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Accessibility of the *N*-ethylmaleimide-unreactive sulfhydryl of human erythrocyte Band 3

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The human erythrocyte anion exchange protein, Band 3, was reacted with *N*-ethylmaleimide (NEM) in cells to a stoichiometry of 5.3 mol NEM per mol Band 3, indicating that all NEM-reactive cysteines in Band 3 were labeled. Quantitatively NEM-blocked Band 3 was still able to bind to and be eluted by reducing agents from a mercurial affinity resin, [*p*-(chloromercuribenzamido)ethylene]amino-Sepharose. Reaction of NEM-blocked Band 3 with *p*-chloromercuribenzoate (pCMB) did not prevent binding to the resin due to exchange of pCMB for the immobilized mercurial. pCMB has been reported to inhibit water and urea permeation across the red cell membrane, and this has been attributed to reaction with a NEM-reactive sulfhydryl in Band 3. The interaction of Band 3 with the immobilized ligand directly demonstrates the reaction of NEM-blocked Band 3 with a mercurial and indicates that the NEM-unreactive, pCMB-reactive sulfhydryl residue is accessible to within ≈ 12 Å (the distance from the solid support to the Hg) of the surface of the solubilized Band 3 protein.

The amino acid sequence of human Band 3 as deduced from the sequence of cDNA contains five cysteine residues [1]. Mouse Band 3 contains six cysteine residues [2], the first cysteine residue in the mouse sequence at position 152 is not found in the corresponding position in the human protein sequence [1–3]. Amino acid analyses of human Band 3 indicate a content of 5–6 cysteine residues [4–6] per molecule, although original estimates were higher [7,8]. All but one of the cysteine residues in human Band 3 can be modified in cells with either NEM or pCMBS [9]. All these NEM-reactive cysteine residues are located on the cytoplasmic side of the membrane [9,10], two being located in the 20 kDa tryptic fragment of the N-terminal cytoplasmic domain [11] and two within a 9 kDa section in the C-terminal 35 kDa fragment [9,12]. The two sulfhydryls in the C-terminal fragment do not react with NEM in ghosts unless the ghosts are first reduced with high concentrations of 2-mercaptoethanol [9], but can be labeled with NEM in inside-out vesicles [12].

An additional cysteine, which is unreactive to NEM both in cells and in ghosts [11,12], and is thus denoted the 'cryptic' sulfhydryl by Solomon [13], has been located in the 17 kDa membrane-spanning fragment produced by chymotrypsin treatment of ghosts, as indicated by both the amino acid compositions of the fragments [4,6,11], and the cleavage with NTCB [4,10,14]. The deduced human sequence [1] contains a cysteine residue located in the 17 kDa fragment (Cys-479). Although unreactive with NEM, this residue appears to react with organic mercurials since, after quantitative reaction of erythrocytes with NEM, treatment with either [^{14}C]pCMB or [^{203}Hg]pCMBS [13,15–18] resulted in association of radiolabel with Band 3 in SDS-polyacrylamide gels. However, the site of attachment of these mercurials within Band 3 has not been determined.

While pCMBS treatment of erythrocytes does not alter the rate of sulfate efflux [19], there is a substantial volume of work suggesting that the binding of pCMBS and, in some cases, other mercurials or DTNB, to the NEM-unreactive sulfhydryl group in Band 3 inhibits the water permeability of the erythrocyte membrane (for example, Refs. 13, 15, 18, 19–23). Furthermore,

Abbreviations: NEM, *N*-ethylmaleimide, pCMB, *p*-chloromercuribenzoic acid; pCMBS, *p*-chloromercuribenzenesulfonate; pCMB-Sepharose, [*p*-(chloromercuribenzamido)ethylene]amino-Sepharose 4B; $C_{12}E_8$, *n*-dodecyl octaethyleneglycol monoether; SDS, sodium dodecyl sulfate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTCB, 2-nitro-5-thiocyanobenzoic acid.

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some of this evidence has been used to develop a model [13] in which Band 3 provides an aqueous channel in the red cell membrane that allows the permeation of water and urea, and controls the passage of cations and anions. The model also proposes that the NEM-unreactive sulfhydryl is located within ≅ 10–15 Å of the external face of the membrane, to the external side of the DIDS site. This aspect of the model is based on evidence that pCMBS is transported by Band 3 and that this transport is 98% inhibited by DIDS [24], yet DIDS does not prevent pCMBS inhibition of urea and water transport [13].

