Conformational Change of Band 3 Protein Induced by Diethyl Pyrocarbonate Modification in Human Erythrocyte Ghosts†

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ABSTRACT: Diethyl pyrocarbonate inhibited the phosphate exchange across the human erythrocyte membrane. The exchange rate was inhibited only when the membranes were modified with the reagent from the cytosolic surface of resealed ghosts. The intracellular modification by diethyl pyrocarbonate inhibited the extracellular binding of [^{3}H]dihydro-4,4′-diisothiocyanostilbene-2,2′-disulfonic acid to band 3 protein. Furthermore, the extracellular 4,4′-dinitrostilbene-2,2′-disulfonic acid protected the membranes from the intracellular modification by diethyl pyrocarbonate. These results suggest that the extracellular binding of 4,4′-dinitrostilbene-2,2′-disulfonic acid to band 3 protein induces the conformational change of the intracellular counterpart of band 3 protein and the diethyl pyrocarbonate susceptible residue(s) is (are) hidden from the cytosolic surface of the cell membrane in connection with the conformational change. Conversely, under the conditions where the diethyl pyrocarbonate modification is confined to the intracellular side of the membrane, the extracellular binding site of [^{3}H]dihydro-4,4′-diisothiocyanostilbene-2,2′-disulfonic acid is hidden from the cell surface.

Greater understanding of molecular mechanisms underlying anion transport in human erythrocyte membranes has been achieved since band 3 has been shown to be the transporter of inorganic anions (Passow, 1986). Lysine, arginine, and carboxyl residues of band 3 have been shown to be essential amino acids for transport activity (Passow, 1986; Wieth et al., 1982; Jennings, 1982; Cabantchik et al., 1975; Nanri et al., 1983). One of the functional lysine residues has been affinity labeled with pyridoxal phosphate (Cabantchik et al., 1975; Nanri et al., 1983; Matsuyama et al., 1983). The labeled lysine residue was located at the 61st residue from the COOH terminal of band 3 (Kawano et al., 1988), suggesting that the COOH-terminal region of band 3 must constitute at least part of the active center for the anion transport system.

Recently, it was suggested that a histidine residue or residues in the band 3 protein participates in anion transport in addition to lysine and arginine residues (Chiba et al., 1986; Matsuyama et al., 1986). In the present study, we investigated the effect of diethyl pyrocarbonate (DEPC),[1] a histidine-oriented reagent, on phosphate transport across the erythrocyte membrane and on the [^{3}H]H_2-DIDS binding to band 3. The functional amino acid(s) for anion transport which is (are) susceptible to DEPC was (were) located at the intracellular surface of the cell membrane, and the modification of the intracellular amino acid inhibited the binding of H_2-DIDS from extracellularly to band 3.

Materials and Methods

Preparation of Resealed Ghosts. Human blood which was freshly drawn or stored for less than 2 weeks was used in these studies. The stored blood was obtained from the Fukuoka Red Cross Blood Center where it had been maintained at 4 °C in a citrate/phosphate/dextrose solution. Experiments were conducted with resealed erythrocyte ghosts which were prepared essentially by following the method of Schwoch and Passow (1973) as described previously (Matsuyama et al., 1986). In brief, washed erythrocytes were lysed osmotically with 30 instead of 10 volumes of lysing solution in order to facilitate removal of the hemoglobin inside the ghosts. Ghosts were resealed at 37 °C for 60 min by adding an equal volume of resealing media. The resealing medium contained 400 mM sucrose, 2 mM sodium fluoride, 20 mM glucose, and 60 mM sodium phosphate at pH 6–8. The degree of resealing was assessed from the DNDS-sensitive portion of the transport rate and from the glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) activity. More than 95% of the ghosts were resealed as right-side-out. When these ghosts were treated with 5 mM DEPC, the degree of resealing was disrupted to some extent, but their integrity was unchanged during the assay of transport rate.

After resealing, ghosts were washed with media made up by diluting the resealing media by 2-fold. The packed ghosts were resuspended in 10 volumes of a phosphate buffer (200 mM sucrose, 1 mM sodium fluoride, 10 mM glucose, and 30 mM phosphate at pH 7.4) in which the transport activity was determined.

