Histidine-834 of Human Erythrocyte Band 3 Has an Essential Role in the Conformational Changes That Occur during the Band 3-Mediated Anion Exchange†

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ABSTRACT: We have shown that diethyl pyrocarbonate (DEPC) inhibits band 3-mediated anion exchange and that the inhibition occurs only when histidine residue(s) is (are) modified with DEPC from the cytosolic surface of resealed ghosts [Izuhara et al. (1989) *Biochemistry 28*, 4725−4728]. In the present study, we have identified the DEPC-modified histidine residue as His834 using liquid chromatography with electrospray ionization mass spectrometry (LC/ESI-MS). This mild, rapid, sensitive, and quantitative method was successfully applied to analysis of the unstable DEPC−histidine adduct. The DEPC modification of His834 was pH dependent and 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) sensitive as previously shown. After DEPC modification, band 3-mediated anion exchange is inhibited. Consistent with previous results, we confirmed that His834 was located on the cytosolic side of the membrane and the DEPC modification of His834 had allosteric effects on the extracellular DNDS-binding site of band 3. Therefore, we conclude that His834 is located at the cytosolic surface of band 3 and is an essential residue for band 3-mediated anion exchange. We will discuss important roles of the region from TM12 to TM14 in the conformational changes that occur during the band 3-mediated anion exchange.

Our understanding of the molecular mechanisms underlying anion exchange in erythrocyte membranes has advanced considerably since it was first demonstrated that band 3 is the red cell inorganic anion transporter (*1*−*5*). The one-to-one exchange of anions across the erythrocyte membranes has been explained by the ping-pong model (*6*). The rate of transport is governed by a single conformational change in band 3. This conformational change leads to the transfer of a single substrate anion across the membrane, and the rates of association and dissociation of the substrate are much faster than the rate of the conformational change that leads to the translocation of the bound anion (*5*). Lysine, arginine, glutamic acid, and histidine residues of band 3 have been shown to be essential amino acids for transport activity (*5*, *7*−*12*). However, little is known about the nature and location of these functionally important amino acids, mainly because of the difficulty in handling the hydrophobic transmembrane peptide portions which include the functional amino acids.

We have shown that histidine residue(s) participate(s) in the anion-exchange process by experiments involving pH titration and diethyl pyrocarbonate (DEPC)[1] modification (*7*, *9*, *13*). The essential histidine residue is located at the cytosolic surface of band 3. Extracellular binding of DNDS to band 3 induces a conformational change in the intracellular portion of band 3 such that the histidine residue is hidden from the cytosolic surface of the cell membrane. Conversely, when the DEPC modification takes place at the intracellular side of the membrane, the extracellular reaction site of H_2DIDS is hidden from the cell surface as a result of the conformational change in band 3. This suggests that the histidine residue is essential for the conformational change during the anion-exchange process (*7*). The essential role of histidine residues of band 3 in anion exchange has been confirmed by Muller-Berger et al. (*14*). However, the nature and location of the histidine residues involved have not been clarified.

In the present study, we have identified the histidine residue that reacts with DEPC using liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS). Our results indicate that only one histidine residue among the six histidine residues in the membrane domain of band 3 is modified with DEPC in a functionally significant manner and the DEPC-modified histidine residue is His834. We discuss the implications of these data on the structure/function relationships of band 3-mediated anion exchange.

EXPERIMENTAL PROCEDURES

Materials. TPCK-trypsin (sequence grade) and *N*-glycosidases were purchased from Roche Diagnostics (Mannleira,

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[1] Abbreviations: DEPC, diethyl pyrocarbonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; H_2DIDS, 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid; LC/ESI-MS, liquid chromatography of electrospray ionization mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; TM, transmembrane spanning portion; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SAO, Southeast Asian ovalocytosis.

