A New Variant of the Anion Transport Protein in Human Erythrocytes†

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ABSTRACT: The major plasma membrane protein of human erythrocytes is the anion transport protein, termed protein 3. We previously reported a variant form of protein 3 that is elongated on the amino-terminal end of the molecule, which is exposed on the cytoplasmic side of the membrane, but otherwise its features are identical with those of the normal molecule. We have termed this molecule protein 3 variant 1. We now report a new variant form, protein 3 variant 2. The erythrocyte donor was a double heterozygote whose red cells possess a normal protein 3 and a protein 3 variant which is elongated and possesses a second variation at the 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) reactive site. Variant 2 reacts with 4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid (H_2DIDS) more readily than does the normal molecule. At high pH values, H_2DIDS acts as a bifunctional cross-linking agent; it cross-links the proteolytic products generated by Pronase (or chymotrypsin) treatment of variant 2 less efficiently than noted for normal protein 3 or the first variant. Thus, the newly identified molecule has an alteration at the DIDS reactive site, which is near the outer surface of the membrane. The results can be interpreted as indicating that the DIDS binding site of variant 2 is more exposed than the normal molecule, but further removed from the site on the carboxyl-terminal fragment involved in cross-linking. Although there is a difference in the reactivity of the two protein 3 chains in variant 2, the reaction of variants 1 and 2 and normal cells with varying concentrations of [^{3}H]H_2DIDS results in the same amount of incorporation in all cells. Since protein 3 exists as a dimer or higher aggregate in the membrane, these results may indicate an interaction between monomers. The anion transport activities of both variants are within the experimental range for the normal protein 3. No hematologic abnormalities appear to be associated with the observed changes in protein 3.

Protein 3 is the major intrinsic glycoprotein of human erythrocytes and has a molecular mass of 95 kilodaltons (kDa)[1] (Fairbanks et al., 1971). It is a multifunctional protein that facilitates the exchange of anions (Cl^-, HCO_3^-, SO_4^{2-}, etc.) across the plasma membrane (Passow et al., 1975; Cabantchik et al., 1978; Knauf, 1979) and anchors the cytoskeleton to the membrane bilayer (Bennett & Stenbuck, 1980; Luna et al., 1979; Yu & Goodman, 1979; Hargreaves et al., 1980). It also binds cytoplasmic proteins, such as hemoglobin (Shaklai et al., 1977a,b), glyceraldehyde-3-phosphate dehydrogenase (Kant & Steck, 1973; Yu & Steck, 1975a,b; Kliman & Steck, 1980), aldolase (Strapazon & Steck, 1977; Murthy et al., 1981), and phosphofructokinase (Higashi et al., 1979). For recent reviews, see Jennings (1984) and Macara & Cantley (1983).

We previously detected a variant of protein 3 with a higher molecular mass than normal (Mueller & Morrison, 1977). This finding was attributed to a change in the amino-terminal portion of protein 3, which is located on the cytoplasmic domain of the protein. No clinical abnormality was found to be associated with this alteration in the protein. Here we report another variant of protein 3, which was detected by labeling intact human erythrocytes with [^{3}H]H_2DIDS, a specific inhibitor of anion transport.

Materials and Methods

Blood from volunteer donors was drawn into an acid–citrate–dextran solution and stored no longer than 1 week at 4 °C. The erythrocytes were isolated by centrifugation and washed 3 times in isotonic saline (155 mM NaCl) and twice in HEPES buffer (10 mM HEPES, 75 mM KCl, 50 mM Na_2SO_4, and 40 mM sucrose, pH 7.4). DIDS was purchased from Pierce Chemical Co. Its tritiated dihydro derivative, [^{3}H]H_2DIDS (from Amersham Corp)., had a specific activity of 1.9 Ci/mmol. Pronase was obtained from Calbiochem-Behring Corp. HEPES, BSA, and PMSF were all from Sigma Chemical Co., and $Na_2{}^{35}SO_4$ (10 mCi/mL) was from New England Nuclear. Other chemicals were reagent grade unless otherwise indicated.

