p-(Chloromercuri)benzenesulfonate Binding by Membrane Proteins and the Inhibition of Water Transport in Human Erythrocytes†

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ABSTRACT: The binding of [^{203}Hg]-*p*-(chloromercuri)benzenesulfonate to the membrane proteins of human erythrocytes and erythrocyte ghosts was examined under conditions where binding to the bulk of membrane sulfhydryl groups was blocked by *N*-ethylmaleimide. Binding was essentially complete within 90 min when approximately 40 nmol was bound per milligram of membrane protein. This binding was correlated with the inhibition of water transport measured by an NMR technique. Maximal inhibition was observed with the binding of approximately 10 nmol of *p*-(chloromercuri)benzenesulfonate/mg of membrane protein. Under these conditions, both band 3 and band 4.5 bound 1 mol of inhibitor/mol of protein. In contrast to previous experiments, these results indicate that band 4.5 proteins as well as band 3 have to be considered as playing a role in water transport.

Despite the transport of water across biological membranes being essential for cell function, very little is known about the process. The evidence indicating that water transport occurs through a channel in the erythrocyte membrane was recently reviewed by Macey (1984). One of its main characteristics is an inhibition by mercurial reagents that bind to sulfhydryl (SH) groups. The reagents that are inhibitory are partly hydrophobic and take 10–20 min to produce maximal inhibition (Macey & Farmer, 1970; Naccache & Sha'afi, 1974; Benga et al., 1982). In contrast, NEM,[1] which binds to SH groups in an aqueous environment, is not inhibitory (Brahm, 1982; Benga et al., 1983). These properties are consistent with their interaction with a sulfhydryl group of a protein and one that is not in an aqueous environment but buried within the lipid bilayer or a hydrophobic domain of a protein. The protein or proteins involved have not been conclusively identified to date. Some studies have suggested that the major erythrocyte protein band 3 is involved (Brown et al., 1975; Sha'afi & Feinstein, 1977; Solomon et al., 1983). This protein is known to function in anion transport (Knauf, 1979). In these studies, the binding of radioactive SH-binding reagents to membrane proteins was examined, but the binding was not correlated with the inhibition of water transport. For instance, Brown et al. (1975) observed almost exclusive binding of DTNB to band 3 and suggested on this basis that band 3 was involved in water transport. However, subsequent studies have revealed that DTNB does not inhibit diffusional water permeability (Brahm, 1982; Benga et al., 1983). Binding of *p*-(chloromercuri)-benzoate to erythrocyte proteins was also examined in the presence of NEM, iodoacetamide, and mersalyl (Sha'afi & Feinstein, 1977). Mersalyl inhibits diffusional water permeability, clouding the interpretation of this experiment (Benga et al., 1983).

NMR offers an accurate method for estimating the rapid water exchange process in erythrocytes (Conlon & Outhred, 1972; Morariu & Benga, 1977). Using this technique, we have correlated the inhibition of water transport with the binding of PCMBS by erythrocyte membrane proteins. We have found that binding to band 3 and the polypeptides in band 4.5 occurs under these conditions, suggesting that proteins in either of these bands could be associated with water channels in erythrocytes.

Materials and Methods

Chemicals. *N*-Ethylmaleimide, PCMBS, bovine serum albumin, β-mercaptoethanol, and dithiothreitol were purchased from Sigma, acrylamide, bis(acrylamide), and sodium dodecyl sulfate were from Bio-Rad, and Hepes was from Research Organics. [^{203}Hg]PCMBS was obtained from Amersham. All other chemicals used were of reagent grade.

Erythrocytes and Resealed Ghosts. Erythrocytes were isolated from fresh, human blood by centrifugation at $2000g_{max}$ for 10 min at 4 °C and washed twice with buffer containing 150 mM NaCl–5 mM sodium phosphate, pH 7.5. Erythrocyte ghosts were prepared and resealed according to the procedure of Schwoch and Passow (1973).

Water Diffusion. Cells or resealed ghosts were resuspended as a 50% suspension in 150 mM NaCl, 5 mM Hepes, 5.5 mM glucose, and 0.5% bovine serum albumin, pH 7.4. An aliquot of 0.2 mL was mixed with 0.1 mL of doping solution (40 mM $MnCl_2$–100 mM NaCl). The water proton relaxation time (T'_{2a}) was measured by the spin-echo method previously described (Conlon & Outhred, 1972; Morariu & Benga, 1977). T'_{2a} is dominated by the exchange process through the membrane, and the water exchange time is inversely related to the water permeability of erythrocytes. Ashley and Goldstein (1981) determined that expressing results in exchange times when water permeability is inhibited by PCMBS in erythro-

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NEM, *N*-ethylmaleimide; NMR, nuclear magnetic resonance; PCMBS, *p*-(chloromercuri)benzenesulfonic acid.