Measurement of Transport Rate in Resealed Ghosts. Rates of phosphate exchange were determined in resealed ghosts by measuring influx rates of phosphate at 37 °C as described previously (Matsuyama et al., 1986). The transport assay was initiated by adding radioactive phosphoric acid (carrier-free). At specified intervals, aliquots of the ghost suspension (1.0 mL) were plunged into 30 mL of ice-cold media containing 100 μM DNDS and 100 mM glucose. DNDS inhibits anion transport, and glucose addition facilitates precipitation of the ghosts (Tomoda et al., 1984). The diluted ghost suspensions were centrifuged at 27000*g* for 5 min at 4 °C during which the

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[1] Abbreviations: DEPC, diethyl pyrocarbonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; GA3PDH, glyceraldehyde-3-phosphate dehydrogenase; [^{3}H]H_2-DIDS, [^{3}H]dihydro-4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; pH_i, intracellular pH; pH_e, extracellular pH.

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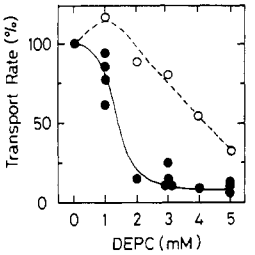

FIGURE 1: DEPC inhibition of rate of phosphate exchange into erythrocyte ghosts. Erythrocyte ghosts were resealed by suspending 1 volume of ghost suspension in an equal volume of medium containing 400 mM sucrose, 2 mM sodium fluoride, 20 mM glucose, and 60 mM sodium phosphate (pH 7.4) with (○) or without (●) 20 mM histidine (final concentration of histidine, 10 mM) as described under materials and methods. Phosphate exchange fluxes were determined in resealed ghosts suspended in a medium containing 200 mM sucrose, 1 mM sodium fluoride, 10 mM glucose, 30 mM sodium phosphate (pH 7.4), and various concentrations of DEPC at 37 °C.

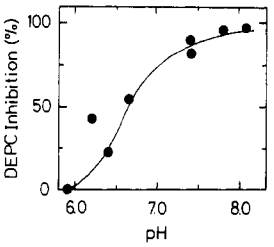

FIGURE 2: Influence of pH on DEPC inhibition of phosphate exchange rates into erythrocyte ghosts. Ghosts were prepared in resealing media at pH 6–8 and pretreated with 5 mM DEPC for 30 min at 0 °C in the absence of a pH gradient. Phosphate exchange rates were measured in DEPC-treated ghosts at pH_e 7.4 after preincubating the ghosts in the same buffer for 20 min at 37 °C.

ghosts were completely precipitated. The ghosts were deproteinized with 0.6 M perchloric acid, and the extracts were used for radioactive counting. Exchange rates were determined from the DNDS-sensitive incorporation of phosphate.

Treatment of Ghosts with DEPC. The influence of DEPC on exchange rates was examined initially at 37 °C in transport media containing 1–5 mM DEPC in the absence of transmembrane pH gradients. In the presence of these pH gradients, ghosts were pretreated with 5 mM DEPC for 30 min at 0 °C in order to maintain the pH gradients during DEPC incorporation. These DEPC-treated ghosts were washed at 4 °C with 6 volumes of a phosphate buffer (200 mM sucrose, 1 mM sodium fluoride, 10 mM glucose, and 30 mM phosphate at pH 6–8) before measuring transport rates at pH 7.4 and 37 °C.

Dihydro-DIDS Binding to Band 3. Resealed ghosts were pretreated with 5 mM DEPC at 0 °C for 30 min in the presence and absence of transmembrane pH gradients. DEPC-treated ghosts were incubated with 25 μM $[^3H]H_2$-DIDS with a specific radioactivity of not less than 200 mCi/mmol for 30 min at 37 °C in the same medium used to determine transport rates (containing 200 mM sucrose, 1 mM sodium fluoride, 10 mM glucose, and 30 mM sodium phosphate, pH 7.4) and were analyzed by SDS–PAGE.

Analytical Procedures. Radioactivity was determined with a liquid scintillation spectrometer by utilizing the Cerenkov effect. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. SDS–PAGE for protein analysis was carried out according to the method of Laemmli (1970).

Materials. Carrier-free radioactive phosphoric acid and $[^3H]H_2$-DIDS were purchased from Japan Atomic Energy Research Institute, Ibaraki, Japan, and from HSC Research Development Corp., Toronto, Canada, respectively. DEPC and DNDS were from Aldrich, Milwaukee, WI, and Tokyo Kasei Kogyo, Tokyo, Japan, respectively. Other reagents were of analytical grade.