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Table 1: Tryptic Peptide Fragments Containing DEPC-Modified or Unmodified Histidine Detected by LC/ESI-MS

no.	start	end	location of histidine	mass of unmodified histidine peptide	retention time (min)	mass of modified histidine peptide	retention time (min)
H1	433	551	His547	1350.9 (+10)[a]	82.66−83.95	1358.1 (+10)	84.06−84.65
H2	647	656	His651	574.6 (+2)	35.92−38.06	610.6 (+2)	40.48−42.45
H3	699	730	His703	1092.0 (+3)	55.95−57.27	1116.0 (+3)	58.62−60.62
H4	731	743	His734	665.0 (+2)	28.04−30.44	701.0 (+2)	32.99−34.80
H5	818	826	His819	559.4 (+2)	28.07−31.02	595.4 (+2)	ND[b]
H6	833	879	His834	1761.8 (+3)	70.03−72.19	1785.8 (+3)	73.76−76.01

[a] The charge states of the most intense ion are presented in parentheses. [b] Not detected.

Germany). Trifluoroacetic acid, DEPC (diethyl pirocarbonate), and other chemical reagents were purchased from Wako Co. Ltd. (Osaka, Japan).

Preparation of Unsealed and Resealed Ghosts. Human blood stored at 4 °C in acid/citrate/dextrose solution was obtained from the Fukuoka Red Cross Blood Center. Erythrocytes stored for less than 2 weeks were used in these studies. Unsealed ghosts were prepared as described previously (*15*). Resealed ghosts were prepared according to the methods of Kuma et al. (*16*). In brief, erythrocytes were washed with 165 mM KCl and lysed on ice in 40 volumes of 4 mM $MgSO_4$ and 1.2 mM acetic acid. Two minutes after lysis ammonium acetate was added to a final concentration of 150 mM. Ghosts were collected by centrifugation and resuspended in 7.5 mL of resealing buffer [200 mM sucrose, 30 mM ammonium acetate, and 10 mM sodium phosphate (pH 6 or 7.4)]. Suspensions were incubated for 30−90 min at 37 °C, diluted with 30 mL of 200 mM sucrose, 30 mM ammonium acetate, and 10 mM sodium phosphate (pH 7.4), and then centrifuged for 10 min at 28000*g*. The pellets were diluted by the same buffer to 2 mg/mL of protein concentration. Resealing was confirmed by trypsin susceptibility to the intracellular 40 kDa domain of band 3. One milliliter of ghosts was incubated with trypsin (2 μg/mL) on ice for 30 min, and the mixture was checked by SDS−PAGE (*17*). The resealed ghosts used in the present experiments were more than 95% sealed right-side-out membranes.

Treatments of Ghosts with DEPC. Unsealed and resealed ghosts were pretreated with 0.5−5 mM DEPC at 4 °C for 30 min under various conditions described previously (*7*). These DEPC-treated ghosts were washed three times with 6 volumes of resealed buffer [200 mM sucrose, 30 mM ammonium acetate, and 10 mM sodium phosphate (pH7.4)] and then washed three times with 5 mM $NaHCO_3$.

Preparation of Tryptic Fragments of Band 3. The preparation of band 3 tryptic fragments for HPLC has been described previously (*18*). Briefly, to remove the NH_2-teminal 40 kDa domain of band 3, DEPC-treated ghosts (1 mg/mL of proteins) were pretreated with a low concentration of trypsin (1 μg/mL) in 5 mM $NaHCO_3$ on ice for 30 min. Peripheral proteins and peptides were stripped by washing with 10 mM NaOH. Trypsin-digested ghosts were washed three times with 5 mM $NaHCO_3$. The washed membranes were dissolved in 0.1 M Tris-HCl (pH 8) buffer containing 0.1% $C_{12}E_8$ at a total volume of 200 μL. Two hundred microliters of protein solution (120 μg) was digested with 4 μg of TPCK-trypsin in 0.1 M Tris-HCl (pH 8) containing 0.1% $C_{12}E_8$ for 2 h at 37 °C.

HPLC Separation of Tryptic Fragments by LC/ESI-MS. Tryptic fragments of DEPC-treated band 3 were analyzed by LC/ESI-MS. LC/ESI-MS was carried out using a combination of HPLC (Waters 600E, Waters) and a LCQ advantage ion trap mass spectrometer (Finnigan, San Jose, CA). Tryptic peptides were separated by HPLC using a gradient of H_2O (solvent A) and 2/1 (v/v) 2-propanol/acetonitrile (solvent B), each containing 0.025% TFA at a flow rate of 1 mL/min. The mobile phase composition was held at 5% solvent B for 5 min, and then ramped from 5% to 85% in 60 min and 85−100% in 20 min. To improve the separation of peptides for LS/ESI-MS, a C_{18} reversed-phase HPLC column was used (2.1 i.d. × 250 mm; Waters symmetry 300 C_{18} 5 μm). The column effluent from the HPLC separation was split and 20% of the flow (200 μL/min) directed into the ESI source. Data were acquired and analyzed using LCQ version 2.0 software. The instrument parameters were as follows: ESI needle voltage, 5 kV; ESI capillary temperature, 260 °C; ion energy, 35%; isolation window, 2 amu; scan range, 550−2000 amu. The theoretical mass of each cleavage peptide was calculated by using Protein Prospector on the World Wide Web (http://prospector.ucsf.edu/).