Despite the bulk of evidence appearing to relate inhibition of water permeability to a residue on Band 3 protein, there is still considerable uncertainty about whether the pCMBS interaction site that is involved in water transport inhibition is equivalent to the pCMBS site on Band 3 protein, and as to what residue on Band 3 provides the pCMBS site [16,17,25–30]. pCMBS labeling of Band 3 in SDS gels was correlated with the inhibitory effects in some of the studies [16–18], and these indicated labeling of Band 3, but also of Band 4.5 proteins. The time course of the labeling of Band 4.5 was, however, much slower than that for the inhibition of urea and water transport [18]. Other studies, which employ the pCMBS-induced quenching of membrane protein tryptophan fluorescence [13,30], or use altered kinetics of 4,4′-dibenzamidostilbene-2,2′-disulfonate [22] as a measure of pCMBS binding, provide only an indirect measure of pCMBS binding. The pCMBS labeling site of Band 3 and the water inhibition site share the qualities of being unreactive to NEM and reactive to pCMBS, but the existing pharmacologic, kinetic and radiation inactivation evidence indicate that the water permeation pathway is distinct from the anion exchange pathway [28–31]. This, however, does not exclude the possibility that they are mediated by separate parts of the same protein, or perhaps by different oligomeric states of the protein.

In this paper we describe an investigation of the reactivity of the NEM-unreactive cysteine residue in Band 3. The reaction with free sulfhydryl reagents and the interaction with a sulfhydryl affinity resin having pCMB as a ligand with detergent-solubilized, NEM-blocked Band 3 were studied.

Methods. Production of NEM-blocked Band 3 *. Treatment of intact erythrocytes with NEM was performed as described by Rao [9], exposing washed erythrocytes at 25% suspension in 0.15 M NaCl, 10 mM Tris (pH 7.4) to 12 mM NEM for 1 h at 37° C. In some preparations cells were treated twice with NEM to ensure blocking of all NEM-reactive groups. Treatment of erythrocytes with [^{3}H]NEM was performed under the same conditions as for unlabeled NEM. [^{3}H]NEM at a specific radioactivity of 120 mCi/mmol in pentane (New England Nuclear) was evaporated in the bottom of a plastic tube, then dissolved in a solution of known NEM concentration, resulting in a specific radioactivity of from 4 to 8 mCi/mmol. Calculations of the stoichiometry of labeling were based on the protein concentration in ghost suspensions, or of purified Band 3, as determined by the method of Lowry et al. [32]. The Band 3 protein determination by the Lowry method agrees quite well with protein concentrations determined by amino acid analyses [33].

Reaction of purified Band 3 with sulfhydryl reagents. Purification of NEM-blocked Band 3 was performed by aminoethyl-Sepharose chromatography [34]. Reaction of purified Band 3 with [^{3}H]NEM in solution was carried out by diluting a freshly prepared stock of 100 mM [^{3}H]NEM (prepared as described above, with a specific radioactivity of 8.2 mCi/mmol) to final concentrations of 0.05 to 2 mM into a solution of 1 mg/ml Band 3 in 50 mM sodium phosphate (pH 7.0), 0.1% $C_{12}E_8$. Reaction was allowed to proceed for 1 h at 37° C, or for 2 h at room temperature. The unreacted NEM was removed by gel filtration on a Sephacryl S-200 or S-300 column in 50 mM sodium phosphate (pH 7.0), 0.1% $C_{12}E_8$. Separation of the protein-bound and unreacted radioactivity was achieved, and any contribution of free radioactivity was subtracted from that in the protein peak before calculation of the stoichiometry of labeling. Measurement of incorporation into proteolytic fragments of Band 3 was made in some cases, isolating the major proteolytic fragments NCh-60 and TrC-55 as described previously [33]. The estimate for TrCH-17 was made from the radioactivity associated with the fragment after SDS gel electrophoresis [35].

A stock of about 5 mM [^{14}C]pCMB was prepared by dissolving 50 μCi [^{14}C]pCMB (Research Products International) in 0.65 ml 50 mM NaCl, alkalinizing the solution with 30 μl 1 M NaOH, and neutralizing with 10 μl 1 M HCl to a pH of about 8. The specific radioactivity of 36300 cpm/nmol was determined from dilutions of this stock into 50 mM sodium phosphate (pH 7.0) or into 0.5 M sodium acetate (pH 4.0), using the known absorption coefficients of pCMB [36]. Band 3 in solution at 1 mg/ml in 50 mM sodium phosphate (pH 7.0), 0.1% $C_{12}E_8$ was treated with 175 μM [^{14}C]pCMB for 2 h at room temperature. Ojcius and Solomon [18] used a similar concentration of pCMBS which resulted in filling of both the water and urea transport inhibition sites, and in labeling of Band 3 to about 1.3 mol/mol, during treatment of erythrocytes. Separation of unreacted pCMB from labeled Band 3 was achieved in three ways: by dialysis, ultrafiltration, or gel filtration.

