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Inhibition of Phosphate Transport across the Human Erythrocyte Membrane by Chemical Modification of Sulfhydryl Groups

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ABSTRACT: Effects of sulfhydryl-reactive reagents on phosphate transport across human erythrocyte membranes were examined using ^{31}P NMR. Phosphate transport was significantly inhibited in erythrocytes treated with sulfhydryl modifiers such as *N*-ethylmaleimide, diamide, and Cu^{2+}/*o*-phenanthroline. Quantitation of sulfhydryl groups in band 3 showed that the inhibition is closely associated with the decrease of sulfhydryl groups. Data from erythrocytes treated with diamide or Cu^{2+}/*o*-phenanthroline demonstrated that intermolecular cross-linking of band 3 by oxidation of a sulfhydryl group, perhaps Cys-201 or Cys-317, decreases the phosphate influx by about 10%. The inhibition was reversed by reduction using dithiothreitol. These results suggest that sulfhydryl groups in the cytoplasmic domain of band 3 may play an important role in the regulation of anion exchange across the membrane.

Human band 3 (M_r 101 791), composed of 911 amino acids (Lux et al., 1989), is the major intrinsic membrane protein of the erythrocyte which catalyzes a one-to-one exchange of anions across the plasma membrane (Passow, 1986). This glycoprotein is composed of two functionally distinct domains (Steck et al., 1976, 1978): a 43-kDa cytoplasmic domain which binds ankyrin (Hargreaves et al., 1980; Bennett & Stenbuck, 1980; Thevenin et al., 1989) and other cytoskeletal (Korsgren & Cohen, 1986) and cytosolic proteins (Strapazon & Steck, 1976), and the C-terminal transmembrane domain which spans the bilayer multiple times (Tanner et al., 1988; Lux et al., 1989).

Amino acids essential for anion transport activity in erythrocyte membranes have been examined using a variety

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of chemical probes (Passow, 1986) and site-directed mutagenesis (Bartel et al., 1989; Garcia & Lodish, 1989). Lysine, arginine, histidine, and glutamic acid have been shown to be candidates for amino acids involved in anion exchange. Human band 3 contains five cysteine residues per molecule (Rao, 1979; Lux et al., 1989). Knauf and Rothstein (1971) demonstrated that sulfhydryl modification by *p*-(chloromercuri)-benzenesulfonate has no direct effect on sulfate efflux. Rao (1979) reported that anion transport appears to be unaffected by *N*-ethylmaleimide (NEM).[1] However, no sufficient data on anion transport are shown in these papers. Therefore, it seems premature to conclude that sulfhydryl groups in band 3 have no effect on anion transport. So, we examined the role of sulfhydryl groups of band 3 on phosphate transport across the erythrocyte membrane using ^{31}P NMR. The present work describes that band 3-mediated anion transport is significantly inhibited by chemical modification of sulfhydryl groups.

Experimental Procedures

Materials. Compounds were obtained from the following sources: diazinedicarboxylic acid bis(*N*,*N*'-dimethylamide) (diamide), Sigma; 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and dithiothreitol (DTT), Wako Chemicals; *N*-ethylmaleimide (NEM), *o*-phenanthroline, and 2,4,6-trinitrobenzenesulfonate (TNBS), Nacalai Tesque. All other chemicals were of reagent grade.

Chemical Modification of Membrane Proteins. Human blood was obtained from the Fukuoka Red Cross Blood Center. The blood was centrifuged at 750*g* for 10 min at 4 °C. The plasma and buffy coat were removed carefully. The erythrocytes were washed three times in phosphate buffer (5 mM sodium phosphate, 150 mM NaCl, pH 7.6), suspended at 10% hematocrit in the same buffer, incubated for at least 30 min at 37 °C, and centrifuged at 1000*g* for 10 min at 25 °C. The packed erythrocytes (1.5 mL) were suspended at 10% hematocrit in the phosphate buffer and then treated for 30 min at 37 °C with reagents such as 0.1–10 mM NEM, 0.1–5 mM diamide, 0.1 mM DIDS, 2 mM DTNB, 1 mM TNBS, or 10 mM DTT. Treatment of erythrocytes (1.5 mL) with 0.2 mM $CuSO_4$/1 mM *o*-phenanthroline was carried out for 30 min at 25 °C, as described by Thevenin et al. (1989). The erythrocytes thus modified were washed two times with the warmed buffer and were finally packed by centrifugation at 1000*g* for 10 min at 25 °C. The packed cells were used for the transport experiment and the preparation of membranes. By chemical modification and subsequent washing of the cells, no hemolysis was observed.

