 502–504
9 Macey, R.I. (1984) Am. J. Physiol. 246, c195–c203
10 Terwilliger, T.C. and Solomon, A.K. (1981) J. Gen. Physiol. 77, 549–570
11 Sha'afi, R.I., Rich, G.T., Mikulecky, D.C. and Solomon, A.K. (1970) J. Gen. Physiol. 55, 427–450
12 Steck, T.L., Koziarz, J.J., Singh, M.K., Reddy, G. and Kohler, H. (1978) Biochemistry 17, 1216–1222
13 Rao, A. and Reithmeier, R.A.F. (1979) J. Biol. Chem. 254, 6144–6150
14 Rao, A. (1979) J. Biol. Chem. 254, 3503–3511
15 Ramjeesingh, M., Gaarn, A. and Rothstein, A. (1983) Biochim. Biophys. Acta 729, 150–160
16 Kopito, R.R. and Lodish, H.F. (1985) J. Cell. Biochem. 29, 1–17
17 Haest, C.W.M., Kamp., D. and Deuticke, B. (1981) Biochim. Biophys. Acta 643, 319–326

18 Czerlinski, G.H. (1966) Chemical Relaxation, Marcel Dekker, New York
19 Rao, A., Martin, P., Reithmeier, R.A.F. and Cantley, L.C. (1979) Biochemistry 18, 4505–4516
20 Dainty, J. (1963) Adv. Bot. Res. 1, 279–326
21 Ginzburg, B.Z. and Katchalsky, A. (1963) J. Gen. Physiol. 47, 403–418
22 Rao, A. (1978) Ph.D. Thesis, Harvard University, The reactive sulfhydryl groups of the band 3 polypeptide of human erythrocyte membranes (see p. 155 and Fig. V-4)
23 Verkman, A.S., Dix, J.A. and Solomon, A.K. (1983) J. Gen. Physiol. 81, 421–449
24 Cantor, C.R. and Schimmel, P.R. (1980) Biophysical Chemistry, p. 921, Freeman, San Francisco
25 Smith, K.R. and Dix, J.A. (1984) Biophys. J. 45, 202a
26 Brahm, J. (1977) J. Gen. Physiol. 70, 283–306
27 Schwarz, J.R., Ulbricht, W. and Wagner, H.H. (1973) J. Physiol. 233, 167–194
28 Ashley, D.L. and Goldstein, J.H. (1981) J. Membrane Biol. 61, 199–207
29 Benga, G., Pop, V.I., Ionescu, M., Holmes, R.P. and Popescu, O. (1982) Cell Biol. Int. Rep. 6, 775–781
30 Benga, G., Pop, V.I., Popescu, O., Ionescu, M. and Mihele, V. (1983) J. Membrane Biol. 76, 129–137
31 Brahm, J. (1982) J. Gen. Physiol. 79, 791–819
32 Jennings, M.L. and Passow, H. (1979) Biochim. Biophys. Acta 554, 498–519
33 Macara, I.G., Kuo, S. and Cantley, L.C. (1983) J. Biol. Chem. 258, 1785–1792
34 Pimplikar, S.W. and Reithmeier, R.A.F. (1986) Biophys. J. 49, 140a
35 Knauf, P. (1979) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 12, pp. 249–363, Academic Press, New York
36 Reithmeier, R.A.F. (1983) Biochim. Biophys. Acta 732, 122–125
37 Brahm, J. (1983) J. Gen. Physiol. 82, 1–23
38 Jay, D.G. (1983) J. Biol. Chem. 258, 9431–9436
39 Katchalsky, A. and Curran, P.F. (1965) Nonequilibrium Thermodynamics in Biophysics, Ch. 10, p. 113, Harvard University Press, Cambridge, MA
40 Chasan, B. and Solomon, A.K. (1985) Biochim. Biophys. Acta 821, 56–62
41 Levitt,, D.G. and Mlekoday, H.J. (1983) J. Gen. Physiol. 81, 239–253
42 Lukacovic, M.F., Verkman, A.S., Dix, J.A. and Solomon, A.K. (1985) Biochim. Biophys. Acta 778, 253–259
43 Jay, D. and Cantley, L. (1986) Annu. Rev. Biochem., in the press
44 Liu, T.-Y. (1977) in The Proteins, Vol. 3, 3rd Edn. (Neurath, H., Hill, R.L. and Boeder, C., eds.), p. 293, Academic Press, New York
45 Watson, H.C., Kendrew, J.C. and Stryer, L. (1964) J. Mol. Biol. 8, 166–169
46 Marchesi, V.T. (1979) Semin. Hematol. 16, 3–20
47 Shelton, R.L., Jr. and Langdon, R.G. (1984) Anal. Biochem. 140, 366–371
48 Lin, S. and Spudich, J.A. (1974) J. Biol. Chem. 249, 5778–5783
49 Whitfield, C.F., Coleman, D.B., Kay, M.B.K., Shiffer, K., Miller, J. and Goodman, S.R. (1984) in Erythrocyte membranes, Vol. 3: Recent Clinical and Experimental Advances, pp. 31–46, Alan R. Liss, New York
50 Nigg, E. and Cherry, R.J. (1979) Nature 277, 493–494
51 Cuppoletti, J., Goldinger, J., Kang, B., Jo, I., Berenski, C. and Jung, C.Y. (1985) J. Biol. Chem. 260, 15714–15717
52 Mueckler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.H., Allard, W.J., Lienhard, G.E. and Lodish, H.F. (1985) Science 229, 941–945
53 Deziel, M.R., Jung, C.Y. and Rothstein, A. (1985) Biochim. Biophys. Acta 819, 83–92
54 Batt, E.R., Abbott, R.E. and Schachter, D. (1976) J. Biol. Chem. 251, 7184–7190
55 Carruthers, A. and Melchior, D.L. (1983) Biochemistry 22, 5797–5807
56 Carruthers, A. and Melchior, D.L. (1984) Biochemistry 23, 6901–6911