* Throughout this paper the phrase NEM-blocked Band 3 is used to refer to preparations of Band 3 originating from erythrocytes that have been treated with NEM.

Measurement of DTNB reaction with ghost proteins was performed as described by Toon et al. [26], using a freshly dissolved stock of DTNB and a concentration (0.5 mM) they found produced maximal reaction. The reaction was measured by determining the concentration of 3-carboxylato-4-nitrothiophenolate produced, using the absorption coefficient at 412 nm of 13600 $M^{-1} \cdot cm^{-1}$ [36]. Reaction of DTNB with purified Band 3 in detergent solution was performed similarly, following the protocol in Glazer et al. [36], using a 20-fold molar excess of DTNB over protein, and correcting for absorbance of the protein solution.

Sulfhydryl affinity chromatography. The interaction of NEM-blocked erythrocyte ghost proteins, or purified Band 3, with pCMB-Sepharose was performed both in columns and in batch. The pCMB-Sepharose had been prepared by carbodiimide activated coupling of pCMB onto aminoethyl-Sepharose, as described in Lukacovic et al. [37]. It should be noted that both sets of conditions are designed to apply a sub-saturating level of protein to the resin, which has a binding capacity of 7 mg of purified, unmodified Band 3 per ml of resin [38]. For initial studies of interaction, detergent extracts of ghost proteins (rather than purified Band 3) were used. To prepare detergent extracts, 1 volume of ghosts was added to 5 volumes of 1% $C_{12}E_8$ in 5 mM sodium phosphate (pH 8), vortexed, and incubated at 4°C for 30 min. The supernatant resulting after centrifugation at 100000 × *g* for 30 min was used as the '$C_{12}E_8$ extract'. Interaction with the pCMB-Sepharose in columns was performed at 0 to 4°C as follows: 2 to 2.5 ml of the $C_{12}E_8$ extract of ghosts, containing approximately 1.5 mg total membrane protein, was applied to 1 ml of pCMB-Sepharose at a flow rate of 6 ml/h. After application of the $C_{12}E_8$ extract, the resin was washed with 0.1% $C_{12}E_8$ in 50 mM sodium phosphate (pH 8), until the effluent had a negligible absorbance at 280 nm, and was then eluted with the rinse buffer containing either 0.1% 2-mercaptoethanol or 1 mM pCMBS. The protein contents of aliquots from each bulk fraction (the $C_{12}E_8$ extract, the unbound column flow through, and the 2-mercaptoethanol eluate) were analyzed by SDS-polyacrylamide gel electrophoresis [35].

The interaction of purified Band 3 with pCMB-Sepharose in-batch was performed as follows: 200 µl of a solution of aminoethyl-purified, NEM-blocked Band 3 at a concentration of approximately 1 mg/ml in 50 mM sodium phosphate (pH 7.0), 0.1% $C_{12}E_8$ was added to 200 µl of washed, packed pCMB-Sepharose in a 1.5 ml microfuge tube. The mixture was mixed end-over-end for 5 min at room temperature, then centrifuged 30 s in a microfuge. The supernatant was removed and the resin washed three times with 1.2 ml 50 mM sodium phosphate (pH 7.0), 0.1% $C_{12}E_8$. Next, 200 µl of 0.1% 2-mercaptoethanol in 50 mM sodium phosphate (pH 7.0), 0.1% $C_{12}E_8$ was added, mixed for one minute, centrifuged to remove the resin, and the supernatant taken as eluate. Aliquots of the samples before addition to the resin, supernatant, and the eluate were taken for protein determination and gel electrophoresis. For calculations of protein recovery in the supernatant and eluate, correction for the two-fold dilution into the resin's included volume was made. Control experiments with Sepharose 4B were also performed, and resulted in no binding of Band 3.

Results. Quantitation of NEM labeling after treatment of cells. We used Band 3 from cells that had been treated with NEM such that the sulfhydryls on Band 3 that are known to react with NEM under these conditions would be blocked. We first confirmed the quantitation of [^{3}H]NEM labeling of Band 3. Treatment of erythrocytes with [^{3}H]NEM as described in Methods resulted in incorporation of 98 nmol NEM per mg total membrane protein, comparable to (1.17 ×), the labeling obtained by Rao [9]. The fraction of the total radioactivity that was associated with Band 3 after SDS-polyacrylamide gel electrophoresis was 0.13, as determined from the reactivity in solubilized gel slices. That is, 12.9 nmol NEM per mg membrane protein was in Band 3. Using 2.4 nmol Band 3/mg ghost protein [33], the calculated stoichiometry is 5.37 mol NEM per mol Band 3, confirming quantitative modification of all four NEM-reactive sulfhydryls in Band 3. The additional NEM incorporated into Band 3 must be in residues other than cysteine. Cysteine-479 has been shown [11,12] not to be modified under these conditions. Purification of Band 3 from [^{3}H]NEM-labeled cells, using an affinity resin composed of a Band 3 inhibitor, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS), attached to an Affi gel 102 matrix [39], provided Band 3 labeled with 53 nmol NEM/mg protein. Using M_r = 100000, this gives 5.3 mol NEM/mol Band 3. These data confirm that our treatment of Band 3 with NEM results in quantitative labeling of all the NEM-reactive cysteine residues in Band 3 described by Rao [9]. The confirmation of the stoichiometry of NEM labeling is clearly important, since the NEM-reactive residues also react with pCMBS [9]. Since the NEM-reactive sulfhydryls are blocked, then our assumption is that the only sulfhydryl in Band 3 left available to react with sulfhydryl reagents free, or on a matrix, is the NEM-unreactive sulfhydryl (Cys-479).

