ENHANCEMENT OF ANION PERMEABILITY IN LECITHIN VESICLES BY HYDROPHOBIC PROTEINS EXTRACTED FROM RED BLOOD CELL MEMBRANES*

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SUMMARY: Triton X-100 extracts of membrane proteins from ghosts of normal and pronase treated cells enhance the anion permeability of lecithin vesicles. With proteins from cells pretreated with DIDS(4,4'-diisothiocyano-2,2'-stilbene disulfonate), a specific inhibitor of anion transport, the anion permeability is not enhanced. On the basis that the Triton X-100 extracts are considerably enriched in a protein component of 95,000 molecular weight (or a 65,000 molecular weight segment in the case of pronase treated cells), and that DIDS is bound almost exclusively to the same proteins, it is suggested that the pronase resistant, 65,000 molecular weight segment of the 95,000 molecular weight protein is directly involved in anion transport.

It has been proposed that a component of the human red blood cell membrane, a protein of apparent molecular weight of 95,000 (usually called band 3 from its position on SDS acrylamide gels (3)), plays a role in anion permeation (1,2). The evidence is derived primarily from studies with disulfonic stilbenes, potent and specific inhibitors of anion transport(2,4). One compound, 4,4'-diisothiocyano 2,2'-stilbene disulfonate (DIDS), binds covalently to the membrane (1), in a linear relationship to the degree of inhibition (2). In the inhibitory range of concentrations, it binds almost exclusively (95%) to proteins in band 3 and it is largely localized in a pronase resistant 65,000 molecular weight segment of this protein. Because the pronase treatment used had no effect on anion permeation, it was suggested that the 65,000 molecular weight segment contains an anion transport site (5).

The band 3 protein is hydrophobically associated with membrane lipid and is extractable with detergents (6,7). In the case of the non-ionic detergents Triton X-100, the extraction is relatively specific(6). To further establish the role of band 3 proteins in anion transport, attempts were made to re-

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constitute Triton X-100 extracted material in lecithin vesicles and to determine the effects of such reconstitution on anion permeability. Parallel procedures were carried out with extracts from cells pretreated with DIDS at inhibitory concentrations and with extracts from cells pretreated with pronase.

MATERIALS AND METHODS. Red blood cells obtained from fresh or recently outdated blood were washed several times with Hepes buffer saline (Hepes 20mM, NaCl 140mM, pH 7.4) at 0°C. Aliquots of the suspension were pretreated for 10 minutes with 10 μM DIDS, 20 μC of {^{3}H}DIDS and/or for 10 minutes with 0.1 mg/ml of pronase at 37°C as previously described(2). The method of detergent extraction was generally similar to that of Yu et al(6). One volume of ghosts in dilute Hepes buffered saline (Hepes 10mM, NaCl 20mM) was treated with 3 volumes of Triton X-100 (Sigma, 1.0% w/v in dilute Hepes buffered saline, pH 7.4 at 0°C) for 10 to 20 minutes followed by centrifugation at 30,000 x g for 10 minutes. Aliquots from the supernatant and resuspended pellet were analyzed for {^{3}H}DIDS, protein content, and protein distribution by SDS acrylamide gel electrophoresis (3).

The Triton X-100 extracted material from normal, DIDS-treated, and pronase treated cells was placed in a buffered isotope solution (Na_2SO_4 5mM, $Na_2{}^{35}SO_4$ 100μCi, KCl 10mM, EDTA 0.01 mM, Hepes 5mM, pH 7.0). One volume was mixed with one volume of toluene containing 1mg/ml of egg lecithin (Applied Sciences) to form a suspension(8). After low speed centrifugation (1000 rpm for 5 minutes), the clear aqueous phase was removed, N_2 was bubbled through the {^{3}H}DIDS-labelled material to remove traces of toluene and the sample was concentrated three to five fold with a Diaflo UM 20 E filter to a final protein concentration of 1.5 to 2.0 mg/ml. One ml was added to 200 g of pure egg or bovine brain lecithin (Applied Sciences) previously dried under vacuum and N_2 and shaken vigorously under an N_2 atmosphere. The protein-phospholipid dispersion was sonicated for 20 to 30 minutes at room temperature under N_2 in a 100W bath sonicator (Heat Systems, Ultrasonics Inc.), to form vesicles. The fluxes of $^{35}SO_4^{--}$ were determined by a method reported previously (9). The external $Na_2{}^{35}SO_4$ was removed from the vesicles by gel filtration on a Sephadex G-50 coarse column eluted with isotope free buffer. The $^{35}SO_4^{--}$ containing vesicles were placed in dialysis bags (0.33 in. flat dry diameter). The flux of $^{35}SO_4^{--}$ into the bathing solution containing isotope free buffer was measured.