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Table I: Correlation of Inhibition of Water Diffusion in Human Erythrocytes and Resealed Ghosts by PCMBS with Binding of [^{203}Hg]PCMBS by Erythrocyte Membrane Proteins

	conditions of incubation			inhibition of water diffusion (%)	mol of PCMBS bound	
	PCMBS (mM)	temp (°C)	time (min)		per mol of band 3	per mol of band 4.5
erythrocytes	0.2	37	30	43.2	1.04	0.93
	0.5	27	30	43.3	1.10	1.01
	0.5	37	15	44.0	1.55	1.50
	1.0	27	30	45.1	1.65	1.50
resealed ghosts	0.1	0	0	0	0.08	0.12
	0.1	0	15	4.8		
	0.1	37	5	32.2	1.35	0.86
	0.1	37	15	36.2		

cytes introduces an error of only 6.6% due to the change in cell volume.

The percent inhibition was calculated from

$$\% \text{ inhibition} = \frac{\frac{1}{T'_{2a}(\text{control})} - \frac{1}{T'_{2a}(\text{sample})}}{\frac{1}{T'_{2a}(\text{control})}} \times 100$$

Measurements were performed on an AREMI-78 spectrometer (Institute of Physics and Nuclear Engineering, Bucharest-Magurele, Roumania), at 37 ± 0.2 °C, with a computer coupled on line with the NMR spectrometer.

PCMBS Binding. Washed erythrocytes or resealed ghosts were resuspended in wash medium containing 2 mM NEM at a cytocrit of 25% and incubated for 60 min at 25 °C. They were then diluted to a 10% suspension with medium containing 2 mM NEM and [^{203}Hg]PCMBS. After completion of the required incubation, resealed ghosts and erythrocytes were washed 3 times in 20 volumes of wash medium containing 2 mM NEM by centrifugation for 10 min at 4 °C at $8000g_{max}$ and $2000g_{max}$, respectively. Purified membranes were prepared from the intact erythrocytes (Fairbanks et al., 1977) to remove [^{203}Hg]PCMBS that may have bound to hemoglobin and other cytoplasmic components. Protein was measured by the method of Lowry et al. (1951). The radioactivity was counted with a Packard multichannel analyzer Model A 9012. Membrane polypeptides were separated by electrophoresis on 7.5% polyacrylamide gels in the discontinuous buffer system described by Laemmli (1970). β-Mercaptoethanol was omitted, and 20 mM NEM was added to prevent the release of PCMBS and its subsequent binding to NEM binding sites. Gels were cut into 2-mm slices, and the radioactivity was measured.

Results

The [^{203}Hg]PCMBS binding to membranes was studied in both erythrocytes and resealed ghosts as the latter potentially offer a more defined system for studying membrane processes and have been used in recent investigations (Schwoch & Passow, 1973; Solomon et al., 1983). As NEM binds to SH groups but does not inhibit water transport (Brahm, 1982; Benga et al., 1983), membranes were pretreated with this reagent to block SH groups not involved in water permeability. The amount of [^{203}Hg]PCMBS bound was dependent upon temperature and the duration of incubation (Figure 1). Although the maximal binding of PCMBS, ~40 nmol/mg of membrane protein, was detected after 90-min incubation at 37 °C in both erythrocytes and resealed ghosts, we found significant inhibition of water diffusion to occur with much lower binding. In varying PCMBS concentration, temperature (27 or 37 °C), and the time of incubation, the inhibition of water diffusion was maximal with a binding of ~10 nmol/mg of protein (Table I and Figure 1). At 0 °C, no inhibition of water transport occurred, and no binding of PCMBS was detected in 15 min (Figure 1), in contrast to the specific binding of PCMBS to band 3 that has been previously reported (Solomon et al., 1983).

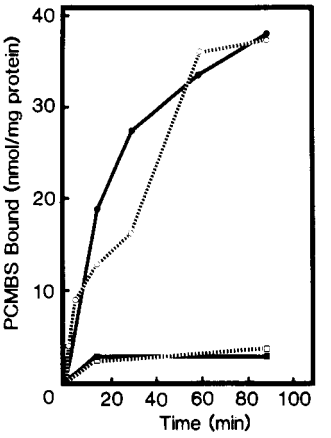

FIGURE 1: Effect of time and temperature on the binding of PCMBS by erythrocytes and resealed ghosts. Erythrocytes were incubated with 0.5 mM [^{203}Hg]PCMBS at 0 (■) or 37 °C (●) and resealed ghosts with 0.1 mM [^{203}Hg]PCMBS at 0 (□) or 37 °C (○).