Membrane Preparation. Erythrocyte ghost membranes were prepared from the cells modified with chemical reagents, according to the method of Dodge et al. (1963). To isolate band 3 from the membane, 1 volume of ghost membrane was incubated with 9 volumes of 0.1 N NaOH for 30 min at 0 °C and then centrifuged at 56000*g* for 30 min at 4 °C. The pellets containing band 3 were washed twice with 5 mM sodium phosphate, pH 8.0, and used for gel electrophoresis and the determination of sulfhydryl groups.

Determination of Membrane Sulfhydryl Groups. Ghost membranes (0.2 mL at pellet) were solubilized by incubating with 0.3 mL of 20% SDS and 2.8 mL of 100 mM sodium phosphate (pH 8.0) for 20 min at 37 °C. Then, 0.1 mL of 10 mM DTNB in 100 mM sodium phosphate (pH 8.0) was added. After the incubation for 15 min at 37 °C, the concentration of membrane sulfhydryl groups from the absorbance at 412 nm was determined using a molar extinction coefficient of 13 600 (Habeeb, 1972). Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

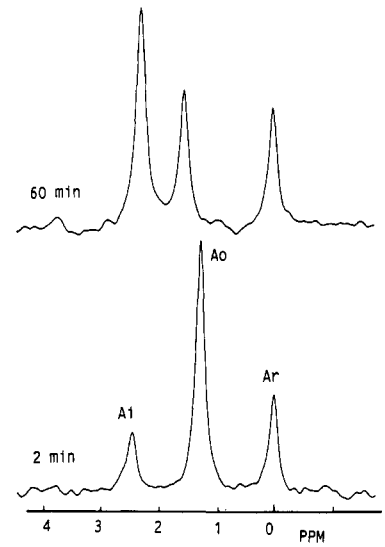

FIGURE 1: ^{31}P NMR spectra of the erythrocyte suspension. Packed erythrocytes (1.5 mL) preincubated in phosphate buffer (5 mM sodium phosphate, 150 mM NaCl, pH 7.6) were mixed with an equal volume of transport buffer (30 mM sodium phosphate, 160 mM sucrose, 22 mM citrate, pH 6.2). The ^{31}P NMR spectra of the erythrocyte suspension were recorded after 2 and 60 min. A_i, A_o, and A_r are the signal areas of phosphate inside the cell and outside the cell and the external reference (85% H_3PO_4), respectively.

NMR Measurement. Erythrocytes obtained by chemical modification of intact cells (1.5 mL) and 1.5 mL of 30 mM sodium phosphate, 160 mM sucrose, 22 mM sodium citrate, pH 6.2 (transport buffer) were preincubated for 10 min at 25 °C, mixed to initiate the transport process, and then incubated at 25 °C. At discrete time intervals, the ^{31}P NMR spectra were recorded at 25 °C with no spinning of a 10-mm round-bottom NMR tube containing both erythrocyte suspension (about 3 mL) and a small glass capillary filled with 85% H_3PO_4. The ^{31}P NMR spectra were run at 161 MHz on a JEOL GSX-400 spectrometer with the following instrument settings: 4096 data points, a 1-kHz spectral width, a 4.0-s pulse repetition, 50 scans, a 45° flip angle. To confirm the results obtained from the NMR method, the concentration of extracellular phosphate was measured spectrophotometrically. Intact cells (0.1 mL) were modified with NEM, mixed with 0.1 mL of transport buffer, incubated for 60 min at 25 °C, and then centrifuged at 1000*g* for 5 min at 4 °C. The phosphate concentration in the supernatant was determined by the method of Ames (1966).