Treatment of NEM-blocked ghosts with 0.5 mM [^{3}H]NEM (for 1 h at 37°C, in 5 mM sodium phosphate (pH 7.0)) resulted in association of 3.2 nmol [^{3}H]NEM/mg membrane protein. Less than 0.1 mol/mol was, however, associated with Band 3 resolved by SDS polyacrylamide gel electrophoresis. Thus, no NEM-reactive groups are available in Band 3 in ghosts prepared from NEM-treated cells.

Reaction of purified NEM-blocked Band 3 in deter-

gent solution with [^{3}H]NEM resulted in incorporation of [^{3}H]NEM into Band 3 reaching a maximum of 2.5–3 moles NEM per mole Band 3. Hydrolysis of the NEM-labelled proteins and amino acid analysis indicated no increase in the level of succinyl-cysteine (the hydrolysis product of NEM-modified cysteines), showing that NEM treatment of solubilized NEM-blocked Band 3 did not result in further modification of cysteine residues. The NEM incorporated into Band 3 in solution is due to modification of other residues, most likely lysine.

[^{14}C]pCMB treatment of purified, NEM-blocked Band 3, with 175 μM pCMB for 2 h at room temperature, resulted in incorporation of 2.2 mol pCMB/mol Band 3. The pCMB stoichiometry is higher than expected and may represent labeling of the cryptic sulfhydryl plus non-specific labeling or absorption of the organic mercurial by the detergent-protein complex. The presence of a second NEM-unreactive sulfhydryl in Band 3 cannot be excluded but is unlikely given the high stoichiometry of NEM labeling of Band 3 in cells (greater than 5 mol NEM per mol Band 3).

We found 3.5 nmol DTNB-reactive sites per mg ghost protein prepared from NEM-treated cells. This would correspond to about 1 mol/mol Band 3, assuming that all the reactive sites are in Band 3. Reaction of purified NEM-blocked Band 3 with DTNB in solution indicated 1.9 mol of DTNB-reactive residues per mol Band 3. The reaction of NEM-blocked Band 3 with sulfhydryl reagents reveals that NEM treatment does not modify the cryptic sulfhydryl, but that this cysteine residue may be able to react with PCMB and DTNB.

Interaction of NEM-blocked Band 3 with a mercurial affinity resin. The Band 3 used to study the interaction of purified Band 3 in $C_{12}E_8$ with pCMB-Sepharose was about 90% pure, as can be seen in the gel lanes before application to the resin. The gel staining patterns of the fractions resulting from the interaction with pCMB-Sepharose are shown in Fig. 1. The amount of protein that did not bind to the resin and remained in the supernatant was very low for both untreated (Fig. 1, lane 1b) and NEM-blocked Band 3 (Fig. 1, Lane 2b): from 8 to 24 μg protein was measured in the supernatant for 200 to 224 μg protein applied to 200 μl resin. The amount contained in the eluate with 0.1% 2-mercaptoethanol (Fig. 1, lanes 1c,2c) was 144 to 152 μg, indicating a recovery of 67–75%. These data show that Band 3 isolated from cells that have been treated with NEM can still bind to, and be eluted from, pCMB-Sepharose.