Acrylamide gel electrophoresis was performed in 1% SDS by the method of Fairbanks et al(3). The {^{3}H}DIDS distribution in gels was obtained by fractionation with a Savant gel extruder, solubilization overnight in a 10% protosol based solvent and counting in a Packard Liquid Scintillation Counter (2). Protein was estimated by a modified microbiuret method(1) that tolerates the presence of 1% Triton X-100. The detergent was measured chemically (10).

RESULTS AND DISCUSSION. As demonstrated previously (6,7), the extraction by Triton X-100 is somewhat specific for band 3(95,000 molecular weight protein) and for glycoproteins. Thus in figure 1, the extract was considerably enriched in band 3 (stained by Coomassie blue) and glycoproteins (stained by periodic acid-Schiff reagent). However, in the case of pronase treated cells, no periodic acid-Schiff stained bands were found and virtually the only band

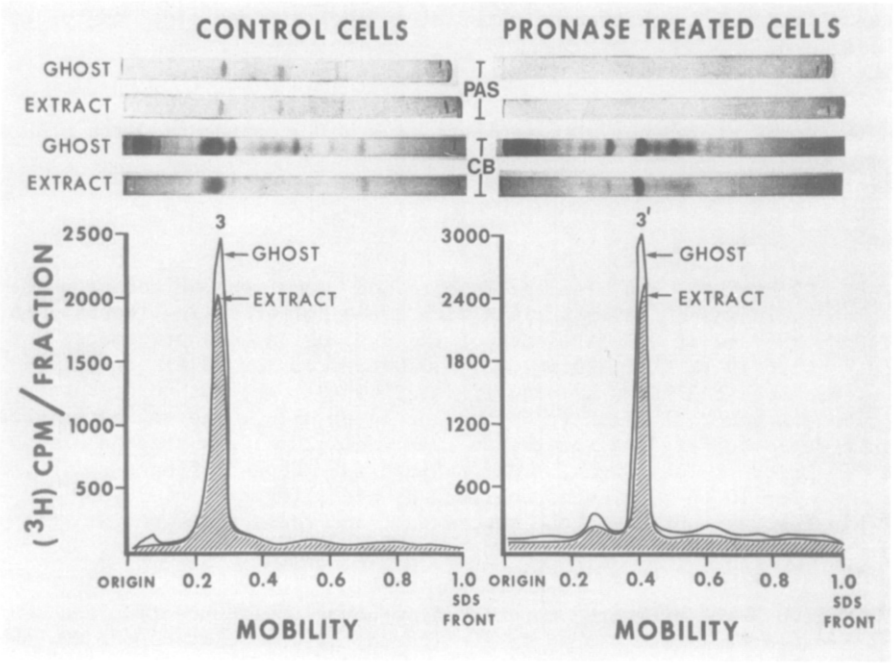

Figure 1. Extraction of {^{3}H}DIDS labelled proteins from normal and pronase treated cells by Triton X-100

The upper sections are photographs of sodium dodecyl sulfate acrylamide gels of proteins derived from ghosts, or extracted by Triton X-100, stained for protein with Coomassie blue (CB) or for carbohydrate by periodic acid Schiff reagent (PAS). The lower sections are counts of {^{3}H}DIDS in slices from gels prepared in parallel. In normal cells the counts are almost all at a position in the gel corresponding to band 3 (95,000 molecular weight) and in pronase treated cells, to band 3' (65,000 molecular weight).

stained by Coomassie blue in the Triton X-100 extract was that at 65,000 molecular weight (band 3').

Over 95% of {^{3}H}DIDS bound covalently to the cell surface is located in band 3, and 85% is recovered in the 65,000 molecular weight segment (band 3') after pronase treatment (2,5). The extraction of the {^{3}H}DIDS labelled material is also illustrated in figure 1. The label in the ghosts was found in a single peak corresponding to band 3, or in pronase treated cells to band 3', and most of it was extracted in Triton X-100.

Quantitative information on the extraction under the particular

TABLE 1 EXTRACTION OF {^{3}H}DIDS-LABELLED GHOST PROTEIN BY TRITON X-100

	CONTROL CELLS		PRONASE TREATED CELLS	
	Protein mg/ml	Radioactivity cpm/ml x 10^{-3}	Protein mg/ml	Radioactivity cpm/ml x 10^{-3}
Suspension	1.92(100)	1.020(100)	1.98(100)	1.299(100)
Extract	0.64(33)	867(85)	0.67(33)	977(77)
Pellet	1.30(68)	141(14)	1.28(65)	259(20)

Values in parentheses denote the % yield in the particular fraction.

conditions is given in Table 1. For either normal or pronase treated cells, 33% of the total ghost protein was solubilized, but the extraction of {^{3}H}DIDS labelled material was much higher, 85% and 77% respectively. Thus the labelled material (bands 3 and 3') was considerably enriched. Band 3 involves a large fraction of the total ghost protein, reported to be about 30%(3). It can therefore be calculated that band 3 must constitute about 85% of the total protein in the Triton X-100 extract. In the case of pronase treated cell, the total protein in the extract was more directly determined. Parallel determinations were carried out by biuret and by densitometry of Coomassie blue stained acrylamide gels using known amounts of albumin to standardize the two procedures against each other. Assuming that the 65,000 molecular weight protein and albumin behave the same with respect to staining, the former accounts for not less than 85% of the total extracted protein.