RESULTS

Effect of DEPC on the Rate of Phosphate Exchange in Erythrocyte Ghosts. Phosphate exchange rates were determined at 37 °C in erythrocyte ghosts resealed to yield a pH_i of 7.4 and suspended in a phosphate buffer (pH_e 7.4) containing DEPC. As shown in Figure 1, the phosphate exchange rate at pH 7.4 was inhibited by DEPC, and half of the activity was inhibited at a medium DEPC concentration of 1.3 mM. When 10 mM histidine was also sealed inside ghosts, the DEPC inhibitory effect was reduced by 33–100% depending on the DEPC concentration (Figure 1).

Table I: Effects of DEPC on Phosphate Exchange Rates in Erythrocyte Ghosts with a pH_i of 7.4 or 6.0 Suspended in Media with a pH_e of 7.4 or 6.0[a]

condition	pH_i	pH_e	exchange rate[b] (% of control)	GA3PDH act. (% of control)
1	6.0	6.0	125.5	6
2	6.0	7.4	100.0	nd[c]
3	7.4	6.0	37.0	nd
4	7.4	7.4	17.0	4

[a] Ghosts were resealed in phosphate buffer with a pH_i value of 6.0 (conditions 1 and 2) or 7.4 (conditions 3 and 4) and were resuspended in an equal volume of the phosphate buffer (pH_e 6.0 or 7.4). The resealed ghosts were pretreated with 5 mM DEPC in a phosphate buffer of pH_e 6.0 (conditions 1 and 3) or 7.4 (conditions 2 and 4) for 30 min at 0 °C. Phosphate exchange rates of these ghosts were measured in a phosphate buffer with pH 7.4 at 37 °C after removing medium DEPC as described under Materials and Methods. Exchange rate and GA3PDH activity were expressed as ratios to those of nontreated ghosts under each condition. [b] Average of two experiments. [c] nd, not determined.

The influence of pH on DEPC inhibition was first evaluated by changing the pH inside the ghosts from 6 to 8. Resealed ghosts were pretreated with 5 mM DEPC at 0 °C for 30 min, and phosphate exchange rates were measured at pH_e 7.4 and 37 °C. When resealed ghosts were treated with DEPC at pH_i 6.0, exchange rates were not inhibited at all whereas the rates in ghosts pretreated at pH_i 8.0 were inhibited as much as at pH_i 7.4 (Figure 2). DEPC reacts principally with deprotonated imidazole as well as with amino groups of proteins (Miles, 1977). The inhibitory effect decreased as the pH_i was lowered from 8 to 6 as if the amino acids interacting with DEPC were being protonated. The apparent p*K* of the DEPC-sensitive residues was 6.6 from Figure 2, which is similar to the p*K* value obtained from pH titration (Matsuyama et al., 1986). This suggests that the amino acid moiety modified by DEPC is the imidazole group of histidine. At the present time, however, we could not eliminate the possibility that the DEPC-sensitive locus was an amino group, because the apparent p*K* of "Lys a" is 7–8 (Passow, 1986). The influence of hydroxylamine in restoring transport activity could not be examined because this agent altered the integrity of the resealed ghosts.

Localization of the DEPC-Susceptible Amino Acids. Two kinds of ghosts were prepared for determining the fluid compartment with which the DEPC-susceptible residues were interfacing: one was prepared by resealing ghosts in a medium with pH 6.0 (conditions 1 and 2, Table I) which resulted in DEPC having no effect on the phosphate exchange rate and the other by resealing ghosts in a medium with pH 7.4 (conditions 3 and 4, Table I) which did permit DEPC to exert its inhibitory effect on the exchange rate (Figure 2). In addition, the influence of DEPC on phosphate transport was examined

Table II: Effect of DEPC on Phosphate Exchange Rates of Erythrocyte Ghosts following DNDS Pretreatment[a]

exchange rate [nmol (mg of protein)$^{-1}$ min^{-1}]		
	DEPC treated	
control	without DNDS pretreatment	with DNDS pretreatment
85.7 ± 7.6 (100%)	16.3 ± 6.2 (19.0%)	80.3 ± 4.5 (93.7%)

[a] Ghosts resealed in a medium with pH 7.4 were preincubated with DNDS for 5 min at 37 °C and for 30 min at 0 °C. The DNDS-treated ghosts were exposed to 5 mM DEPC at 0 °C for 30 min. After removing DNDS by washing with a phosphate buffer containing 0.5% bovine serum albumin, phosphate exchange rates were measured in these ghosts at pH 7.4 and 37 °C. Values are mean ± SD (n = 3).

in media with pH_e of either 6.0 and 7.4. These ghosts were pretreated with 5 mM DEPC for 30 min at 0 °C instead of 37 °C in order to maintain the transmembrane pH gradients during the DEPC incorporation, and the transport rates were subsequently examined in a phosphate buffer of pH 7.4 at 37 °C after removing extracellular DEPC.