RESULTS

Identification of the DEPC-Susceptible Histidine. Anion-exchange activity is located in the transmembrane 55 kDa domain of band 3 (from Gly361 to Val911) which contains six histidine residues. All the tryptic peptide fragments of the band 3 transmembrane domain that contain histidine residues were identified by LC/ESI-MS as shown in Table 1. The molecular mass of the DEPC−histidine adduct is increased by 72 (*19*). We were able to identify individual DEPC-modified fragments of band 3 as new peptide fragments with an increase of molecular mass of 72 divided by charge state (Table 1). For example, the charge state of the most intense ion of the H1 peptide (from Asn433 to Lys551) was +10. Therefore, the apparent molecular mass of the DEPC-modified H1 peptide was increased by 7.2. As shown in Table 1, we were able to identify the DEPC-modified H1 peptide at position 1358.1 whose molecular mass is increased by 7.2 from the unmodified H1 peptide. Figure 1 shows the selected ion chromatograms of H4 (from Ser731 to Lys743) and H6 (from Met833 to Arg879) peptides containing His734 and His834, respectively. The HPLC retention time of the unmodified H4 peptide was 28.04−30.44 min, while that of the modified H4 peptide was 32.99−34.80 min (Table 1). As shown in Figure 1, there was no selected ion peak at the expected retention time of the DEPC−H4 adduct, indicating that His734 was not modified by DEPC under the conditions used in this experiment. In contrast to His734, His834 was almost completely modified with DEPC (Figure 1). Likewise, we calculated the modifications of the other individual