[^{3}H]H_2DIDS Labeling of Intact Red Cells. Washed cells were labeled with [^{3}H]H_2DIDS by the method of Cabantchik & Rothstein (1972), suspended in HEPES buffer that contained concentrations of [^{3}H]H_2DIDS to 25% hematocrit, and then incubated for 30 min at 37 °C under dim white light. They were subsequently washed with cold HEPES buffer, 0.5% BSA in HEPES buffer, and HEPES buffer alone.

Pronase Digestion of Intact Red Cells. The cells were digested with Pronase in a manner similar to that reported by Mueller & Morrison (1977). They were digested with 0.1 mg/mL Pronase for 1 h at 37 °C and 10% hematocrit in HEPES buffer. The digestion was stopped by adding PMSF (final concentration 0.4 mM) to the cell suspension and washing the cells with cold 1% BSA in HEPES buffer, followed by HEPES buffer alone.

Membrane Preparation and $NaDodSO_4$–Polyacrylamide Gel Electrophoresis. The membranes of red cells were isolated by hemolyzing the cells and washing membranes repeatedly

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[1] Abbreviations: kDa, kilodalton(s); DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; H_2DIDS, 4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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with cold hypotonic 7 mM sodium phosphate buffer (pH 7.4) containing 0.4 mM PMSF. NaOH extraction of membranes was performed by adding 9 volumes of 0.1 N NaOH solution to 1 volume of membranes at 0 °C (Steck & Yu, 1973), centrifuging at 30000*g* for 30 min, and then washing the pellet twice with hypotonic sodium phosphate buffer. Membranes were then solubilized by heating at 100 °C for 5 min in 1 volume of 2% $NaDodSO_4$ and 15 mM dithiothreitol. Membrane proteins were separated on a gradient slab gel (5–15% acrylamide) with the discontinuous buffer system of Laemmli (1970); the sample buffer consisted of 2% $NaDodSO_4$, 10% glycerol, 80 mM dithiothreitol, 0.002% bromphenol blue, and 0.0625 M Tris-HCl (pH 6.8). The staining and destaining of gels and the preparation of fluorographs followed procedures described by Mueller & Morrison (1977). The amount of membrane proteins was estimated by intrinsic fluorescence (excitation at 285 nm, emission at 332 nm, and 8-nm bandwidths) with Monitrol used as the standard.

An Ortec densitometer interfaced with a Hewlett Packard Model 87 computer was used to scan the Coomassie blue stained gels or fluorographs. The radioactivity in the gels was then determined as follows: After the gels were destained, the areas that contained 60- or 63-kDa fragments were carefully excised, collected in scintillation vials, and dried at 45 °C. The dried gels were incubated in 0.1 mL of 30% H_2O_2 overnight at 45 °C and digested with 0.4 mL of protosol overnight in the dark at room temperature. Radioactivity was counted in the same vial to which 10 mL of scintillation fluid (5 g of PPO/0.1 g of POPOP/970 mL of toluene/30 mL of protosol) had been added.

$^{35}SO_4^{2-}$ Self-Exchange Efflux Rate Constant of Red Blood Cells. The method used to measure the $^{35}SO_4^{2-}$ efflux rate constant of red cells was based on that described by Cabantchik & Rothstein (1972). Prewashed cells were loaded $^{35}SO_4^{2-}$ by incubating cells in HEPES buffer containing a tracer amount of $Na_2{}^{35}SO_4$ for about 2 h at 37 °C and 25% hematocrit. The well-washed, $^{35}SO_4^{2-}$-loaded cells were then suspended in HEPES buffer at 5% hematocrit. P_t (the radioactivity at different times) was determined by removing 1 mL of cell suspension at 5, 10, 20, 30, and 45 min, centrifuging each sample at 4 °C, and counting 0.2 mL of supernatant in 6 mL of Bray's solution in a Packard liquid scintillation spectrometer. P_∞ (the radioactivity at infinite time) was determined by adding 0.2 mL of Cl_3CCOOH solution (30% w/v) to 1 mL of the cell suspension and counting the radioactivity of the supernatant after centrifugation. The rate coefficient of $^{35}SO_4^{2-}$ efflux from cells was determined by the linear least-squares method from the slope of the line obtained by plotting $[\ln (1 - P_t)]/P_\infty$ vs. time.

