Biochimica et Biophysica Acta, 942 (1988) 73–82
Elsevier

BBA 74031

Sites of *p*-chloromercuribenzenesulfonate inhibition of red cell urea and water transport

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(Received 17 September 1987)
(Revised manuscript received 21 March 1988)

Key words: Erythrocyte; Sulfhydryl group; Urea transport; Water transport; Band 3; *p*-Chloromercuribenzenesulfonate

The mercurial sulfhydryl reagent, *p*-chloromercuribenzene sulfonate (pCMBS), inhibits water and urea fluxes across the human red blood cell membrane. The kinetics and affinities for pCMBS binding to separate water transport and urea transport inhibition sites were previously determined by Toon and Solomon ((1986) Biochim. Biophys. Acta 860, 361–375) in red cells that had been treated with *N*-ethylmaleimide (NEM) to block five of the six sulfhydryls on the red cell anion exchange protein, band 3. We have used autoradiographs of gels from NEM-treated cells, labeled with ^{203}Hg-pCMBS, to localize these water and urea transport inhibition binding sites separately and find that both are on band 3. Each site is saturable and the time course of each uptake can be fitted to the equation for a bimolecular association (with negligible dissociation) with time constants in agreement with those of Toon and Solomon. Determination of the binding stoichiometry shows one urea inhibition site and three water inhibition sites for every four band 3 molecules. These results indicate that band 3 plays a role in both urea and water transport and suggest that the functional unit may be a tetramer.

Introduction

The initial observation of Macey and Farmer [1] that the flux of water and urea into the human red cell was inhibited by 0.4 mM of the mercurial sulfhydryl reagent, *p*-chloromercuribenzenesulfonate (pCMBS), was followed by the demonstration of Knauf and Rothstein [2] that 0.1 mM pCMBS induced a large cation leak in the human red cell but had little or no effect on anion exchange. Sha'afi and Feinstein [3] characterized the effects of a large number of sulfhydryl reagents on red cell water flux and showed by gel electrophoresis that *p*-chloromercuribenzoate (pCMB), which is also an effective inhibitor of red cell water flux, was bound primarily to the red cell anion exchange protein, band 3, when appropriate precautions were taken to block other sulfhydryl groups by reagents that had no effect on red cell water transport. On the basis of studies with another sulfhydryl reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), Brown et al. [4] suggested that band 3 was the locus of the red cell water channel. Solomon et al. [5] found that pCMBS also bound primarily to band 3, when similar precautions were taken, and suggested that band 3 was the locus not only for the water and urea

Abbreviations: pCMB, *p*-chloromercuribenzoate, pCMBS, *p*-chloromercuribenzenesulfonate; NEM, *N*-ethylmaleimide; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate.

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transport pathway, but also for anion exchange and the passive cation leak characterized by Knauf and Rothstein [2]. However, Macey [6] pointed out that the kinetics for pCMBS inhibition of urea transport were much faster than for water inhibition and argued that, since there were different sites for pCMBS inhibition of water and urea transport, solute and solvent traversed different channels.

Recently, each site for pCMBS inhibition of water and urea flux was fitted to its own single site binding curve by Toon and Solomon [7], who determined the binding affinities and kinetics of pCMBS binding to red cells that had been treated with *N*-ethylmaleimide (NEM), which blocks five of the six sulfhydryls (Kopito and Lodish [8] and Rao [9]) on band 3 but has little effect on water or urea transport. Since there appeared to be only a single sulfhydryl group free to react with the two separate pCMBS sites, Toon and Solomon [7] suggested that one of these sites was on a different protein. Benga et al. [10] used ^{203}Hg-pCMBS gel electrophoresis to study the binding and reported two predominant peaks, under conditions of maximum water and urea transport inhibition, one each on band 3 and band 4.5, the glucose transport protein, with a stoichiometry of 1 pCMBS per site. They were unable to find the peak on band 3 which had been rapidly labelled (2 min, 0 °C) by Solomon et al. [5]. Benga et al. [11] confirmed and extended these results in a subsequent gel electrophoresis study.

In this paper, we have also used ^{203}Hg-pCMBS gel electrophoresis to characterize the two binding sites, which can be separated on gels by taking advantage of the very great difference in binding affinities and kinetics. Our results show that both sites are on band 3, but the stoichiometries of the two sites differ by a factor of approx. 3. The specific precautions we took to minimize pCMBS loss and migration from protein to protein during preparation of the gel, and also to confine pCMBS to extracellular sites, account for the substantial differences between our results and those of Benga et al. [10,11].

Materials and Methods

Materials. pCMBS and NEM were obtained from Sigma Chemical Co. (St. Louis, MO). 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) was from Molecular Probes (Junction City, OR) and ^{203}Hg-pCMBS was from Amersham Co. (Arlington Heights, IL). Tris, glycine, sodium dodecyl sulfate (SDS), molecular weight standards and the usual electrophoresis reagents were from Bio-Rad (Richmond, CA). Recently outdated blood was kindly donated by the Children's Hospital, Boston, MA.