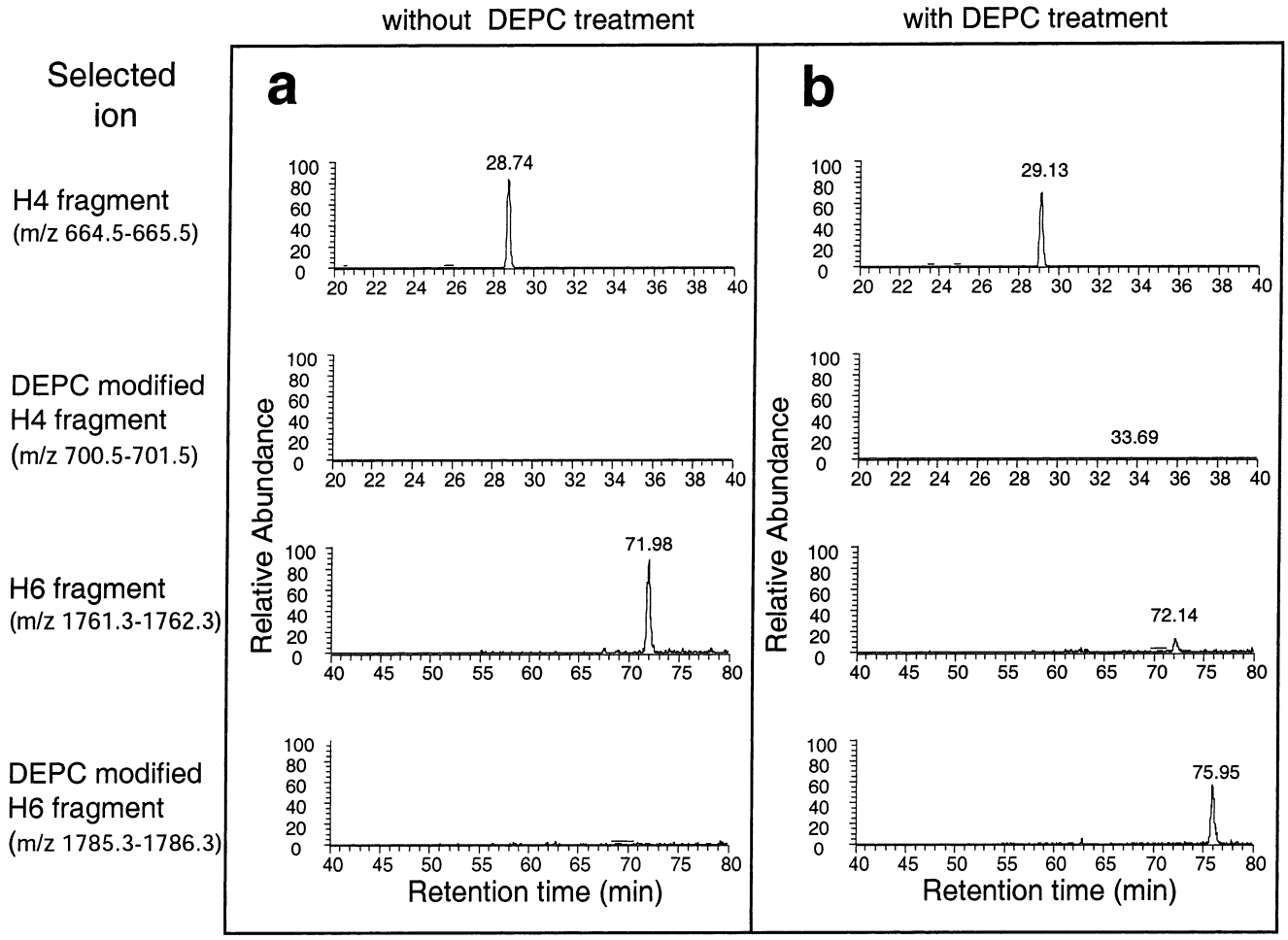

FIGURE 1: Selected ion chromatograms from ESI-MS detection of peaks eluting from HPLC separation of tryptic peptide fragments. Membranes were treated with or without 0.5 mM DEPC at pH 7.4. The treated membrane were dissolved in 0.1 M Tris-HCl (pH 8) buffer containing 0.1% $C_{12}E_8$ and digested by trypsin. The tryptic peptides in the solubilized membrane were separated by HPLC using a reversed-phase column (Waters symmetry 300 C_{18} 5 μm, 2.1 i.d. $\times$ 250 mm; Waters, Milford) with a water/acetonitrile/2-propanol eluant system containing 0.025% trifluoroacetic acid as described under Experiment Procedures. These chromatograms of (a) DEPC-modified and (b) unmodified H4 and H6 fragments were generated by the most intense molecular ion charge state within the mass/charge range of the instrument.

Table 2: DEPC Modifications of Each Histidine Calculated by the Peak Area of LC/MS after 0.5 mM DEPC Treatment at 4 °C and pH 7.4

His residue	modification (%)[a]	His residue	modification (%)[a]
His547	38.9 ± 4.5	His734	2.0 ± 0.9
His651	3.0 ± 1.5	His819	0
His703	1.5 ± 0.9	His834	83.3 ± 6.0

[a] The modification of all histidines calculated by the modified and unmodified peak areas using selected ion chromatograms from ESI-MS detection of peaks eluting from HPLC separation of tryptic fragments containing histidine. Values are the mean ± SD (n = 3).

histidine residues at His547, His651, His703, and His819 from the peak area ratio of the modified and unmodified peptide peaks. Table 2 shows the DEPC modification ratios of individual histidine residues. His547 and His834 were modified with DEPC by 38.9% and 83.3%, respectively, while negligible (less than 3%) modification with DEPC occurred at His651, His703, His734, and His819 under the conditions used (0.5 mM DEPC at 4 °C and pH 7.4 for 30 min).

Figure 2 shows the effects of pH and concentration on the DEPC modification at His834. The modification of His834 with DEPC was complete at pH 7.4. However, at pH 6.0 the DEPC modification was drastically decreased (Figure 2) as reported in our previous study (*7*). Only partial modification of His547 occurred with DEPC at pH 7.4 (Table 2).