The Triton X-100 extracts of ghosts from normal, DIDS-treated and pronase treated cells were reconstituted in lecithin vesicles by the

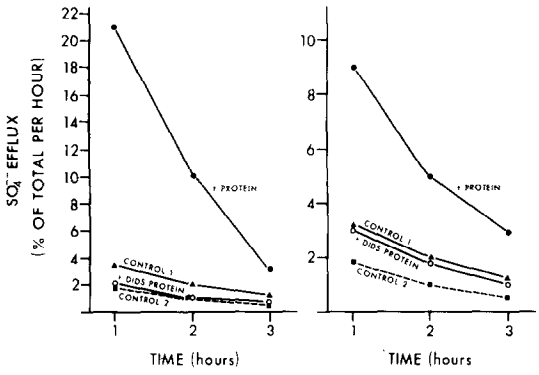

Figure 2. Anion permeability of lecithin vesicles constituted with Triton X-100 extracted fractions.

1.2 to 2.0 mg of total protein derived from normal and DIDS-treated cells were reconstituted in lecithin vesicles ($500_{\mu}g$) as described in Methods. Control 2 contains pure phospholipids whereas Control 1 contains in addition 0.03% Triton X-100, the same concentration as that found in the protein reconstituted systems.

procedure described in the Methods. Two extractions with toluene-lecithin mixtures reduced the Triton concentration from 0.8% to about 0.03%. Although the latter concentration has only a small effect on $^{35}SO_4^{--}$ fluxes from lecithin vesicles, the amount of detergent carried over by protein reconstitution was determined in each case and the same amount of detergent was added to control vesicles (no protein) at the time of sonication. In addition, in each experiment an aliquot of cells to be extracted with detergent was pretreated with DIDS to specifically and irreversibly block the anion permeation system (2). The proteins from normal and DIDS-treated cells were extracted, reconstituted in parallel, and the sulfate fluxes of the vesicles was measured. Because the DIDS treatment did not alter the amount of protein extracted, the acrylamide gel patterns, or the amount of detergent carried over, the DIDS-treated protein served as an internal control for non-specific effects of proteins and of detergent on anion fluxes. The 95K- and 65K-rich fractions increase the $^{35}SO_4^{--}$ fluxes substantially as illustrated by the representative experiment of figure 2.

The extent of enhancement was variable (from three to ten fold) in seven different experiments depending on the particular preparation and the amount of protein added. Fractions obtained from DIDS-treated cells caused no increase over controls containing the same concentration of Triton X-100 (control 1), but were somewhat higher than controls with no Triton (control 2). The inhibitory effect could also be demonstrated by adding the DIDS to vesicles reconstituted with proteins from normal and pronase treated cells immediately before or after the gel filtration step. In this case the inhibition was considerably less and somewhat variable but averaged about 50%. The partial inhibition results presumably from the inaccessibility of some DIDS susceptible anion transport sites. Some of the sites may be oriented inward in the vesicles or may be located in the interior of multilamellar vesicles. DIDS had no effect on the sulfate permeability of pure lecithin vesicles.

The results point strongly to the 95,000 molecular weight protein (band 3) as the membrane component responsible for the enhanced anion fluxes. Firstly, this protein (or the 65,000 segment resulting from pronase treatment) accounts for over 85% of the protein in the extracts. Secondly, the failure of protein from DIDS-treated cells to enhance the anion fluxes, limits the effect to those particular components that are labelled with DIDS. The specificity of DIDS for the 95,000 molecular weight protein is striking, as illustrated in figure 1. It has been reported that in the inhibitory range over 95% of the membrane bound DIDS is located in that protein (2). The only other component with identifiable amounts of DIDS (less than 4%) is the major sialoglycoprotein (5). This component of DIDS binding is, however, eliminated by pronase treatment without any effects on anion permeability or on DIDS inhibition (5). It could not, therefore, account for the reconstitution observed from proteins extracted from pronase treated cells. Some lipids are also extracted by the Triton X-100 (6), but these membrane components are not significantly

labelled by DIDS under the conditions of the present experiments (2), and cannot therefore be directly involved in the permeation function.

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