Methods. After washing three times with Tris-buffered saline (150 mM NaCl, 5 mM KCl, 17 mM Tris-HCl (pH 7.4)), red cells were incubated at 25% hematocrit for 1 h at 37 °C with 12 mM NEM and 20 μM DIDS (in Tris-buffered saline). The cells were diluted to 10% hematocrit with a Tris-buffered saline solution containing 12 mM NEM and the appropriate (radioactive or unlabelled) pCMBS concentration. The reaction with pCMBS was stopped by osmotically lysing 1 : 20 (v/v) for 5 min with 1 mM NEM, 5 mM NaP_i (pH 8.0), centrifuging immediately at approx. $20\,000 \times g$ for 10 min at 4 °C, and then washing the pellet three or four times (until white) with osmotic lysis medium.

To label separately the inhibition sites for urea and water transport, advantage was taken of the fact that both the binding affinities and kinetics for the two sites differ by about two orders of magnitude [7]. The urea site, with negligible contribution from the water site, was labelled by reacting the cells with 25 μM ^{203}Hg-pCMBS (23.4 mCi/g) for 10 min at 21–24 °C, the temperature for all the labelling reactions (except those specified at 0 °C). In order to label the water site, the urea site was first filled with 25 μM unlabelled pCMBS for 10 min; the water site was then labelled with 175 μM ^{203}Hg-pCMBS for 2 h. Alternatively, both sites were labelled by reacting the cells with 175 μM ^{203}Hg-pCMBS for 2 h.

The membrane pellets (5 mg protein/ml) were diluted 1 : 5 (v/v) in 20 mM NEM, 80 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 0.001% (w/v) Bromophenol blue. After heating for 8 min at 80 °C, the proteins (approx. 50 μg/lane) were separated using the discontinuous SDS-polyacrylamide gel system, as described by Laemmli [12], except that mercaptoethanol was left out. Unless otherwise noted, the fixing, staining and destaining steps were also omitted, and

instead the slab gels were immediately dried under vacuum at 80 °C for 3 h and subsequently used for autoradiography (using Kodak X-AR5 film and a Dupont Cronex Lightning Plus fluorescent screen) at −80 °C for periods ranging from one to two weeks. The autoradiographs were scanned in a scanning densitometer (Model GS 300, Hoefer Co., San Francisco, CA), which was coupled to a Hewlett Packard Model 217 computer. The densitometer output was calibrated against a Kodak Step Tablet No. 1A and the response was found to be linear over the range from 0.05 to 1.8 *A*, which encompasses the density range of our autoradiographs. Programs were written to average 5–10 scans and to integrate the areas under the peaks.

In order to determine the ratio of pCMBS sites per band 3 monomer, a 50 μl aliquot of ^{203}Hg-pCMBS labelled ghost membranes was solubilized in 5 ml of Ultrafluor, placed in a Tracor scintillation counter (Model Delta 300, Elk Grove Village, IL) and its radioactivity (counted to 0.25%) was calibrated with an equal aliquot of a known concentration of ^{203}Hg-pCMBS; hence, the number of pCMBS sites per total protein was estimated. The mg total protein in the membrane sample was converted to mg of band 3 by staining a gel with Coomassie blue and measuring the percentage of the stain under the band 3 peak, not including the stain under the tracking dye (which Rao [13] also does not include). Typically, the percentage of band 3 in untreated ghost membranes is 25% [14], but after pCMBS treatment Rao [13] found the percentage increases to as much as 48%. Our value (approx. 35%) is lower than Rao's because DIDS treatment limits the pCMBS access to extracellular sites only. Combining the results from the scintillation counting and the Coomassie blue staining, and assuming a band 3 molecular weight of 100 kDa (Kopito and Lodish [8]), the number of pCMBS sites per band 3 can be calculated.

Results and Discussion

Lability of pCMBS binding to proteins

It is well known that the pCMBS bond to red cell membrane proteins is labile. Sutherland et al. [15] observed that uptake of pCMBS by human red cells was followed by a release phase which they attributed to leakage of pCMBS binding reagents from the cell. Knauf and Rothstein [16] pointed out that pCMBS initially taken up by the red cell membrane subsequently migrates to the cytoplasm where virtually all of the pCMBS is found after 2–4 h. Rega et al. [17] found that extracellular albumin reversed the pCMBS-induced red cell cation leak, consistent with the observation of Sutherland et al. [15] that albumin removes pCMBS from the red cell membrane.