When ghosts were pretreated with 5 mM DEPC at pH 7.4 and 0 °C for 30 min, the transport rate was inhibited by 83% (condition 4, Table I), whereas no inhibition or slightly acceleration was noted with ghosts pretreated with DEPC at pH 6.0 (condition 1, Table I). Moreover, DEPC markedly inhibited the transport rate of only those ghosts that were resealed in a medium with pH 7.4 (conditions 3 and 4, Table I) but not those resealed with pH 6.0 (conditions 1 and 2, Table I). When the pH_e was different from the pH_i (condition 3, Table I), DEPC inhibitions tended to be weaker than that in condition 4 of Table I. It must be due to the difficulty to keep the pH_i at 7.4 for 30 min at 0 °C under this condition. It appears that DEPC entered the ghosts and achieved concentrations high enough to modify DEPC-susceptible residues, because intracellular GA3PDH, which requires a functional histidine residue for enzyme activity (Buehner et al., 1974), was inhibited to a similar extent at pH 7.4 and 6.0 (Table I). It was, therefore, concluded that the essential amino acid residues for phosphate transport faced the intracellular fluid.

Influence of Extracellular DNDS on DEPC Modification of the Intracellular Amino Acids. Stilbene compounds such as DIDS and DNDS inhibit anion transport competitively but bind preferentially to the *outward* configuration of the transport system in erythrocyte membranes (Barzilay et al., 1979; Shami et al., 1978). We examined the possibility that functional amino acid residues are exposed to the cytosolic surface of the membranes only when the transporter is present in the *inward* configuration. Ghosts, which had been resealed in a medium with a pH of 7.4, were preincubated with DNDS for 5 min at 37 °C and for 30 min at 0 °C in order to convert the band 3 molecules into the *outward* configuration. The DNDS-pretreated ghosts were then incubated with 5 mM DEPC at 0 °C for 30 min, under conditions which should inhibit 80–90% of the transport rate (Figure 2). After the excess DNDS was washed out of the medium, the anion transport rate was measured in the resealed ghosts. The transport rate in the DNDS-pretreated ghosts was 93.7% of the control (Table II), indicating that *extracellular* DNDS protected the DEPC-susceptible residue in the *intracellular* fluid from DEPC modification. DNDS did not inactivate the reactivity of DEPC itself, because GA3PDH was inhibited by DEPC in the presence of DNDS, which is not an inhibitor of GA3PDH (data not shown).

Influence of DEPC Modification on Dihydro-DIDS Binding to Band 3. Since DNDS blocks the DEPC inhibition of anion transport (Table II), a reverse relationship, viz., an influence

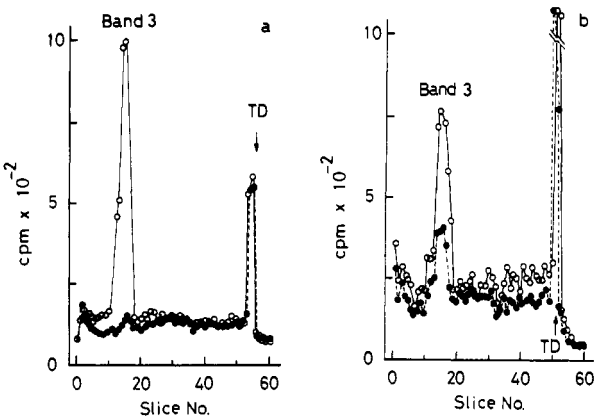

FIGURE 3: Influencing of DEPC on [^{3}H]H_2-DIDS binding to band 3 protein. Resealed ghosts (a) were prepared in a pH 7.4 medium and pretreated with (●) or without (○) 5 mM DEPC for 30 min at pH_e 7.4 and 0 °C or (b) were prepared in a medium at pH 6.0 (pH_i 6.0) (○) or 7.4 (pH_i 7.4) (●) and pretreated with 5 mM DEPC in a pH 6.0 medium (pH_e 6.0). Each DEPC-pretreated ghost preparation was incubated with 25 μM [^{3}H]H_2-DIDS for 5 min at pH 7.4 and 37 °C. For SDS–PAGE analysis on 9% gels (Laemmli, 1970), 30 μg of ghosts was placed in each lane, and the gels were stained with Coomassie brilliant blue. Gel radioactivities were determined with a liquid scintillation counter after the gels were sliced into 60 fractions and soaked with protosol at 56 °C overnight. TD refers to the tracking dye. Distribution of radioactivities between band 3 and TD fractions varied with different lots of [^{3}H]H_2-DIDS.