Results

Pronase treatment of erythrocytes cleaves protein 3 into two fragments with apparent molecular masses of 35 and 60 kDa. The former contains the C-terminus and the latter the N-terminus of protein 3, both of which remain firmly attached to the membrane (Bender et al., 1971; Bretscher, 1971; Phillips & Morrison, 1971; Hubbard & Cohn, 1972; Triplet & Caraway, 1972; Cabantchik & Rothstein, 1974; Whiteley & Berg, 1974; Mueller & Morrison, 1975; Reichstein & Blostein, 1975). Figure 1 summarizes the results of Pronase digestion of the normal and variant forms of protein 3 in the membranes of control or $[^3H]H_2DIDS$-labeled red cells. In the samples prepared from cells that contain the normal 95-kDa protein 3, Pronase digestion generated a single well-defined 60-kDa band. However, in the samples prepared from cells obtained from two heterozygotes, whose red cell membranes contain

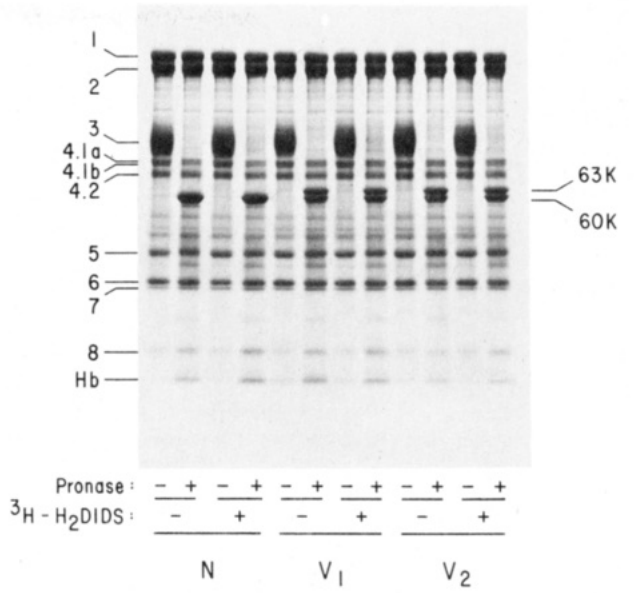

FIGURE 1: Coomassie blue staining profile of the membrane proteins isolated from the red cells of the normal donor (N) and those from donors whose cells contain a variant protein 3 (V_1 and V_2 heterozygotes). Cells were labeled with 2 μM $[^3H]H_2DIDS$ and then digested with 0.1 mg/mL Pronase. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed on a 5–15% acrylamide slab gel using the discontinuous buffer system of Laemmli (1970). The peptides were labeled according to the system of Fairbank et al. (1971). Hb = hemoglobin.

both the normal and the variant forms of protein 3, Pronase digestion yielded two distinct bands with molecular masses of 60 and 63 kDa. The 60-kDa band is generated from the normal protein 3, and the 63-kDa band is generated from the variant (Mueller & Morrison, 1977). The other proteolytic product, the 35-kDa fragment, is not clearly apparent in the Coomassie blue stained gel because of a high carbohydrate content. A scan of a Coomassie blue stained gel of the proteolytic products shows that the two amino-terminal fragments (60 and 63 kDa) in both of the heterozygote cells occur in equal quantities as is apparent in Figure 1.