That the binding of Band 3 to the resin is mediated by covalent attachment of the protein to the immobilized pCMB is shown by the ability of the protein to be eluted from the extensively *washed* resin by the simple addition of sulfhydryl reducing agents to the binding buffer. Several sulfhydryl reducing agents in addition to 2-mercaptoethanol were effective in eluting the Band 3, including 1 mM dithiothreitol, 10 mM cysteine, and 0.1% thioglycolic acid. The possibility that Band 3 is bound noncovalently to the pCMB is unlikely, since the structurally dissimilar 2-mercaptoethanol would not be expected to displace Band 3 by binding to a pCMB site in the protein. In addition, a non-sulfhydryl analog of 2-mercaptoethanol, ethyleneglycol, at a concentration of

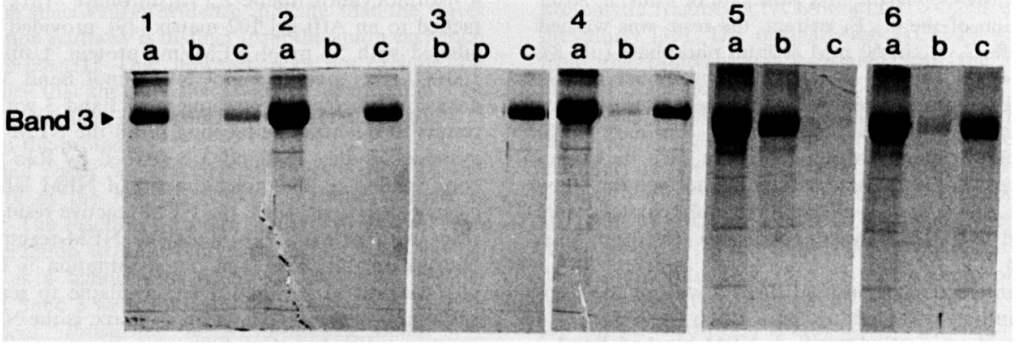

Fig. 1. Interaction of purified Band 3 with pCMB-Sepharose after treatments with sulfhydryl reagents. Aliquots of equal volumes of the fractions resulting from interaction in-batch with pCMB-Sepharose were run on SDS-polyacrylamide gels (10% acrylamide), and stained with Coomassie brilliant blue. The three fractions in all except group 3 are: (a) Band 3 in 0.1% $C_{12}E_8$, 50 mM sodium phosphate (pH 7.0) before application to the resin; (b) supernatant after incubation with the resin (the unbound fraction); (c) eluate with 0.1% 2-mercaptoethanol added to the rinse buffer of 50 mM sodium phosphate (pH 7.0) and 0.1% $C_{12}E_8$. Six different samples were tested for their interaction with the resin, each having been treated with a different combination of reagents, as such: (1) untreated Band 3; (2) NEM-blocked Band 3 (erythrocytes were twice treated with 12 mM NEM at pH 7.4, for 1 h at 37°C, prior to purification of Band 3); (3) NEM-blocked Band 3 (as in set 2), for which the first fraction (a) is not shown, and where (p) is the eluate produced by 1 mM pCMB added to the rinse buffer, and (c) is 2-mercaptoethanol elution after the pCMB elution; (4) [^{14}C]pCMB-treated Band 3 (NEM-blocked Band 3, in $C_{12}E_8$ solution, treated with 175 μM [^{14}C]pCMB for 2 h at 22°C, and dialyzed before application to the resin); (5) NEM-blocked Band 3 with 0.1% 2-mercaptoethanol present during the interaction with the resin; (6) NEM-blocked Band 3, with 12 mM pCMB present during interaction with the resin.

1%, produced no elution of Band 3. Displacement of Band 3 is likely due to reaction of the free sulfhydryls with the immobilized PCMB.

After application of NEM-blocked Band 3 to the resin, washing with 1 mM pCMBS did not release any protein (Fig. 1, lane 3p). Subsequent treatment with 2-mercaptoethanol-containing buffer eluted Band 3, as before. Free pCMBS up to a concentration of 10 mM was not able to elute the bound Band 3 from the pCMB-Sepharose. 50 mM pCMBS, however, eluted about 40% of the amount of protein relative to the amount eluted by 2-mercaptoethanol (data not shown). The ineffectiveness of pCMBS in eluting Band 3 could represent an enhanced stability of interaction with the immobilized ligand compared to the free ligand. Alternatively, perhaps once the NEM-unreactive residue has reacted with the *immobilized* pCMB its accessibility is limited, such that the larger organic mercurial pCMBS has limited access to the pCMB attachment site on Band 3. A similar phenomenon has been described, in which a putative conformational change renders the pCMBS site less accessible to glutathione than to cysteine [27].

To further characterize the interaction between Band 3 and the mercurial resin, the effect of treatment of Band 3 with free pCMBS prior to interaction with the resin, on the attachment of Band 3, was investigated. Ghost membranes containing NEM-blocked Band 3 were treated with pCMBS (1 mM, for 90 min, at 22° C) and the ghosts were washed before detergent extraction. When this detergent extract was applied to pCMB-Sepharose, Band 3 appears to bind and be eluted as in untreated ghosts (data not shown). Similarly, purified Band 3 that had been treated with 175 μM [^{14}C]pCMB to a stoichiometry of labeling of 2.2 mol/mol Band 3 was bound to and eluted from pCMB-Sepharose (Fig. 1 lanes 4,a–c). In other words, attachment to the resin was not blocked by pre-treatment with pCMB or pCMBS. This observation is not fully unexpected, since the covalent mercaptide bond that a sulfhydryl forms with pCMBS is subject to competition by sulfhydryl reducing reagents and to exchange with other mercurials [40].