of DEPC on the binding of a radioactive analogue of DNDS (dihydro-DIDS) to band 3, might be demonstrable. When resealed ghosts were treated with [^{3}H]H_2-DIDS at pH 7.4, the radioactivity of [^{3}H]H_2-DIDS in a pH 7.4 medium was incorporated into band 3 (open circles, Figure 3a). This incorporation was completely inhibited by pretreating ghosts with DEPC at pH 7.4 (closed circles, Figure 3a). Essentially the same results were obtained when the treatment was performed in the presence of transmembrane pH gradients. The radioactive incorporation was inhibited almost completely when resealed ghosts with pH_i 7.4 were pretreated with a pH 6.0 medium containing 5 mM DEPC for 30 min at 0 °C (closed circles, Figure 3b), i.e., under conditions which inhibited the transport in resealed ghosts (condition 3, Table I). In contrast, [^{3}H]H_2-DIDS incorporation was not inhibited when ghosts were resealed at pH 6.0 and pretreated with DEPC as described above (open circles, Figure 3b), i.e., under conditions which did not inhibit the transport in the ghosts (condition I, Table I). The incomplete inhibition by DEPC in the presence of pH gradients must have been due to the incomplete maintenance of pH_i (see above). [^{3}H]H_2-DIDS binding to band 3 was therefore inhibited only when intracellular functional residues were modified by DEPC. Under these conditions, the transport rate in the ghosts was also inhibited (conditions 3 and 4, Table I). Thus, when functional amino acids were modified by DEPC at the cytosolic membrane surface, the configuration of band 3 protein assumed the *inward* form to which the extracellular stilbene compounds could not bind.

Discussion

We suggested previously that intracellular histidine residues may participate in phosphate transport across the erythrocyte membrane (Matsuyama et al., 1986). This was based on the observation that phosphate efflux was inhibited as histidine residues were presumably protonated by acidifying the intracellular fluid of ghosts from pH 6.8 to 6. The participation of histidine in anion transport has also been suggested by the influence of pH titration on eosin 5-isothiocyanate binding to

band 3 (Chiba et al., 1987). In the present paper, we have used a histidine-oriented reagent, DEPC, to modify the expected histidine residues, and by using resealed ghosts, we could readily modify intracellular amino acids by DEPC as well as other experimental conditions such as intracellular pH. The influence of DEPC on phosphate exchange rates varied by changing the pH: the inhibitory effects decreased as the pH in the reactive environment decreased from 8 to 6 (Figure 2), suggesting that the reactivity of DEPC decreases as the protonated form of histidine residues increases. DEPC, however, reacts not only with histidine but also with lysine, cysteine, and tyrosine residues, and we could not examine the restoration effect of hydroxylamine on DEPC inhibition. At the present time, therefore, we could not conclude that the DEPC-susceptible amino acid is histidine. DEPC inhibited more effectively the fluxes of resealed ghosts with pH_i 7.4 (conditions 3 and 4, Table I) than with pH_i 6.0 (conditions 1 and 2, Table I), and its inhibitory effects was reduced by resealing free histidine residues inside the ghosts (Figure 1), indicating that the DEPC-susceptible residues mediating anion transport are located inside the membrane, which is consistent with our previous results (Matsuyama et al., 1986).

Extracellular DNDS protected the intracellular amino acids from DEPC modification (Table II), and the DEPC modification apparently inhibited dihydro-DIDS binding to band 3 in turn (Figure 3). Stilbene compounds bind only to the outward conformation of band 3 (Barzilay et al., 1979; Shami et al., 1978). Therefore, it appears highly conceivable that the DEPC-susceptible residues are hidden from the cytosolic surface in the *outward* form and make their appearance in the cytosolic compartment only when the membrane protein is in the *inward* conformation.

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