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of membrane proteins was performed on 5.6% gel using the continuous buffer system of Fairbanks et al. (1971).

Results

Figure 1 shows the time course of the ^{31}P NMR spectra of the erythrocyte suspension. The two peaks appeared downfield relative to the external reference signal (85% H_3PO_4). Phosphate (pH 6.2) outside the cell gives an upfield signal compared to that (pH 7.6) inside the cell (Brauer et al., 1985). The spectrum after 60 min shows the influx of phosphate.

[1] Abbreviations: NEM, *N*-ethylmaleimide; diamide, diazinedicarboxylic acid bis(*N*,*N*'-dimethylamide); DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; TNBS, 2,4,6-trinitrobenzenesulfonate.

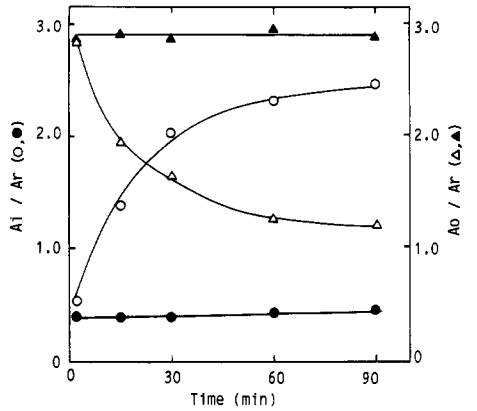

FIGURE 2: Time course of phosphate influx into erythrocytes at 25 °C. Intact (open symbols) or 0.1 mM DIDS-treated (closed symbols) erythrocytes were mixed with an equal volume of transport buffer. The ^{31}P NMR signals of phosphate inside (circles) and outside (triangles) the cell were recorded versus time at 25 °C. A_i, A_o, and A_r are shown in Figure 1.

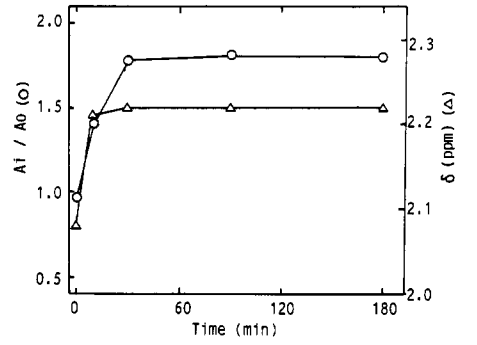

FIGURE 3: Effects of preincubation time at 37 °C of human erythrocytes on A_i/A_o and chemical shift of intracellular phosphate. Intact erythrocytes suspended at 10% hematocrit in phosphate buffer were preincubated at 37 °C and then centrifuged. The pellets were mixed with transport buffer and incubated for 60 min at 25 °C. Values are the mean for two experiments.

Figure 2 shows the time course of A_i/A_r and A_o/A_r, where A_i, A_o, and A_r are the signal areas of phosphate inside the cell and outside the cell and the external reference, respectively. The values of A_i and A_o changed steeply up to about 30 min, as demonstrated by Brauer et al. (1985). On the other hand, the values of A_i and A_o in DIDS-treated erythrocytes remained constant (Figure 2). Moreover, the response of signal area to the concentration of phosphate was examined. In DIDS-treated erythrocytes, the values of A_o changed in proportion to the concentration of phosphate outside the cell (data not shown). These results indicate that signal area sensitively reflects the concentration of phosphate. So, the Donnan ratio, i.e., the concentration ratio of phosphate inside and outside the cell, is expressible as the ratio (A_i/A_o) of both signal areas. Thus, we can use A_i/A_o as a parameter of phosphate transport.