H_2DIDS is a specific, irreversible anion transport inhibitor in human red blood cells, and the ^3H-labeled form has been used extensively to study the structure and function of protein 3 (Cabantchik et al., 1972, 1978; Cabantchik & Rothstein, 1974; Passow et al., 1975; Lepke et al., 1976; Ship et al., 1977; Jennings & Passow, 1979; Ramjeesingh et al., 1980; DuPre & Rothstein, 1981). When normal, variant 1, and variant 2 red cells were labeled with 2 μM $[^3H]H_2DIDS$ and then digested with Pronase, the membrane protein labeling profile (Figure 2A) indicated that protein 3 is equally well labeled in all of the cells. The amount of H_2DIDS incorporated was within experimental error the same in normal, variant 1, and variant 2 cells as indicated in Table II. This is also substantiated in Table IV which shows that anion transport is inhibited by DIDS to the same extent in all three types of cells. After Pronase digestion of the intact cell, $[^3H]H_2DIDS$ was found in the 60- and 63-kDa bands of the variant cells. However, the amount of $[^3H]H_2DIDS$ was not distributed in the same manner in the two variants. In variant 1, the 60- and 63-kDa fragments were labeled equally, while in variant 2 there was more of the label contained in the 63-kDa fragment as shown in Table I. Thus, the results suggest that the variant 2 form of protein 3 is different from variant 1, although both variants have similar amounts of normal and variant protein 3 as is evident from the Coomassie blue staining profiles in Figure 1.

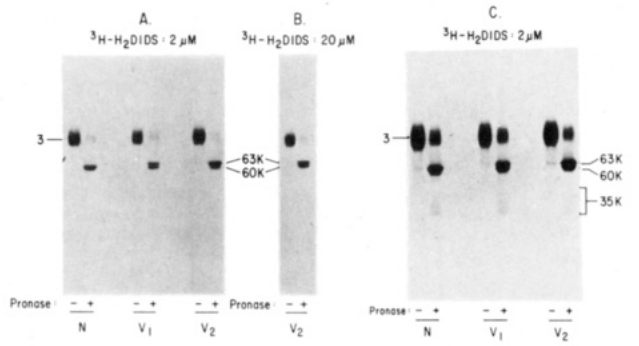

FIGURE 2: Fluorographs of NaDodSO$_4$–polyacrylamide gel electrophoresis of membrane proteins isolated from [^{3}H]H$_2$DIDS-labeled normal (N) and variant (V$_1$ and V$_2$, heterozygous) red blood cells. Cells were labeled with 2 (A) or 20 μM (B) [^{3}H]H$_2$DIDS and then digested with 0.1 mg/mL Pronase. (C) is the same as (A) except that the exposure time was longer. NaDodSO$_4$–polyacrylamide gel electrophoresis was performed on a 5–15% acrylamide slab gel using the discontinuous buffer system of Laemmli (1970).

Table I: Differential Labeling of Variants 1 and 2[a]

	ratio of [^{3}H]H$_2$DIDS labeling at [^{3}H]H$_2$DIDS concn (μM) of [b]			
cell type	2	5	10	20
variant 1	1.1 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
variant 2	2.4 ± 0.5	1.4 ± 0.3	1.3 ± 0.3	1.2 ± 0.1

[a] Membranes were isolated from cells that had been labeled with 2–20 μM [^{3}H]H$_2$DIDS and then digested with 0.1 mg/mL Pronase. The membrane proteins were separated by NaDodSO$_4$–polyacrylamide gel electrophoresis on a 5–15% polyacrylamide slab gel by using the discontinuous buffer system of Laemmli (1970). After gel staining, the areas that contained 63-kDa fragments and 60-kDa fragments were excised, and the radioactivity of the gel slices was measured. [b] Ratio of the radioactivity of the 63- to the 60-kDa fragment after being labeled with various concentrations of [^{3}H]H$_2$DIDS. All ratios are expressed as the mean ± SD for the cpm of the 63-kDa fragment/cpm of the 60-kDa fragment.