The proteins binding PCMBS under conditions where maximal inhibition of water diffusion was first obtained were identified by polyacrylamide gel electrophoresis (Figure 2). The bulk of PCMBS was bound to the major membrane protein band 3. However, the proteins migrating as band 4.5 also bound significant amounts of the water diffusion inhibitor (20% of radioactivity with resealed ghosts). The clearest result was obtained with resealed ghosts that were depleted of some of the peripheral proteins bound to the erythrocyte membrane, notably band 6 (glyceraldehyde-3-phosphate dehydrogenase) and a protein migrating near actin (band 5). This latter protein, band 4.9, recently identified as a trimeric actin bundling protein (Branton et al., 1984), did bind significant amounts of PCMBS in intact erythrocytes (Figure 2b), but it was not present in resealed ghosts (Figure 2a). Assuming a molecular weight of 95 000 for band 3 and an average molecular weight of 55 000 for band 4.5, we found approximately 1 mol of PCMBS bound per mole of each of these proteins under these incubation conditions.

Discussion

Current knowledge of water transport in erythrocytes is consistent with it occurring through a channel created by membrane-spanning proteins. Although it has been suggested that water permeability may be entirely accounted for by flow through kinks or defects in the lipid bilayer (Trauble, 1972), current assessments are that it accounts for no more than 10% of the water flow (Sha'afi, 1981; Macey, 1984). This is based on differences in water flow across erythrocyte membranes and artifical lipid bilayers. Under osmotic pressure, water flow

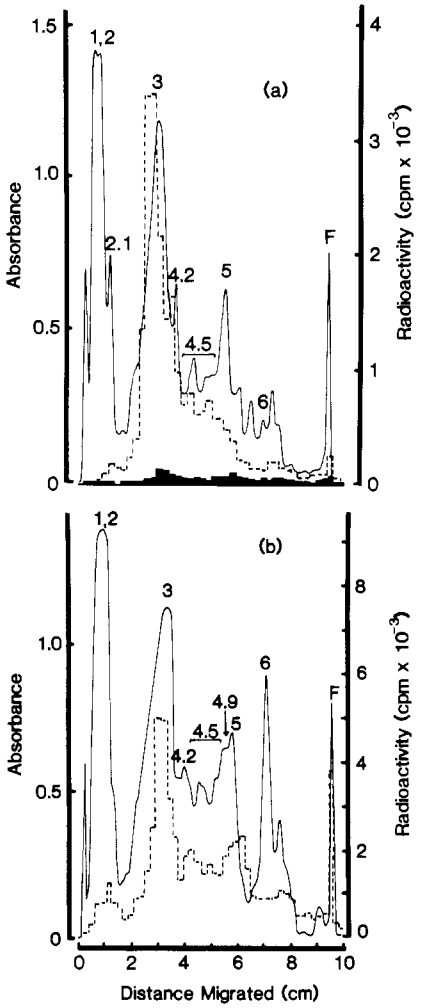

FIGURE 2: Binding of PCMBS to membrane proteins of resealed ghosts and erythrocytes resolved by SDS–polyacrylamide gel electrophoresis. (a) Resealed ghosts were incubated with 0.1 mM [^{203}Hg]PCMBS for 5 min at 37 °C (broken bar graph) or for 15 min at 0 °C (solid bar graph). (b) Erythrocytes were incubated with 1 mM [^{203}Hg]-PCMBS for 30 min at 37 °C and purified membranes prepared before electrophoresis. The bar graphs represent radioactivity estimated in gel slices, and the continuous line represents the densitometric tracing obtained by scanning gels stained with Coomassie Blue as described by Fairbanks et al. (1971). The nomenclature devised by Fairbanks et al. (1971) is used to identify membrane proteins, and F represents the migration of the tracking dye.

across erythrocyte membranes increases whereas the rates of osmotic and diffusional flow are similar in artifical membranes. Also, the activation energy is much higher for flow across artifical lipid bilayers than for flow across erythrocyte membranes.

A number of sulfhydryl-reactive reagents inhibit the transport of water in certain mammalian erythrocytes and other cell types (Macey, 1979; Sha'afi, 1981). In the presence of many of these inhibitors the osmotic flow equals the diffusional flow, suggesting that membrane pores or channels are blocked by these agents and that the residual flow observed in the presence of these inhibitors is due to flow through the lipid bilayer. However, these reagents only inhibit up to 60% of diffusional flow, indicating that other factors have to be considered. Mercurial reagents are stronger inhibitors than disulfide reagents (Sha'afi, 1981), and their potency depends on their having some hydrophobicity. We have observed the dimercurial reagent fluoresceinmercuric acetate to be 5 times more potent than PCMBS (Benga et al., 1982). Furthermore, its inhibition was not reversed by cysteine. We have suggested that this irreversibility of fluoresceinmercuric acetate inhibition may result from its tight association with two sulfhydryl groups, possibly those in a dimeric complex of two membrane-spanning monomeric proteins.