It seems likely that the values of A_i/A_o are affected by intracellular ionic distribution before initiation of the transport measurement. So, effects of the preincubation at 37 °C of erythrocytes on phosphate transport were examined. When the erythrocytes were preincubated at 37 °C in phosphate buffer (5 mM sodium phosphate, 150 mM NaCl, pH 7.6) and then used for the transport experiment, the values of A_i/A_o increased until 30 min and then remained almost constant (A_i/A_o = 1.8) (Figure 3). A similar increase of A_i/A_o was also obtained by using 150 mM NaCl instead of the phosphate buffer (data not shown). However, no such increment was observed upon preincubation for 60 min at 37 °C in Cl^--free medium (205 mM sucrose, 28.5 mM sodium citrate, pH 7.6);

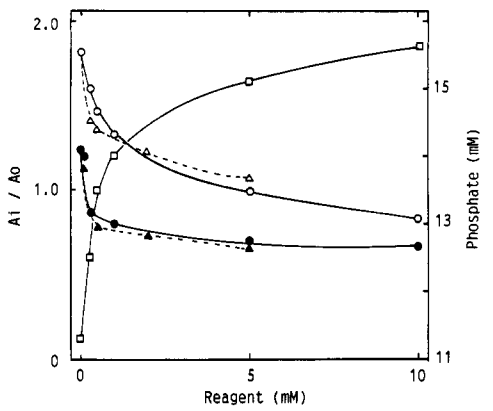

FIGURE 4: Concentration effects of NEM and diamide on phosphate transport. To examine the effects of NEM (circles) or diamide (triangles) on A_i/A_o, erythrocytes (1.5 mL at pellet) preincubated for at least 30 min at 37 °C in phosphate buffer were modified using these reagents for 30 min at 37 °C. The modified cells were mixed with transport buffer and incubated for 30 (closed symbols) and 60 min (open symbols) at 25 °C. To determine phosphate influx by the spectrophotometric method (□), erythrocytes (0.1 mL at pellet) were modified with NEM as in NMR samples, incubated in transport buffer for 60 min at 25 °C, and then centrifuged. The phosphate concentration in the supernatant was determined by the method of Ames. Values are the mean for three experiments.

i.e., A_i/A_o = 1.0. These results suggest that the Cl^- influx upon preincubation is of importance for an increment of the A_i/A_o value. Moreover, intracellular ionic distribution is reflected as the changes in pH. The ^{31}P NMR chemical shift is very sensitive to pH. Upon pretreatment in the phosphate buffer, the chemical shifts of the ^{31}P resonance of intracellular phosphate changed in a manner similar to that of A_i/A_o (Figure 3), suggesting that preincubation for 30 min at 37 °C allows intracellular ions to reach equilibrium distribution. Therefore, the erythrocytes were incubated in the phosphate buffer for at least 30 min at 37 °C before chemical modification of erythrocyte membranes and the transport experiment.

To examine whether the signal areas of the ^{31}P NMR resonance are affected by such factors as oxidation or reduction of hemoglobin which may be generated from the treatment of the cells with chemical modifiers, DIDS- (0.1 mM) treated cells were modified using agents such as NEM (10 mM), diamide (5 mM), DTNB (2 mM), TNBS (1 mM), DTT (10 mM), and $CuSO_4$ (0.2 mM)/*o*-phenanthroline (1 mM). These modified cells were mixed with transport buffer, and then NMR spectra were measured. In all cases, the values of A_i/A_o or A_i/A_r were almost the same as that of DIDS-treated cells (0.18 or 0.52, respectively). These results suggest that the value of A_i/A_o, under our experimental conditions, is unaffected by other factors except for phosphate transport.