Our exploratory pCMBS experiments were carried out without special precautions to avoid pCMBS loss from the gel, using Coomassie blue staining and destaining in methanol/acetic acid, the same technique that was used by Benga et al. [11]. When gels were prepared from ghosts that had been reacted with 1 mM pCMBS for 1 h, there was a peak at band 3 and most of the other membrane proteins were labelled, in agreement with Benga et al. (Fig. 6 in Ref. 11). When we labelled the ghosts for very short times, which should have filled the urea transport inhibition site, we found no label on band 3, in agreement with Benga et al. [11], but in disagreement with our own earlier results (Solomon et al. [5]) in which the radioactivity measurements were made on tube gels that not been fixed and stained.

Rao [13] studied the effects of pCMBS lability on results obtained by gel electrophoresis and reported that radioactivity was lost slowly from the gels during electrophoresis and rapidly during staining and destaining, so she omitted the staining and destaining steps, a procedure which we have adopted, but a precaution which was not taken by Benga et al. [11]. Rao [13] prepared ^{203}Hg-pCMBS treated (without NEM treatment) red cell ghost samples for gel electrophoresis by washing them in 1 mM NEM and dissolving them in SDS containing 5 mM NEM to prevent pCMBS exchange. Under these conditions Rao recovered 75% of the added radioactivity in her gels and found 2.8 mol of pCMBS/mol band 3 in the primary peak (at the band 3 location) for ^{203}Hg-pCMBS (2 mM) binding to unsealed ghosts that had been incubated for 10 min at 0 °C.

We have reported [5] that pCMBS inhibition of water and urea transport is not affected by NEM which binds to five of the six band 3 SH groups present in red cells and to the three intracellular

SH groups that remain in ghosts. Rao [9] has shown that reaction of the band 3 SH groups with NEM protects them against pCMBS binding. Rao's measurements [9] of NEM binding to ghosts showed saturation after 1 h incubation with 0.5 mM NEM at 37°C; red cells required 12 mM NEM for 1 h at 37°C. Consequently we blocked the NEM-reactive sites in red cells by incubation with 12 mM NEM under Rao's conditions and kept 1 mM NEM in all subsequent wash and resuspension media to avoid pCMBS migration; Benga et al. [10] used 2 mM NEM for 1 h at 25°C for incubation and subsequent manipulations for both ghosts and red cells.

pCMBS is an anion which permeates the red cell membrane through the anion exchange pathway, as shown by Rao [13] who found that the anion exchange inhibitor, 1-isothiocyano-4-benzenesulfonic acid, increased the half-time for pCMBS permeation into resealed ghosts from a control value of 40 min to 190 min (presumably at room temperature). Rothstein [18] reports that more than 98% of the pCMBS flux into the red cell occurs via a DIDS-sensitive pathway. We have shown that DIDS does not block pCMBS inhibition of urea and water fluxes [5], which indicates that the pCMBS sites that inhibit these transport systems are on the extracellular side of the DIDS inhibition site. Consequently we have treated the cells with 20 μM DIDS to keep pCMBS from reacting with intracellular sites, even though stilbene anion exchange inhibitors have some small effects on the kinetics of pCMBS inhibition of water transport. In NEM-treated red cells, 10 μM DIDS inhibits water flux [19] by 11% at pH 7.4. We have also found that another stilbene inhibitor, DBDS (4,4′-dibenzamidostilbene-2,2′-disulfonate), extends the time course of pCMBS inhibition of water transport by about 20%. Though DIDS has a similarly small, though variable, effect on pCMBS inhibition of urea transport, it has no measurable effect on the pCMBS inhibition of water transport (Toon and Solomon, private communication). The advantage of confining the binding sites that we study to the outside of the cell outweighs the relatively small effects that stilbene inhibitors exercise on the pCMBS inhibition process.

Benga et al. [10,11] did not use a stilbene anion exchange inhibitor; the possible contribution of intracellular pCMBS binding may be estimated from Rao's control pCMBS permeability. Motais [20] has pointed out that the activation energy for organic ion permeation is very close to that for inorganic ions, such as Cl^- self-exchange for which Brahm [21] gives an activation energy of 20 kcal · mol^{-1} over the range 15–38°C. This value leads to an estimated half-time for pCMBS permeation into resealed ghosts of 10 min at 37°C. Since Benga et al. [10,11] incubated resealed ghosts with pCMBS for 5 min at 37°C, and red cells for 30 min at 37°C, intracellular labelling could have contributed to the peaks on their gels, particularly the label on bands 1 and 2 of their red cell gel (Fig. 2b, Ref. 10). This may well be the explanation for the peak Benga et al. reported for band 4.5, since Deziel et al. [22] have shown that there is only a single pCMBS-reactive site on the external face of reconstituted band 4.5 vesicles and that site is blocked * by NEM (15 mM NEM, 1 h, 37°C).