Direct evidence for exchange was obtained by applying [^{14}C]pCMB-modified Band 3 from NEM-treated cells (in which free [^{14}C]pCMB had been removed by dialysis) to 1 ml of pCMB-Sepharose in a column (Fig. 2). The Band 3 protein bound to the column, with no protein detectable in the flow-through, while over half of the radioactivity eluted with the flow-through. The evidence that the radiolabel found in the column flow-through was not simply noncovalently associated comes with the fact that when the same Band 3 preparation was bound to SITS-AffiGel [39], greater than 80% of the radioactivity also bound. Addition of buffer containing 0.1% 2-mercaptoethanol to the pCMB column eluted Band 3 protein, as well as most of the remaining radioactivity, from the resin. The relative amounts of radioactivity and protein eluted corresponded to a stoichiometry of 0.96 mol [^{14}C]pCMB/mol Band 3. Our interpretation is that the pCMB on the resin would provide a ligand to compete with that attached to the residue on Band 3, and would displace a certain fraction of the pCMB (or pCMBS) on Band 3, thus binding the Band 3. Considering that the stoichiometry of [^{14}C]pCMB labeling of solubilized Band 3 was 2.2 mol/mol Band 3, the eluted Band 3 would be expected to have label still associated if each Band 3 molecule interacted with the resin by a single reactive group. The pCMB remaining associated with the eluted Band 3 may represent non-specifically absorbed reagent to the Band 3-detergent complex.

Demonstration of competition for Band 3 binding to pCMB-Sepharose, by inclusion of competing sulfhydryl reducing agents, would further support that the interaction is due to mercaptide bond formation. When the Band 3 solution applied to the resin contained 0.1% 2-mercaptoethanol the interaction of Band 3 with the resin was fully prevented (Fig. 1, lanes 5 a–c). 10 mM cysteine and 1 mM dithiothreitol were as effective as 0.1% 2-mercaptoethanol in blocking binding. These reagents are effective blockers of Band 3 binding, since they can react covalently with the immobilized mercurial.

NEM treatment of solubilized NEM-blocked Band 3 did not prevent Band 3 binding to the resin. This confirms that NEM treatment of solubilized Band 3 does not modify the cryptic sulfhydryl. When 1 mM pCMBS or 12 mM pCMB were present in the Band 3 solution during its application to the pCMB-Sepharose the amount of Band 3 not bound to the resin, and appearing in the supernatant, was slightly increased, and elution from the resin was correspondingly somewhat diminished, relative to a sample without pCMB or pCMBS present (Fig. 1, lanes 6a–c). The amount of protein not binding to the resin represented 17% of that recovered (Fig. 1, lanes 6a–c), compared to 8% unbound in only NEM-blocked (Fig. 1, lanes 2a–c). A partial interference with binding, but never a complete prevention of binding, was observed in four such interactions. A concentration of pCMBS as high was 50 mM could only partially block Band 3 binding to the resin. A more complete prevention of binding was anticipated, but it is possible that the immobilized ligand is at high enough concentration that substantial concentrations (up to 50 mM) of free ligand do not compete effectively to completely prevent binding. It is also important to consider that the expected affinity of the ligand for Band 3 may be altered by immobilization.

Band 3 from cells that had been treated with 100 μM DIDS (at 37° C, for 30 min, at pH 7.4, producing complete reaction) in addition to the NEM treatment of

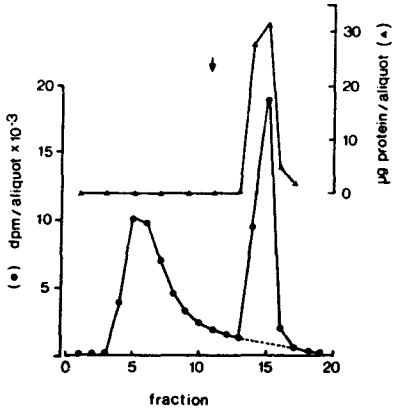

Fig. 2. Displacement of [^{14}C]pCMB from Band 3 during chromatography on pCMB-Sepharose. Purified, NEM-blocked Band 3 was labeled with [^{14}C]pCMB to a stoichiometry of 2.2 mol/mol Band 3, and the free [^{14}C]pCMB removed by dialysis (as described in Methods). A portion of the [^{14}C]pCMB-modified Band 3 (0.4 ml at 1 mg protein/ml) was applied to a column containing 1 ml of pCMB-Sepharose, which had been washed at 4°C with 50 mM sodium phosphate (pH 7.0), containing 0.1% $C_{12}E_8$. Application of the sample was followed by elution with 50 mM sodium phosphate (pH 7.0) containing 0.1% $C_{12}E_8$, then (as indicated by the arrow) with the same buffer containing 0.1% 2-mercaptoethanol. Aliquots of 50 μl of the fractions were taken for scintillation counting (●) and protein determination (▲). The extrapolated baseline (------) from the first peak was subtracted from the second before calculation of the stoichiometry.