If cells were labeled with 20 μM [^{3}H]H$_2$DIDS to completely inhibit anion transport activity and to saturate H$_2$DIDS binding sites, a similar labeling pattern was obtained for both variant 1 and variant 2; that is, both the 63- and 63-kDa bands were labeled with [^{3}H]H$_2$DIDS to the same extent (Figure 2B).

Parts A and B of Figure 2 do not show the minor components that have been reported to be labeled with [^{3}H]H$_2$DIDS and assigned to glycophorin, an unidentified "satellite" band and 35-kDa fragment (Cabantchik & Rothstein, 1974; Lepke et al., 1976; Ship et al., 1977). However, these components can be seen when the exposure time of the fluorograph is increased. From the results shown in Figure 2C, it would appear that the small, unidentified (or satellite) band found by Lepke et al. (1976) is derived from protein 3. When these cells were labeled with 2 μM [^{3}H]H$_2$DIDS, one such minor band with a molecular mass of 60 kDa was found in normal cells, two minor bands with molecular masses of 60 and 63 kDa were found in variant 1 cells, and only one minor band with a molecular mass of 63 kDa was identified in variant 2 cells. Both 60- and 63-kDa minor bands were equally labeled in variant 1 and variant 2 when cells were treated with 10–20 μM [^{3}H]H$_2$DIDS (data not shown).

Table I shows the ratio of radioactivity of 63- vs. 60-kDa fragments in the membranes of variant 1 and variant 2 cells, after labeling with various concentrations of [^{3}H]H$_2$DIDS (2–20 μM) and subsequent digestion with Pronase. In variant 1, this ratio remained constant (0.9–1.1) when the concentration of [^{3}H]H$_2$DIDS was increased. However, in variant 2, it decreased from 2.4 to 1.2 if the [^{3}H]H$_2$DIDS concentration was increased from 2 to 20 μM. No significant differences in the total amount of membrane-bound [^{3}H]H$_2$DIDS were observed in these cells (Table II). The results suggest that all of the cells contain the same number of H$_2$DIDS binding sites in their membranes. Yet, in variant 2 cells, the H$_2$DIDS binding sites of the 63-kDa fragment and of the 60-kDa fragment appeared to react differently with the inhibitor.

Table II: [^{3}H]H$_2$DIDS Bound to Membranes of Normal and Variant Erythrocytes[a]

	sp act. (cpm/μg of protein)			
	2 μM [^{3}H]H$_2$DIDS		20 μM [^{3}H]H$_2$DIDS	
cell type	−Pronase	+Pronase	−Pronase	+Pronase
normal	899 ± 50[b]	840 ± 45	1932 ± 495	1939 ± 305
variant 1	937 ± 30	910 ± 51	2026 ± 216	1922 ± 402
variant 2	926 ± 53	948 ± 21	2089 ± 170	1845 ± 315

[a] Membranes were isolated from cells prelabeled with 2 or 20 μM [^{3}H]H$_2$DIDS and then digested with 0.1 mg/mL Pronase. [b] Radioactivity is expressed as the mean ± SD for cpm per microgram of membrane protein.

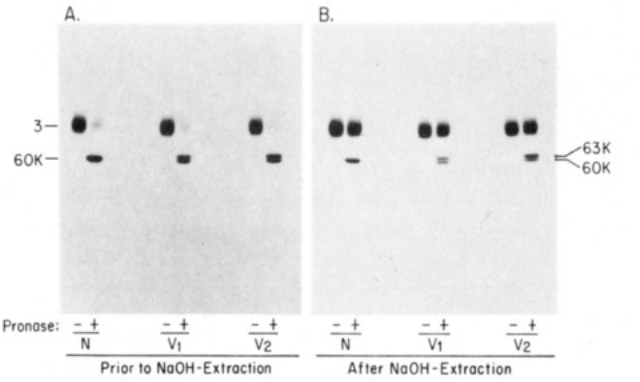

FIGURE 3: Fluorographs of NaDodSO$_4$–polyacrylamide gel electrophoresis of NaOH-extracted membranes prepared from normal and variant (V$_1$ and V$_2$, heterozygous) red blood cells. Cells were labeled with 20 μM [^{3}H]H$_2$DIDS and then digested with 0.1 mg/mL Pronase. After the membranes were isolated, they were extracted with 0.1 N NaOH solution. Membrane proteins were separated by NaDodSO$_4$–polyacrylamide gel electrophoresis on a 5–15% acrylamide slab gel using the discontinuous buffer system of Laemmli (1970).