pCMBS binding to high- and low-affinity sites

The binding of pCMBS to the water and urea transport inhibition sites was characterized phenomenologically by Toon and Solomon [7], who measured the time constants and binding affinities for inhibition of the transport processes. The bimolecular association site for urea transport inhibition has an apparent inhibition constant, $K_{i,app} = 90 \pm 60$ nM and an on-rate constant, $k_1 = 310 \pm 200\ M^{-1} \cdot s^{-1}$. The binding of pCMBS to the water transport inhibition site is very much weaker with $K_{i,app} = 160 \pm 30\ \mu M$ and a very slow on-rate constant, $k_1 = 1.77 \pm 0.03\ M^{-1} \cdot s^{-1}$, two orders of magnitude slower than for urea. These differences mean that it is possible to differentiate binding to the two different sites by suitable choices of pCMBS concentrations and reaction times. However, the uptake of ^{203}Hg-pCMBS by the gel measures binding, rather than flux inhibi-

* Deziel et al [22] used 15 mM NEM to be sure NEM would react with the two NEM sites inside the bilayer. Since the extrafacial SH groups generally react easily and quickly with SH reagents, it is likely that the extrafacial SH group on band 4.5 was blocked by the 2 mM NEM concentration used by Benga et al. [10,11].

tion, so that delays could ensue between binding and inhibition, though our results indicate that this is not the case.

Our initial gel studies were made under conditions when both sites should have been labelled. Fig. 1A shows a densitometric scan of a typical Coomassie blue stained gel. Comparison of Fig. 1B, in which the gel was prepared in the conventional way (NEM-treated cells, normal staining and destaining steps; no treatment with DIDS), with Fig. 1C shows how important it is to use the protocol described in the previous section. Fig. 1B is in qualitative agreement with Benga et al.'s red cell gel (Fig. 2b, Ref. 10) in which bands 1 and 2, 4.5 and 5 are labelled, in addition to band 3. The gel in Fig. 1C, which was prepared according to our final protocol, is strikingly different. The peak for band 3 is very much higher because we have suppressed the radioactivity loss in the staining procedure and bands 1, 2, and 4.5 have disappeared because we have blocked access to the intracellular binding sites. Comparison with molecular weight standards gives a M_r of approx. 46 000 for the second peak in Fig. 1C, slightly larger than the 43 000 (Haest [23]) for band 5. Our peak may be related to the peak identified as band 4.9 by Ralston and Crisp [24] in their studies of ^{203}Hg-pCMBS (1 mM, 1 h, 0 °C) labelling of NEM-treated (5 mM, 1 h, 0 °C) human red cells. As Fig. 1C shows, there is no trace of a peak at band 4.5 in this gel, though in other gels there are occasional small peaks, as will be discussed.

Since the approx. 46 kDa peak was apparent only after long exposure to ^{23}Hg-pCMBS, we studied the binding kinetics after blocking the urea transport inhibition site with unlabelled pCMBS. Fig. 2 compares the binding of 175 μM pCMBS at 30 min (Fig. 2A) with that at 2 h (Fig. 2B; the results for the three other gels in this series at 10 min, 75 min and 180 min are not shown). In this set of experiments, the relative height of the peak at approx. 46 kDa at 2 h in Fig. 2 is smaller