the cells, also bound to and was eluted from the resin (data not shown). Labeling Band 3 with both NEM and DIDS therefore does not prevent the attachment of Band 3 to immobilized pCMB. This is consistent with the findings by Solomon et al. [13] of lack of effect of DIDS on pCMBS inhibition of water transport, and with a location of the pCMBS-reactive residue outside the DIDS binding region.

Discussion. This study was designed to address the reactivity and accessibility of the NEM-unreactive cysteine residue in Band 3. The interactions with immobilized pCMB that we have shown are direct measurements of covalent attachment of sulfhydryl reagents to Band 3, as distinct from noncovalent binding, or binding measured indirectly. The first important point is that this confirms that there is a residue in NEM-blocked Band 3 that reacts covalently with pCMB, and which is likely a sulfhydryl. Our finding of reaction between Band 3 and immobilized pCMB at 4°C, as well as at room temperature, agrees with the finding by Solomon et al. of [^{203}Hg]pCMBS labeling by treatment at 4°C [13]. Benga and co-workers [17], however, reported an absence of any labeling when reaction was at 0°C; some of their data actually show about 2 nmol pCMBS per mg ghost protein for reaction at 0° for 15 min. It is possible that the low level of labeling that occurs at 4°C may be significant in terms of the reactivity of a residue in Band 3, even though it is not correlated with the inhibition of water transport [17,18].

The quantitative interaction of Band 3 with matrix-bound pCMB also indicated that essentially all the Band 3 molecules contained a pCMB-reactive residue. However, the number of reactive residues per Band 3 molecule could not be determined from the studies of interaction with matrix-bound reagent. The measurement of reaction of free [^{14}C]pCMB with Band 3 indicated reaction of 2 mole pCMB per mole Band 3. We suggest that the simplest interpretation of these results is that the cysteine-479 can mediate the interaction with pCMB-Sepharose, and that it is not reactive with NEM in cells, ghosts or after solubilization of Band 3. Sha'afi and Feinstein [15] observing the inability of even hydrophobic maleimides to cause the inhibition of water transport that the mercurials produce, suggested that the sulfhydryl groups involved are accessible, but not reactive. Our data demonstrate this prediction of the accessibility of the NEM-unreactive residue in Band 3. We suggest that there may be a microenvironment within the protein that, although relatively accessible, makes this residue unreactive to NEM, but quickly reactive to even matrix-bound pCMB.

The initial reaction of pCMB with Band 3 may not be the step that results in inhibition of water permeability. The possibility has been suggested that the slow development of inhibition could be related to subunit rearrangement of Band 3 (i.e., dissociation of tetramers), based on evidence from: the sublimation of water from beneath intramembrane particles (IMPs) in freeze-fractured membranes [41]; the changes in both IMP distribution and water permeability noted in pathophysiological conditions (summarized by Benga [17]); and the single-channel conductances measured after incorporation of Band 3 into planar bilayers, which were attributed to Band 3 tetramers [42]. In the same vein, it has been suggested [43] that the NEM-unreactive cysteines might be located in the contact regions between oligomers. Until a complete understanding of the nature and reactivity of the NEM-unreactive cysteine in Band 3 is known, the interpretation of investigations addressing any functional roles of the cysteine residue will be vague.

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References

1 Tanner, M.J.A., Martin, P.G. and High, S. (1988) Biochem. J. 256, 703–712.
2 Kopito, R.R. and Lodish, H.F. (1985) Nature (London) 316, 234–238.
3 Kaul, R.K., Murthy, S.N.P., Reddy, A.G., Steck, T.L. and Kohler, H. (1983) J. Biol. Chem. 258, 7981–7990.