The results obtained from 0.1 N NaOH extraction of membrane isolated from cells treated with H$_2$DIDS and then digested with Pronase also support the difference between variants 1 and 2. Jennings & Passow (1979) reported that H$_2$DIDS is a bifunctional cross-linking reagent. When red cells are treated with H$_2$DIDS and then digested with chymotrypsin, H$_2$DIDS is able to reconstitute protein 3 by cross-linking the 60-kDa fragment to the 35-kDa fragment at high pH. Figure 3, a fluorograph prepared from gel electrophoresis of NaOH-extracted membranes isolated from cells treated with 20 μM [^{3}H]H$_2$DIDS and then digested with Pronase, indicates that [^{3}H]H$_2$DIDS cross-linking of Pronase-digested protein 3 does occur when membranes are extracted with 0.1 N NaOH, although it is not complete (≈80%). Comparison of the amount of labeling present in the remaining 60- and 63-kDa fragments after NaOH extraction of membranes isolated from [^{3}H]H$_2$DIDS-labeled variant 1 cells suggests that H$_2$DIDS cross-links the normal and variant fragments of protein 3 at the same rate (Table III). Thus, in variant 1, the amount of [^{3}H]H$_2$DIDS in the two fragments is the same both before (Table I) and after cross-linking (Table III). However, in variant 2 cells, the cross-linking of the two forms of protein 3 by H$_2$DIDS is not the same. The ratio of radioactive label increases from 1.2 (Table I) to 1.6 (Table

Table III: [^{3}H]H_2DIDS Cross-Linking of Pronase-Cleaved Protein 3 in Normal (N) and Variant (V_1 and V_2) Erythrocytes[a]

cell type	[^{3}H]H_2DIDS concn (μM)	% radioactivity: cross-linked protein 3	% radioactivity: fragment not cross-linked, 60 kDa	% radioactivity: fragment not cross-linked, 63 kDa
N	2	82 ± 2	16 ± 1	
	20	81 ± 1	18 ± 1	
V_1	2	85 ± 1	7 ± 1	7 ± 1
	5	81 ± 1	9 ± 1	9 ± 1
	20	81 ± 1	9 ± 1	9 ± 1
V_2	2	73 ± 4	<1	24 ± 1
	5	74 ± 2	6 ± 1	18 ± 1
	20	72 ± 2	10 ± 1	16 ± 1

[a] Erythrocytes were labeled with 2, 5, or 20 μM [^{3}H]H_2DIDS and then digested with 0.1 mg/mL Pronase. Membranes were isolated and extracted with NaOH solution. Membrane proteins were separated by $NaDodSO_4$–polyacrylamide electrophoresis on a 5–15% acrylamide slab gel using the discontinuous buffer system of Laemmli (1970). The amounts of radioactivity present in protein 3 and its fragments were estimated by densitometric scanning of the fluorographs.

III) after the alkaline extraction of membrane. Densitometric scans of the Coomassie blue stained gel prepared from cells treated with 20 μM [^{3}H]H_2DIDS or DIDS gave the same type of results.