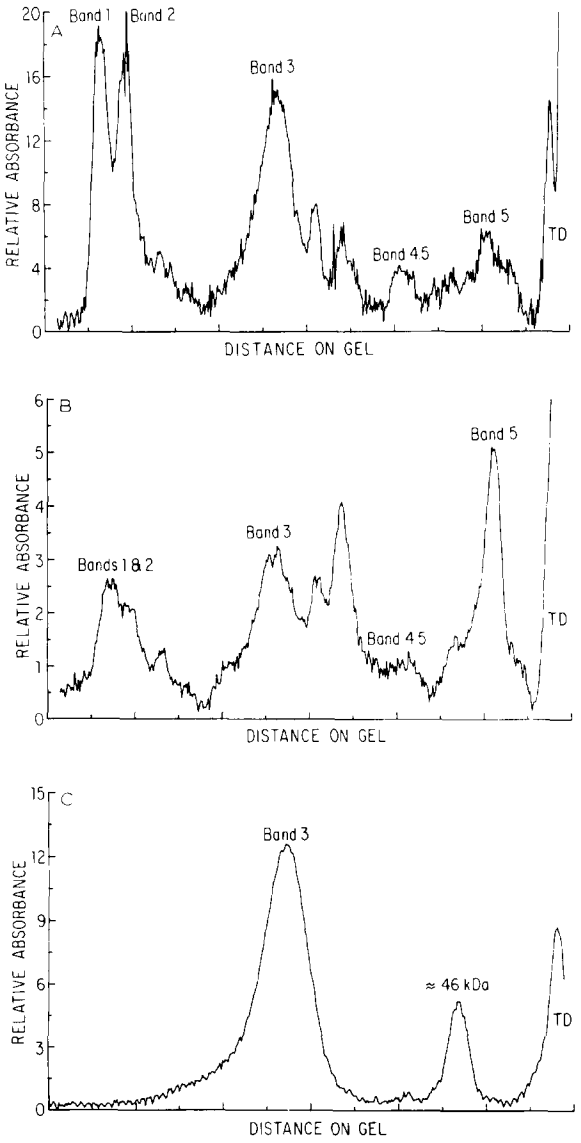

Fig. 1. (A) Densitometric scan of Coomassie blue stained gel of ghosts proteins prepared from ^{203}Hg-pCMBS labelled red cells. Proteins were applied to nonreducing 7.5% polyacrylamide Laemmli SDS-PAGE under the identical conditions used for autoradiography. All figures showing gels or autoradiographs are averaged densitometric scans, as described under Methods. (B) Autoradiograph of proteins reacted with 1 mM ^{203}Hg-pCMBS for 60 min. Cells had been pretreated with 2 mM NEM for 1 h, in the absence of DIDS. Before drying for autoradiography, the gels were fixed and stained in 40% methanol/10% acetic acid and destained in 10% methanol/7.5% acetic acid. (C) Autoradiograph of proteins reacted with 175 μM ^{203}Hg-pCMBS for 2 h at 21–24 °C, showing a predominant peak on band 3 and the ≈ 46 kDa protein. The cells had been pretreated with 12 mM NEM and 20 μM DIDS, and the fixing, staining and destaining steps were omitted. Under these conditions, the inhibition sites for both the urea and water permeability are filled. TD represents the tracking dye.

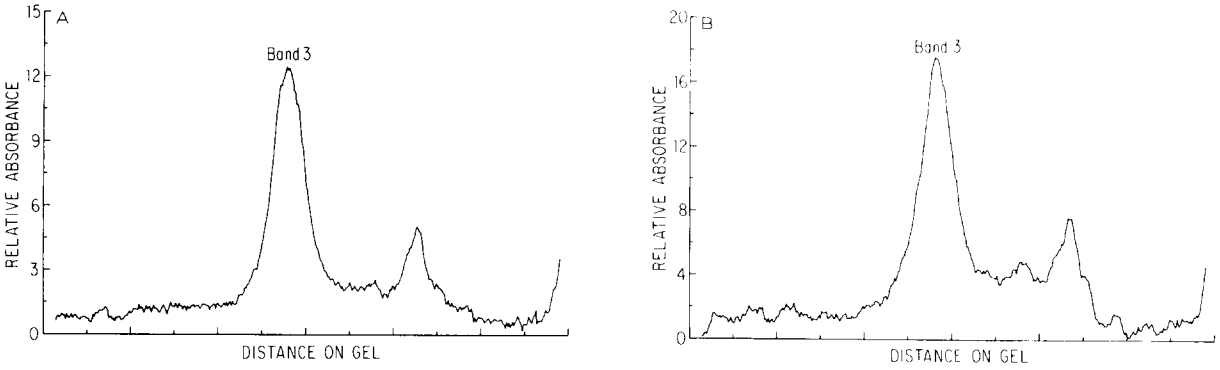

Fig. 2. Autoradiographs of membrane proteins whose urea inhibition site had been prefilled with 25 μM unlabelled pCMBS for 10 min. Cells were then reacted with 175 μM ^{203}Hg-pCMBS for (A) 30 min and (B) 120 min, different absorbance scale. The autoradiographs for 10, 75 and 180 min are not shown. By 180 min, the intensity of the band 3 peak begins to decrease.

than in Fig. 1C and there is a small amount of uptake by band 4.5. Fig. 3 Compares the time course of 175 μM ^{203}Hg-pCMBS binding to band 3, with the time course for the other two peaks in this experiment. The peak height data have been fit by non-linear least squares to the following equation for a bimolecular association with negligible dissociation, consistent with the kinetics used by Toon and Solomon [7].

$$[\text{pCMBS-band 3}] = [\text{pCMBS-band 3}]_{\text{final}}(1 - e^{-t/\tau}) \quad (1)$$

τ for the water inhibition site is 15 ± 2 min at this [pCMBS] which is in fair agreement with the value of 28 ± 5 min computed from the data of Toon and Solomon [7] for the same [pCMBS]. The observation that the peak saturates indicates that there is a single site and the reasonable agreement with the kinetics for pCMBS inhibition of water flux strongly suggests that this site on band 3 modulates red cell water transport.

We have fitted the peak heights for the ≈ 46 kDa peak to Eqn. 1 to give the curve in Fig. 3 for which $\tau = 98 \pm 7$ min. In these autoradiographs, there is a small peak at band 4.5, which has also been fitted to the same equation with $\tau = 78 \pm 65$ min, as shown in Fig. 3. Since the time constants for the two subsidiary peaks are very much greater than that for the water transport inhibition peak and since they are both similar, the peaks at ≈ 46 kDa and band 4.5 may be artifacts resulting from the slow leakage of pCMBS into the cell.