Figure 4 shows the values of A_i/A_o obtained by incubating NEM- (0.1–10 mM) and diamide- (0.1–5 mM) treated erythrocytes with transport buffer for 30 and 60 min at 25 °C. Irrespective of the incubation time, the transport of phosphate was inhibited steeply at low concentrations of NEM and diamide. Although the results described above indicate that ^{31}P NMR can be a powerful method for measuring phosphate transport, the usefulness of the NMR method was further confirmed by measuring the concentration of extracellular phosphate by the spectrophotometric method. NEM-treated erythrocytes were incubated in the transport buffer for 60 min at 25 °C. The concentration of phosphate in the supernatant after centrifugation was increased with increasing NEM concentration (Figure 4), indicating that the phosphate influx is inhibited by the NEM treatment of red cells. So, the degree of inhibition of phosphate influx obtained by both methods

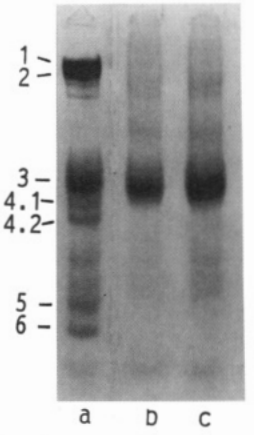

FIGURE 5: SDS–PAGE of erythrocyte membrane proteins. Preparation of band 3 from ghost membranes was performed by using 0.1 N NaOH as described under Experimental Procedures. Lane a: control ghost membrane. Lane b: band 3 prepared from intact cells. Lane c: band 3 prepared from 1 mM NEM-treated cells.

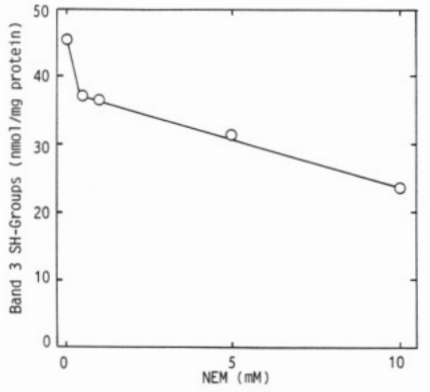

FIGURE 6: Modification of sulfhydryl groups of band 3 by NEM. Preparation of band 3 from NEM-treated erythrocytes was carried out as described under Experimental Procedures. The content of sulfhydryl groups was determined by using DTNB. Values are the mean for at least two experiments.

was compared in the following example. In the case of intact, 0.5 mM NEM- and 0.1 mM DIDS-treated erythrocytes, the extracellular phosphate concentrations determined photometrically were 11.3, 13.5, and 24.6 mM, respectively, whereas the values of A_o/A_r from the NMR spectra were 1.18, 1.46, and 2.89. Thus, the inhibition of phosphate influx by about 9% obtained photometrically is in good agreement with that (10%) from the NMR method. NEM is a sulfhydryl-reactive reagent. So, in NEM-treated erythrocyte membranes the concentration of sulfhydryl groups of band 3 was determined. Stripping of band 3 from NEM-treated erythrocyte membranes was performed by the method of Steck and Yu (1973). Figure 5 demonstrates that band 3 protein is isolated from other membrane proteins. The concentration of sulfhydryl groups in band 3 was decreased with increasing NEM concentration (Figure 6). NEM is specific for sulfhydryl groups, but it can react with other groups such as amino groups under certain conditions (Riordan & Vallee, 1972). To examine a possibility of the reaction of NEM with amino groups of amino acids essential in anion transport, erythrocyte membranes were labeled first with DIDS and then with NEM. The reaction of sulfhydryl groups with NEM in band 3 was unaffected by DIDS (Table I). This indicates that no NEM reacts with amino groups which DIDS binds. As with diamide, Cu^{2+}/*o*-phenanthroline, which is an oxidizing agent of sulfhydryl groups, also inhibited phosphate transport (Table I). When one sulfhydryl group in band 3 was modified with Cu^{2+}/*o*-phenanthroline or NEM, the values of A_i/A_o were decreased by about 22% (Table I). This value is corresponding to 10% inhibition of phosphate influx. Figure 7 demonstrates a relationship between phosphate transport and sulfhydryl content in band 3. The transport of phosphate was inhibited linearly with decreasing sulfhydryl groups in band 3. These results suggest that sulfhydryl groups in band 3 may also affect phosphate transport.