The data show that cells possessing the normal protein or either of the two variants have similar [^{3}H]H_2DIDS binding capacities when exposed to various concentrations of [^{3}H]-H_2DIDS (Table II). When variant 2 cells were treated with [^{3}H]H_2DIDS at concentrations less than 2 μM, most of the label was found in the 63-kDa fragment (Figure 2A and Table I). A comparison of the ability of [^{3}H]H_2DIDS to cross-link the 60- or 63-kDa and 35-kDa fragments is shown in Table III. The data indicate that, in normal or variant 1 cells treated with low concentrations (2 or 5 μM) of H_2DIDS, the fragments of protein 3 are cross-linked similarly at high pH values. In variant 2, however, the cross-linking is not as efficient. More of the 63-kDa fragment of variant 2 remains after exposure to alkali than of the 60-kDa fragment. At 2 μM, these results are exaggerated. This is probably due to the low concentration of [^{3}H]H_2DIDS employed. We conclude that cross-linking of the 63-kDa protein 3 fragment by [^{3}H]H_2D-IDS in variant 2 cells is less complete than that in normal or variant 1 cells.

Because protein 3 is the anion transport protein of erythrocytes, it was important to establish whether or not the observed alteration(s) had functional significance. When the SO_4^{2-} self-exchange efflux rates in normal, variant 1, and variant 2 cells were compared (Table IV), the SO_4^{2-} transport activity of neither variant 1 nor variant 2 was impaired. Even in the presence of various concentrations of DIDS, the rate constants were comparable. In addition, Pronase digestion of control or DIDS-treated cells did not change the SO_4^{2-} self-exchange efflux rate in any of the cell types (data not shown). Thus, variant 2 cells appear to have a normal anion transport system and the same sensitivity toward Pronase digestion as do the normal and variant 1 cells.

Discussion

Normal erythrocytes contain protein 3 which, when treated with proteases such as Pronase or chymotrypsin, is cleaved into two fragments of 60 and 35 kDa. The 60-kDa fragment is readily observed by Coomassie staining while the 35-kDa fragment is not readily observed with this stain. The 60-kDa fragment contains the DIDS binding site and is the amino-terminal end of the molecule which for the most part extends into the cytoplasm. The 35-kDa fragment contains the carboxyl-terminal end of the molecule and contains the oligosaccharide chain. An elongated variant form of protein 3 has been identified (Mueller & Morrison, 1977). In cells containing this form of protein 3, treatment with protease yields a 63-kDa fragment. We have demonstrated that the group of individuals with elongated variant protein 3 in their erythrocytes can be subdivided. In most cases, the elongated protein 3 has the same properties as normal protein 3. Using [^{3}H]H_2DIDS, an anion transport inhibitor, as a probe, we have been able to distinguish a second variant.

Variant 1 of protein 3 is identical with normal protein 3 in its interactions with [^{3}H]H_2DIDS and other reagents. Variant 2, by contrast, shows altered reactivity with [^{3}H]H_2DIDS. In intact cells, the elongated variant 2 chain reacts more readily with [^{3}H]H_2DIDS than does the normal chain. However, at high pH values, when H_2DIDS acts as a bifunctional cross-linking agent, it is less effective cross-linking the proteolytic fragments generated by Pronase digestion of intact cells in variant 2 chains.

H_2DIDS reacts with an amino acid residue at or near the outer face of the cell membranes (Cabantchik & Rothstein, 1972, 1974; Lepke et al., 1976; Ship et al., 1977; Cabantchik et al., 1978; Jennings & Passow, 1979; Ramjeesingh et al., 1980; DuPre & Rothstein, 1981). The different sensitivity toward H_2DIDS binding and the difference in the cross-linking with H_2DIDS of the two fragments generated by Pronase digestion suggest that the structure and/or conformation of the outer portion of variant 2 differs from that of the normal or variant 1 protein. The cause of the alteration is unknown.

Variant 2 cells may have a different peptide sequence and/or conformation in the region of the molecule that binds H_2DIDS. The fact that H_2DIDS is incorporated more readily into the variant 2 elongated protein 3 but cross-links the 63- and 35-kDa fragments at high pH values less readily suggested that this may be the case. The binding site for DIDS may be more exposed than normal but more distant from the site or the 35-kDa fragment involved in cross-linking. Although the elongated chain of variant 2 reacts with DIDS more readily than the normal chain, the amount of DIDS incorporated into variant 2 cells is the same as that incorporated into variant 1 or normal cells under the conditions employed. Since protein 3 exists as at least a dimer in the membrane, these results suggest that there may be an interaction between monomers.