Under conditions in which the urea site alone should be filled, there is only a single peak at band 3, with no evidence of any other peaks, as shown in Fig. 4A. We chose the conditions in Fig. 4A (0 °C, 2 min) to show that our present results confirm the gel previously published by Solomon et al. [5]. The time course of pCMBS uptake to the urea transport inhibition site was studied at room temperature by incubating red cells with 2.5 μM ^{203}Hg-pCMBS for up to 100 min at 21–24 °C, sufficient to fill 99% of the urea flux inhibition site, while filling only 3% of the water flux inhibition site (Fig. 4B). These data have been fitted by non-linear least squares to Eqn. 1 with $\tau = 38 \pm 11$ min, which agrees with the figure of 21 ± 19 min

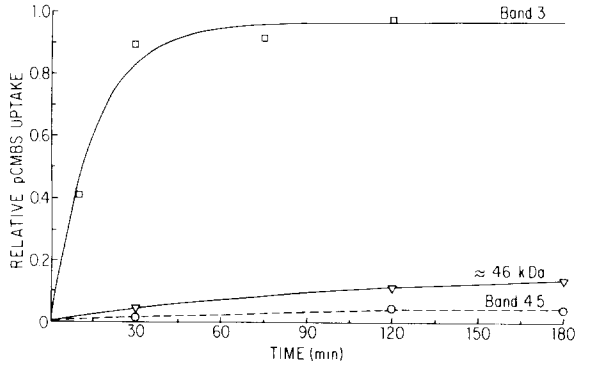

Fig. 3. Time course of pCMBS uptake by the water inhibition site. The heights of the various peaks in the autoradiographs of Fig. 2 were plotted as a function of time and the points were fitted by non-linear least-squares to Eqn. 1. For band 3, $\tau = 15 \pm 2$ min; for band 4.5, $\tau = 78 \pm 65$ min; and for the ≈ 46 kDa protein, $\tau = 98 \pm 7$ min.

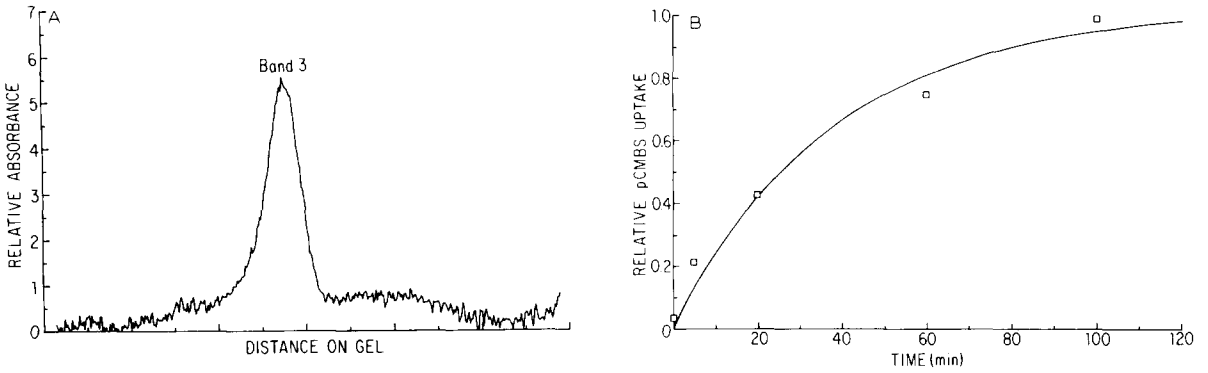

Fig. 4. Time course of pCMBS uptake by the urea inhibition site. (A) Autoradiograph of cells reacted with 100 μM ^{203}Hg-pCMBS for 2 min at 0° C. (B) Cells were reacted with 2.5 μM ^{203}Hg-pCMBS at room temperature and the uptake of label by band 3 was plotted as a function of time. The line was fit to Eqn. 1 with $\tau = 38 \pm 11$ min.

calculated from the data of Toon and Solomon [7]. This agreement strongly suggests that a site on band 3 is responsible for the pCMBS inhibition of urea transport and indicates that, though the inhibition sites have different binding affinities and kinetics, a single protein is involved in the pCMBS inhibition of both water and urea transport.

It would be possible for ^{203}Hg-pCMBS to bind to sites on proteins present in amounts too small to be visible in an autoradiograph. However, the number of channels for urea and water transport is in the range of $(0.6–1.3) \cdot 10^5$/cell, as computed by Toon and Solomon [19], a site density great enough to be seen. Hence, any sites for pCMBS modulation present in approximately equimolar proportion to the number of channels should be visible in Fig. 1C.