The binding of PCMBS by bands 3 and 4.5 after incubation with NEM suggests that cryptic SH groups in these proteins not accessible to NEM bind the mercurial reagent. Consistent with this, Haest et al. (1981) reported that 1.2 SH groups of band 3 and 3.6 SH groups of band 4.5 do not bind NEM. The cryptic SH group in band 3 is most probably located on the 17 000-dalton, hydrophobic proteolytic fragment of band 3 that spans the membrane (Rao & Reithmeier, 1979; Ramjeesingh et al., 1983). The nature of the SH groups in band 4.5 polypeptides is not as yet well defined.

As resealed ghosts are fully active in water transport (Brahm, 1982), this indicates that peripheral proteins are not involved in the process as they are removed in the procedure used for the preparation of resealed ghosts (Darmon et al., 1983) but not in the preparation of purified erythrocyte membranes (Fairbanks et al., 1971). In contrast to the report by Macey (1984), we have not observed in this or previous studies that PCMBS inhibition of water diffusional permeability in erythrocytes can be reversed by saline washes.

Previous labeling experiments with sulfhydryl-reactive reagents did not correlate binding with an inhibition of water transport. The binding pattern of PCMBS we have observed in correlation with the inhibition of water diffusion suggests that either or both band 3 and 4.5 proteins could be associated with water channels. Polypeptides migrating in these regions have already been identified in other transport functions, notably anion exchange and the transport of glucose and nucleosides (Darmon et al., 1983; Wheeler & Hinkle, 1981; Young et al., 1983). To date, however, there is no evidence that a specific inhibitor of one of these processes will inhibit water transport. It remains possible that a minor membrane protein that binds PCMBS is involved in water transport. Estimates of the number of channels are consistent with one of the major membrane proteins being involved (Solomon et al., 1983), although these estimates are not direct and involve assumptions of unknown validity. We believe the best way to clarify the role of bands 3 and 4.5 in water transport will ultimately be through studies on the reconstitution of purified proteins in liposomes. A better understanding of the transport process in human erythrocytes is clearly warranted by reports that water permeability is decreased in erythrocytes from individuals with epilepsy (Benga & Morariu, 1977) and Duchenne muscular dystrophy (Ashley & Goldstein, 1983; Serbu et al., 1985).

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N-Hydroxysulfosuccinimido Active Esters and the L-(+)-Lactate Transport Protein in Rabbit Erythrocytes†

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ABSTRACT: Esters of *N*-hydroxysulfosuccinimide strongly inhibit L-(+)-lactate transport in rabbit erythrocytes, probably by acylating amino groups on the transport protein. Lactate transport studies using bis(sulfosuccinimido) suberate (BS^3), bis(sulfosuccinimido) adipate (BS^2A), bis(sulfosuccinimido) dithiobis(propionate), and a variety of monocarboxylate esters suggest that an exofacial amino group of the lactate transport protein is essential for lactate transport. Also, reductive methylation studies show that even when positive charge is preserved in modified amino groups, the transport is strongly inhibited. At pH <6, band 3 mediated inorganic anion transport is enhanced in BS^3-treated cells, while at pH >6, it is inhibited. BS^3-induced inhibition of L-(+)-lactate transport does not have this pH dependence. BS^3 reduces the labeling of a 40–50-kDa membrane polypeptide (band R) by tritiated 4,4′-diisothiocyanato-2,2-dihydrostilbenedisulfonate ($[^3H]H_2DIDS$) and by tritiated bis(sulfosuccinimido) adipate ($[^3H]BS^2A$). Tritiated sulfosuccinimido acetate ($S^2[^3H]$acetate) also labels band R, over a range of concentrations where lactate transport is inhibited in a dose-dependent manner by S^2acetate. BS^3 is a known impermeant protein cross-linker. S^2acetate permeates rabbit red cell membranes by an H_2DIDS-inhibitable mechanism. BS^3 cross-links the proteolytic fragments of rabbit band 3 produced by extracellular chymotrypsin. These labeling experiments support an association between band R and specific monocarboxylate transport.

Evidence of protein-mediated monocarboxylate transport exists for a wide variety of biological systems. Specific monocarboxylate transporters are present in bacteria (Harold & Levin, 1974), mitochondria (Thomas & Halestrap, 1981) and the plasma membranes from a spectrum of mammalian tissues, including erythrocytes (Halestrap, 1976; Dubinsky & Racker, 1978; Deuticke et al., 1978; Deuticke, 1982), Ehrlich ascites tumor cells (Spencer & Lehninger, 1976; Johnson et al., 1980), thymocytes (Anderson et al., 1978; Regen & Tarpley, 1978), renal brush border (Barac-Nieto et al., 1980), small intestine (Lamers & Hülsmann, 1975), liver (Schwab et al., 1979; Fafournoux et al., 1985), brain capillary epithelium (Oldendorf, 1973), and smooth (Kutchai et al., 1978), skeletal

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