To confirm the contribution of sulfhydryl groups in phosphate transport, additional chemical modification of membrane

Table I: Phosphate Transport and Sulfhydryl Content of Band 3 in Erythrocytes Treated with Chemical Modifiers[a]

reagent	A_i/A_o	sulfhydryl groups in band 3 (nmol/mg of protein)
NT[b]	1.83 ± 0.04 (n = 11)	47.6 ± 3.2 (n = 5)
NEM	1.46 ± 0.01 (n = 3)	39.0 ± 4.6 (n = 3)
diamide	1.38 ± 0.04 (n = 3)	34.1 ± 0.4 (n = 3)
Cu^{2+}/*o*-phenanthroline	1.44 ± 0.02 (n = 3)	41.4 ± 1.6 (n = 3)
DIDS/NEM[c]	0.15 ± 0.01 (n = 2)	38.4 ± 1.4 (n = 2)

[a] Treatment of erythrocytes (at 10% hematocrit) with sulfhydryl modifiers (0.5 mM NEM, 0.5 mM diamide, and 0.2 mM $CuSO_4$/1 mM *o*-phenanthroline) and 0.1 mM DIDS was carried out for 30 min at 37 °C except for Cu^{2+}/*o*-phenanthroline (25 °C). Thus, modified cells were used for phosphate transport (60 min at 25 °C) and determination of sulfhydryl content on band 3. Values are the mean ± SD of n, the number of experiments. [b] NT, no treatment. [c] DIDS (0.1 mM) treated erythrocytes were incubated with 0.5 mM NEM.

Table II: Effects of Sulfhydryl- and Amino-Reactive Reagents on A_i/A_o[a]

primary treatment	secondary treatment	A_i/A_o control	A_i/A_o DTT
NT[b]		1.82	1.80
NEM		1.43	1.37
TNBS		0.89	
NEM	TNBS	0.82	
diamide		1.37	1.55
DTNB		1.87	
DTNB	diamide	1.35	1.58
Cu^{2+}/*o*-phenanthroline		1.42	1.75

[a] Erythrocytes (at 10% hematocrit) were incubated with the primary chemical modifiers, washed, and treated sequentially with the secondary modifiers. In addition to chemical modifiers used under the same conditions as in Table I, cells were treated for 30 min at 37 °C with 1 mM TNBS, 2 mM DTNB, and 10 mM DTT. Phosphate transport after 60 min at 25 °C was measured. Values are the mean for at least two experiments. [b] NT, no treatment.

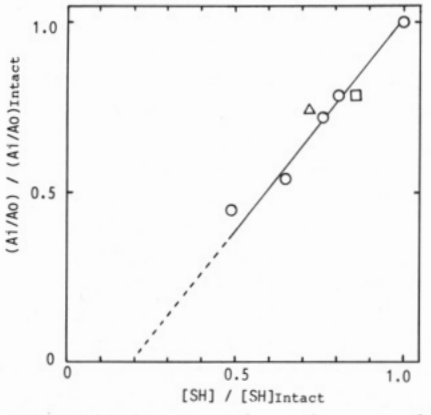

FIGURE 7: Relationship between A_i/A_o and content of sulfhydryl groups of band 3. Values of A_i/A_o or sulfhydryl contents of band 3 in NEM-treated (○), diamide-treated (△), and Cu^{2+}/*o*-phenanthroline-treated (□) erythrocytes are represented relative to those in intact erythrocytes.

proteins was carried out (Table II). Phosphate transport in diamide- or Cu^{2+}/*o*-phenanthroline-treated erythrocytes was restored considerably by reduction of the disulfide bond with DTT. As demonstrated by Reithmeier (1983), the inhibition of phosphate transport in DTNB-treated cells was reversed by washing the cells with buffer. TNBS, an amino-reactive reagent, greatly inhibited phosphate transport. The value of A_i/A_o in TNBS-treated erythrocytes was almost the same as that of NEM-treated ones followed with TNBS. This suggests that in double labeling with NEM and TNBS the inhibition effect of TNBS on phosphate transport is more predominant than that with NEM. This may be attributed to the binding of TNBS to the amino groups of lysine and arginine essential for anion transport.