Stoichiometry

In order to determine the stoichiometry of the pCMBS binding sites, the urea and water sites were saturated with ^{203}Hg-pCMBS for up to 1 h at 21–24° C in separate aliquots in the same experiments, and the ratio of the bound radioactivity was determined. In four experiments of this kind the average value of the ratio was 3.1 ± 0.2 (mol pCMBS bound to the water inhibition site per mol pCMBS bound to the urea inhibition site).

Another set of experiments was used to determine the total number of pCMBS sites per band 3 molecule. This number can be determined indirectly by first measuring the radioactivity in the band 3 peak as a fraction of the total radioactivity in the gel, both in the peaks and in the tracking dye, in an autoradiograph in which both sites are saturated. Then the percentage of the total membrane protein in band 3 must be calculated. As discussed under Methods, both Rao [13] and Ralston and Crisp [24] have pointed out that pCMBS causes protein loss from red cell membranes. We determined the loss under our conditions of NEM, DIDS and pCMBS treatment by measuring the relative areas on a Coomassie blue stained gel. Under our conditions, band 3 comprises approx. 35% of the total area in the gel. These procedures lead to a stoichiometry of 1.3 ± 0.3 (mol (water + urea) transport inhibition sites/mol band 3). By combining these ratios, the number of urea sites/band 3 can be estimated as 0.3 ± 0.1.

The simplest membrane structure which is consistent with these stoichiometric ratios is a band 3 tetramer which contains one urea transport inhibition site and three water transport inhibition sites. Band 3 monomers are generally considered to be bound together in the membrane as non-covalent dimers and further evidence, reviewed by Jennings [25], suggests that tetramers exist in the native membrane, possibly as a result of dimer-dimer association. Bennett [26], who also suggests that band 3 is a tetramer in the membrane, advances arguments that band 3 tetramers bind to a single ankyrin and thence to the cytoskeleton. We have recently found (Ojcius and Solomon [27]) that the

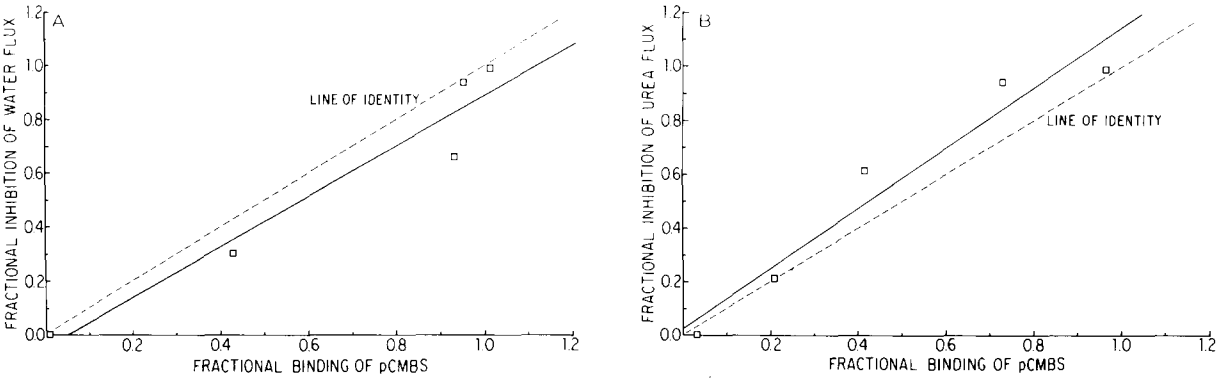

Fig. 5. (A) Fractional inhibition of water permeability as a function of the fractional binding of pCMBS to the water inhibition site. The full line has been fitted by least squares ($r = 0.97$) to inhibition $= -(0.05 \pm 0.1) + (0.94 \pm 0.14) \times$(binding), not significantly different from the dotted line of identity with a slope of 1.0. (B) Fractional inhibition of urea permeability as a function of the fractional binding of pCMBS to the urea inhibition site. The full line has been fitted by least squares ($r = 0.97$) to inhibition = $(0.02 \pm 0.1) + (1.1 \pm 0.2) \times$(binding), not significantly different from the dotted line of identity with a slope of 1.0.

membrane disruption induced by the formation of white ghosts is sufficient to suppress not only normal urea permeability but also the ability of pCMBS to inhibit water permeability. These results in white ghosts suggest that the quaternary structure of band 3 is requisite for pCMBS inhibition and that associations with the cytoskeleton may play an important role. Furthermore, an intact cytoskeleton may be necessary for the preservation of the tetrameric structure. It is interesting that evidence from the radiation inactivation studies of Dix et al. [28] indicates that the basic unit required for urea transport in the red cell has a molecular mass of 496 ± 36 kDa, which is consistent with the approx. 400 kDa molecular mass of a band 3 tetramer.