It is also possible that other membrane components (lipids or proteins) in the vicinity of protein 3 may be different in variant 2 cells. Two observations indicated that sialoglycoproteins were not involved. First, all cell types had similar

Table IV: Effect of DIDS Treatment on the $^{35}SO_4^{2-}$ Self-Exchange Efflux Rate Constant (k_t) in Normal and Variant Erythrocytes[a]

cell type	$-k_t$ (min^{-1}) at [DIDS] (μM) of: 0	5	10
normal	0.0391 ± 0.0017[b]	0.0173 ± 0.009 (55.75)[c]	0.0006 ± 0.0003 (98.47)
variant 1	0.0401 ± 0.0004	0.0164 ± 0.0019 (59.10)	0.0008 ± 0.0002 (99.07)
variant 2	0.0429 ■ 0.0055	0.0176 ± 0.0011 (58.97)	0.0004 ± 0.0001 (98.00)

[a] Cells were treated with DIDS (0–10 μM) according to the procedure of Cabantchik & Rothstein (1972). [b] The rate constants are expressed as the mean ± SD. [c] Percent inhibition = 100(k_t of control cells − k_t of DIDS-treated cells)/k_t of control cells.

sialoglycoprotein labeling patterns when cells were labeled by mild periodate oxidation, followed by reduction with NaB^3H_4 (Mueller et al., 1976). Second, pretreatment of cells with trypsin did not abolish the different sensitivity toward [^{3}H]-H_2DIDS binding of protein 3 and the different rate of [^{3}H]-H_2DIDS cross-linking of Pronase-generated fragments in variant 2 cells (data not shown).

It has been reported that both the 60- and 35-kDa fragments generated by chymotrypsin digestion of red blood cells are involved in anion transport activity and that both fragments can be labeled by reductive methylation using formaldehyde and reducing with tritiated borohydride (CHO/NaB^3H_4) (Jennings, 1982) or with pyridoxal 5′-phosphate/NaB^3H_4 (Nanri et al., 1983). DuPre & Rothstein (1981) and Ramjeesingh et al. (1980) have shown that if membranes prepared from chymotrypsin-digested cells are extracted with NaOH solution and then retreated with chymotrypsin, the 60-kDa fragment is further cleaved to 17- or 15-kDa fragments, and the 35-kDa fragment is cleaved to a 9-kDa fragment. Using those reported procedures, we found that extensive chymotrypsin digestion of NaOH-extracted membranes isolated from CHO/NaB^3H_4-labeled or pyridoxal 5′-phosphate/NaB^3H_4-labeled, chymotrypsin-digested red cells generates 17-, 15-, and 9-kDa fragments in normal and variant cells and that all these small fragments are labeled as well (L. Hsu and M. Morrison, unpublished results). Because a genetic variant protein can be a useful tool for study of the structure–function relationship of the normal protein, further chemical characterization of membrane components and peptide maps of protein 3 in this new variant is currently under investigation.

Variant 2, like variant 1, does not appear to be associated with hematologic abnormalities. Nevertheless, we investigated the erythrocytes of the parents of the donor with variant 2 containing cells. The protein 3 in the mother's cells was normal, while the father was a heterozygote. His cells have a protein 3 that is identical with variant 2, as judged from its altered reactivity with H_2DIDS, but he shows no hematologic features that would be considered abnormal. It is clear from our work that a number of variations of protein 3 will be detected. The high prevalence of protein 3 containing an elongated peptide chain (4–5% of a random population) (Morrison et al., 1981) suggests that it may have biologic importance. It was only because the variant 2 donor was a heterozygote that we were able to detect the change involving the DIDS binding site. It would have been much more difficult to detect the difference in an individual in whom both chains of proteins were identical.

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