Discussion

The NMR technique has been widely used to investigate the structure and function of biological membranes. Usually, in order to discriminate the probe molecules inside and outside the membrane, NMR shift reagents (Pettegrew et al., 1987) and NMR line broadening ones (Ashley & Goldstein, 1981) are used. Therefore, results obtained thus should be analyzed carefully, considering the effect of reagents added. On the other hand, the ^{31}P NMR spectra utilized in the present work show the separated signals of phosphate due to the difference in pH inside and outside the cell. The signal area corresponds to the concentration of phosphate. Thus, the parameter A_i/A_o, which corresponds to the Donnan ratio, was used here and it reflected more sensitively than A_i/A_r or A_o/A_r the phosphate transport across the membrane.

The values of A_i/A_o were increased upon preincubation of erythrocytes in the buffer containing 150 mM NaCl at 37 °C (Figure 3). One possible mechanism is the following. Upon preincubation, extracellular Cl^- ions present at high concentration enter the cells in exchange for intracellular HCO_3^-. The amount of Cl^- diffused into erythrocytes largely depends on that of HCO_3^- present initially inside the cells. The exchange of HCO_3^- and Cl^- is a fast process. When Cl^--loaded erythrocytes are incubated in Cl^--free transport buffer, the Cl^- efflux coincides with the influx of phosphate and the rate of this exchange depends on the intracellular Cl^- concentration. Thus, the values of A_i/A_o are expected to change depending on preincubation under our conditions.

Human band 3 is the anion channel protein for which the relationship between the structure and function has been studied in more detail. By means of chemical modification of specific amino acid residues and site-directed mutagenesis, amino acids involved in the anion exchange of band 3 have been determined. Stilbenedisulfonate derivatives such as DIDS and H_2DIDS bind irreversibly with lysine residues and specifically inhibit anion transport (Passow, 1986). Phenylglyoxal (Bjerrum et al., 1983; Zaki, 1984), diethyl pyrocarbonate (Matsuyama et al., 1986), and Woodward's reagent K (Jenning & Anderson, 1987) were used to modify arginine, histidine, and glutamate, respectively. All these reagents inhibit the anion exchange.

In the present work, we have demonstrated that phosphate transport across the erythrocyte membrane is significantly inhibited by chemical modification of sulfhydryl groups in band 3. Human erythrocyte band 3 contains five cysteine residues (Rao, 1979). As deduced from the sequence of a cDNA for human band 3, cysteines are present at positions 201, 317, 479, 843, and 885 (Lux et al., 1989). All cysteine residues except for Cys-479 are accessible from the cytoplasmic side of the membrane. Cys-201 and Cys-317 are located in the N-terminal cytoplasmic domain of band 3 and react readily with sulfhydryl reagents. Cys-479 and Cys-843 are located in the membrane-spanning domain, and the former is unreactive to NEM in cells (Solomon et al., 1983). In the erythrocyte membrane treated mildly with Cu^{2+}/*o*-phenanthroline (Reithmeier & Rao, 1979; Thevenin et al., 1989) and diamide (Kitajima et al., 1990), cross-linking of band 3 monomers into dimers occurs. A disulfide bond is formed between the intersubunits of the band 3 dimer. The sites of cross-linking have been identified to be Cys-201 and Cys-317 (Reithmeier & Rao, 1979; Thevenin et al., 1989). The number of cross-links is one (Reithmeier & Rao, 1979) or two (Thevenin et al., 1989), and the discrepancy was ascribed to the difference in experimental conditions. Similarly, the reaction of erythrocyte membranes with NEM also showed that Cys-201 and Cys-317 are readily modified with NEM (Thevenin et al., 1989). In the erythrocyte treated with NEM (0.5 mM) or Cu^{2+} (0.2 mM)/*o*-phenanthroline (1 mM), one sulfhydryl group in band 3 was modified (Table I). These facts suggest that the reaction site of band 3 with sulfhydryl reagents under mild conditions is Cys-201 or Cys-317. Thus, we conclude that chemical modification of Cys-201 or Cys-317 in band 3 inhibits phosphate influx by about 10% under our conditions.