Since there are three water transport inhibition sites per urea transport inhibition site, pCMBS binding to the water sites could be cooperative. This seems unlikely because Toon and Solomon [7] showed that pCMBS inhibition of water transport fitted a single site binding curve very well, which means either that all the sites are identical or that the differences are so small that they can be treated operationally as though they were identical. This observation is consistent with the time course of pCMBS binding in Fig. 3 which fits a single-exponential time course. Since the kinetics of both processes have been determined, it is possible to plot fractional inhibition of water and urea transport as a function of fractional pCMBS binding to yield the linear plots shown in Fig. 5. Fractional inhibition of the fluxes of both water and urea is linearly dependent upon the fraction of binding sites filled, with a slope of unity, as given in the caption. This figure indicates that each pCMBS molecule bound to its site has the same fractional effect on water (or urea) transport inhibition so that the sites are entirely independent.

Are the pCMBS binding sites sulfhydryl groups?

Under our conditions in NEM-treated cells, only one of the six SH groups in band 3, the sixth SH group in the 17 kDa membrane fragment [5], has not reacted with NEM and may be available for reaction with pCMBS. Toon and Solomon [7] showed that the water transport inhibition site is slowly reversible by cysteine, which is consistent with binding to an SH group, while the urea transport inhibition site is not reversible by cysteine. It was suggested that binding of pCMBS to the urea transport inhibition site was followed by a conformational change which moved the binding site into a lipid or other environment where cysteine could not reach it. Before the stoichiometry had been determined, we thought [7] that two separate binding sites were required, one for each inhibition process. Now, a straightforward interpretation would assign the sixth SH group to the

water and urea transport inhibition sites *. The sixth SH is cysteine 498 in the notation of Kopito and Lodish [8], which is just at the right place, either at, or just inside, the junction of helix No. 3 with the extracellular face of the cell (see also Jay and Cantley [29]).

Oligomeric model for transport system

Even though the anion exchange process has been studied in great detail, there is still no agreement as to whether the anion exchange channel goes through a monomer, as Falke and Chan [30] suggest, or whether the anion traverses a route between the two monomers of a non-covalent dimer, for which Jay and Cantley [29] summarize the arguments. It is clear that interactions of a stilbene inhibitor with one monomer can affect the conformation of the second monomer in the dimer [29]. There is also increasing evidence for the existence of band 3 tetramers in the membrane, as has been discussed.

In the case of the aqueous channel there is controversy as to whether water and urea share the same channel and there is no consensus that the aqueous channel lies between two monomers in band 3 as Solomon et al. [5] have proposed. This paper does not address the locus or characteristics of the channel. It shows that the sites for pCMBS inhibition of water and urea transport are located on band 3 and that the stoichiometry is one urea plus three water transport inhibition sites for every four band 3 molecules.

We have chosen to discuss the properties of the system in terms of a tetramer, in which the channel consists of four amphipathic α-helices, one from each monomer, as components of a pore which comprises at least six α-helical crossings (see Ref. 5). Other models, particularly dimers and monomers, could also be formulated, but the tetramer has the virtue of simplicity. In the initial step, when the first pCMBS molecule approaches the channel, it will bind to the site with the highest affinity and the fastest kinetics, that is, to the urea transport inhibition site. Toon and Solomon [7] have already surmised that this binding step causes a conformational change that isolates the bound pCMBS from further reaction with a cysteine. The present results appear to require a further conformational change in each of the three partners in the tetramer such that the urea binding site changes its characteristics and is transformed into a water transport inhibition site. Since there appears to be no cooperativity in these three sites, they must have equivalent binding characteristics. The very slow k_{on} which characterizes this water transport inhibition step may be a consequence of some concerted action that might be necessary to accomplish the change in all three monomers.

Since inhibition of the urea flux does not affect water flux, the site of inhibition could be presumed to be at the interface where urea has to exchange its water of hydration for hydrogen bonds with the channel wall. Since occupation of each pCMBS site, as shown in Fig. 5, causes an equivalent decrease in water flux, these water inhibition sites could function by steric hindrance, with each site blocking an equal fraction of the channel. These presumed characteristics of the tetrameric model may provide a plausible construct to view an inhibition process which depends upon four band 3 molecules acting in concert.

* A possible problem with this interpretation comes from studies with another SH reagent, DTNB, whose labelling profile (Brown et al. [4]) shows a primary peak at band 3 in NEM-treated red cells and virtually no binding to any other membrane protein. Since DTNB does not effect pCMBS inhibition of water and urea transport [7], DTNB binding to NEM-treated band 3 would pre-empt the pCMBS site. However, the gel presented by Brown et al. [4] was published before the determination of the conditions required to saturate all the NEM sites on the red cell by Rao [13]. Toon and Solomon (private communication) have measured DTNB binding under the conditions of Brown et al. and find that there are a significant number of unreacted SH sites per mg ghost protein, so that there could have been vacant NEM sites on band 3, under the conditions used in the gel from Brown et al. and the peak that Brown et al. identified need not have been the 6th SH group.

Acknowledgment

This study was supported in part by National Institutes of Health Grant GM 34099.

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