4 Drickamer, K.L. (1977) J. Biol. Chem. 252, 6909–6917.
5 Fukada, M., Eshdat, Y., Tarone, G. and Marchesi, V.T. (1978) J. Biol. Chem. 253, 2419–2428.
6 Steck, T.L., Koziarz, J.J., Singh, M.K., Reddy, G. and Kohler, H. (1978) Biochemistry 17, 1216–1222.
7 Ho, M.K. and Guidotti, G. (1975) J. Biol. Chem. 250, 675–683.
8 Jenkins, R.E. and Tanner, M.J.A. (1977) Biochem. J. 161, 139–147.
9 Rao, A. (1979) J. Biol. Chem. 254, 3503–3511.
10 Ramjeesingh, M., Gaarn, A. and Rothstein, A. (1983) Biochim. Biophys. Acta 729, 150–160.
11 Rao, A. and Reithmeier, R.A.F. (1979) J. Biol. Chem. 254, 6144–6150.
12 Ramjeesingh, M., Gaarn, A. and Rothstein, A. (1981). J. Bioenerg. Biomembr. 13, 411–423.
13 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.
14 Ramjeesingh, M., Gaarn, A. and Rothstein, A. (1980) Biochim. Biophys. Acta 599, 127–139.
15 Sha'afi, R.I. and Feinstein, M.B. (1977) Adv. Exp. Med. Biol. 84, 67–80.
16 Benga, G., Popescu, O., Pop, V.I. and Holmes, R.P. (1986) Biochemistry 25, 1535–1538.
17 Benga, G., Popescu, O., Borza, V., Pop, V.I., Muresan, A., Mocsy, I. Brain, A. and Wrigglesworth, J.M. (1986) Eur. J. Cell Biol. 41, 252–262.
18 Ojcius, D. and Solomon, A.K. (1988) Biochim. Biophys. Acta 942, 73–82.
19 Knauf, P.A. and Rothstein, A. (1971) J. Gen. Physiol. 58, 190–210.
20 Macey, R.I. and Farmer, R.E.L. (1970) Biochim. Biophys. Acta 211, 104–106.
21 Brown, P.A., Feinstein, M.B. and Sha'afi, R.I. (1975) Nature (London) 254, 523–525.
22 Lukacovic, M.F., Verkman, A.S., Dix, J.A. and Solomon, A.K. (1984) Biochim. Biophys. Acta 778, 253–259.
23 Yoon, S.C., Toon, M.R. and Solomon, A.K. (1984) Biochim. Biophys. Acta 778, 385–389.
24 Rothstein, A. (1981) In The Function of Red Blood Cells: Erythrocyte Pathobiology (Wallach, D.F.H., ed.), pp. 105–131, A.F. Liss, New York.
25 Verkman, A.S., Lukacovic, M.F., Tinklepaugh, M.S. and Dix, J.A. (1986) Membr. Biochem. 6, 269–289.
26 Toon, M.R., Dorogi, P.L., Lukacovic, M.F. and Solomon, A.K. (1985) Biochim. Biophys. Acta 818, 158–170.
27 Toon, M.R. and Solomon, A.K. (1986) Biochim. Biophys. Acta 860, 361–375.
28 Macey, R.I. (1984) Am. J. Physiol. 246, C195–C203.
29 Pitterich, H. and Lawaczeck, R. (1985) Biochim. Biophys. Acta 821, 233–242.
30 Verkman, A.S., Skoreck, K.L., Jung, C.Y. and Ausiello, D.A. (1986) Am. J. Physiol. 251, C541–C548.
31 Jones, S.C. and Frohlich, O. (1984) Biophys. J. 45, 199a.
32 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
33 Oikawa, K., Lieberman, D.M. and Reithmeier, R.A.F. (1985) Biochemistry 24, 2843–2848.
34 Lieberman, D.M. and Reithmeier, R.A.F. (1983) Biochemistry 22, 4028–4033.
35 Laemmli, U.K. (1970) Nature (London) 227, 680–685.
36 Glazer, A.N., Delange, R.J. and Sigman, D.S. (1975) Chemical Modification of Proteins: Selected Methods and Analytical Procedures, North Holland/Elsevier, Amsterdam.
37 Lukacovic, M.F., Feinstein, M.B., Sha'afi, R.I. and Perrie, S. (1981) Biochemistry 20, 3145–3151.
38 Boodhoo, A. and Reithmeier, R.A.F. (1984) J. Biol. Chem. 259, 785–790.
39 Pimplikar, S.W. and Reithmeier, R.A.F. (1986) J. Biol. Chem. 261, 9770–9778.
40 Means, G.E. and Feeney, R.E. (1971) Chemical Modification of Proteins, Holden-Day, San Francisco.
41 Pinto de Silva, P. and Branton, D. (1973) Proc. Natl. Acad. Sci. USA 70, 1339–1343.
42 Benz, R., Tosteson, M.T. and Schubert, D. (1984) Biochim. Biophys. Acta 775, 347–355.
43 Haest, C.M.W., Kamp, D. and Deuticke, B. (1981) Biochim. Biophys. Acta 643, 319–326.