Band 3 is connected to the cytoskeleton via the linkage protein ankyrin (Bennett & Stenbuck, 1980). Translational and rotational diffusion of such a band 3 may be considerably restricted, compared with that of ankyrin-free band 3 (Tsuji et al., 1988). Ueno et al. (1987) demonstrated a possibility that a rapid exchange between ankyrin-bound band 3 and ankyrin-free band 3 may occur in intact erythrocytes. In binding of ankyrin to band 3, Cys-201 and Cys-317 on the cytoplasmic domain of band 3 are important: alkylation and oxidation of these sulfhydryl groups inhibit ankyrin binding to band 3 (Thevenin et al., 1989). This inhibition was considered to be due to the conformational changes of the cytoplasmic domain of band 3 by sulfhydryl modification. From a functional point of view, it is interesting to examine the difference in the transport activity between ankyrin-free band 3 and ankyrin-bound band 3. Using inside-out vesicles in which cytoskeletal proteins and linking proteins such as ankyrin are released from the membrane, Grinstein et al. (1978) demonstrated that sulfate efflux from the vesicles is not affected by the removal of the 43-kDa cytoplasmic domain of band 3 by trypsinization. Similar results were obtained by Lepke and Passow (1976). However, from the results obtained by using ankyrin-free band 3, we cannot conclude that the cytoplasmic domain of band 3 has no effect on anion transport in intact cells. There is circumstantial evidence that the ankyrin–band 3 interactions may modulate the rate of anion exchange. Kay et al. (1988) showed that band 3 with an insertion of 2–4 kDa in the 17-kDa transmembrane anion transport segment reveals a decrease in the number of high-affinity ankyrin-binding sites and an increase in the rate of anion transport. On the other hand, our results have demonstrated that the modification of the sulfhydryl group in Cys-201 or Cys-317 reduces the transport rate of phosphate. Taking these results into consideration, it may be possible that ankyrin associates with cysteine residues of the cytoplasmic domain of band 3 (Willardson et al., 1989). Thus, it seems likely that anion transport of band 3 is mediated by the binding and unbinding of ankyrin to Cys-201 and Cys-317.

Conformational changes in band 3 occur with ligand binding to the active site of transport. Amino acids participating in anion transport bear their charged groups inside the channel spanned through the lipid bilayer. The binding of ligands to charged groups of amino acids induces a conformational

change of band 3 from inward- to outward-facing forms and vice versa. However, such reversible conformational changes are blocked by the binding of anion inhibitors to active sites. Binding of PLP at the inner surface of red cells reduces external DIDS binding to band 3 (Rothstein et al., 1976), whereas the conformational changes in band 3 induced by external DIDS binding decrease the affinity of hemoglobin binding to the cytoplasmic domain of band 3 (Salhany et al., 1980) or strengthen the interaction of ankyrin with the cytoplasmic domain of band 3 (Hsu & Morrison, 1983). Salhany and Cassoly (1989) demonstrated that hemoglobin binding to the cytoplasmic domain of band 3 decreases the reaction rate of *p*-(chloromercuri)benzoate with Cys-201 and Cys-317. This suggests that hemoglobin binding to the N-terminal domain of band 3 induces the conformational changes of band 3. Cys-201 and Cys-317 would not be active sites for ligands in the anion exchange. However, an interaction of ankyrin with these cysteines and chemical modification of the sulfhydryl groups would induce the conformational changes of band 3. Conformational changes induced in this manner may disturb native conformational changes which are essential for anion transport. On the other hand, from a structural point of view, Cys-201 and Cys-317 are of importance in maintaining the membrane structural integrity, as seen in the interaction of band 3 with ankyrin. Thus, Cys-201 and Cys-317 may play an important role in the structure and function of band 3.

Registry No. Cys, 52-90-4; PO_4^{3-}, 14265-44-2.

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