Influence of DNDS on DEPC Modification of His834. It has been shown that a histidine residue of band 3 participates in anion exchange (*7*, *9*, *20*) and that the functional histidine residue is susceptible to DEPC modification. The intracellular modification by DEPC inhibits the extracellular reaction of H_2DIDS to band 3, and the extracellular binding of DNDS to band 3 protects the functional histidine residue(s) from DEPC modification (*7*). This suggests that the DEPC-susceptible histidine residue has a pivotal role in the conformational change of inward/outward forms during anion exchange (*7*, *9*, *13*). To assess whether His547 and His834 are the functional histidine residue(s) involved, we examined the effects of DNDS on DEPC modification. As shown in Figure 3, DNDS protected His834 from DEPC modification in a concentration-dependent manner, and 100 μM DNDS almost completely protected His834 from DEPC modification. However, His547 was not protected from DEPC modification by DNDS (data not shown).

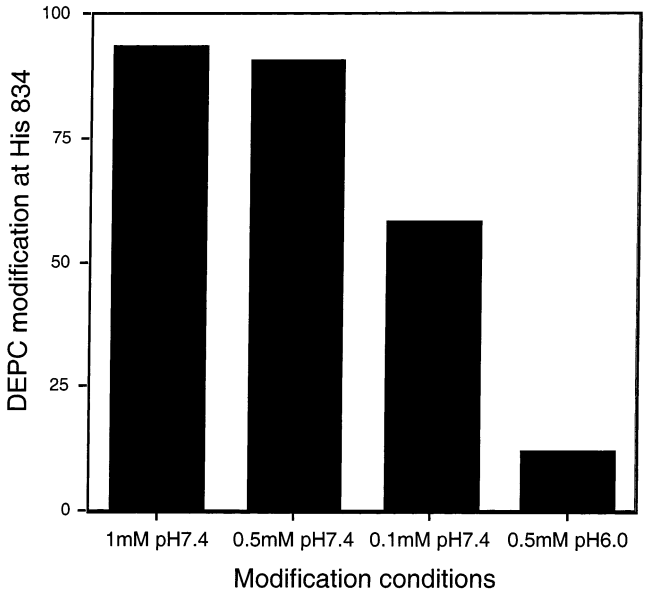

FIGURE 2: DEPC modification in various conditions. Membranes were treated with 0.1, 0.5, and 1 mM DEPC at pH 7.4 and with 0.5 mM DEPC at pH 6.0, respectively. The treated membranes were dissolved in 0.1 M Tris-HCl (pH 8) buffer containing 0.1% $C_{12}E_8$, digested by trypsin, and analyzed by the LC/MS system. The DEPC modifications of His834 were calculated by the reacted and unreacted peak area using selected ion chromatograms of H6 fragments treated with each condition. The modifications of each condition were 93.6%, 90.6%, 58.0%, and 12.0%, respectively.

Location of His834 in the Cell Membrane. Two kinds of resealed ghosts were prepared for determining the location of His834 in the cell membrane: one was prepared by resealing ghosts in a medium with pH 6.0 and the other by resealing ghosts in a medium with pH 7.4. These resealed ghosts were incubated in a medium with pH 7.4 containing 0.5 mM DEPC for 30 min at 4 °C. The DEPC-treated membranes were solubilized with 0.1% $C_{12}E_8$ and digested with trypsin. Trypic peptides in the solubilized membrane were analyzed by the LC/ESI-MS system. As shown in Figure 4, the majority (73.1%) of His834 was modified with DEPC when the intracellular pH was 7.4, while the DEPC modification decreased to 26.1% when the intracellular pH was 6.0, indicating that His834 is located at the cytosolic membrane surface.

DISCUSSION

It is well-known that arginine, lysine, and glutamic acid residues have an essential role in band 3-mediated anion exchange (*1*−*5*, *11*, *12*, *21*). In addition to the above amino acids, we have shown that at least one histidine residue of band 3 also participates in the anion exchange (*7*, *9*, *13*). The participation of the histidine residue has been confirmed by site-directed mutagenesis of band 3 (*14*). However, it has not been established as to which of the six histidine residues in the membrane domain of band 3 is modified by DEPC and which has a pivotal role to the conformational change of inward/outward forms relevant to the anion exchange (*7*, *9*, *13*). In the present study, LC/ESI-MS peptide mapping was used to show that His834 is the key residue for the inward/outward conformational change.

DEPC−histidine adducts are not stable enough for protein chemical analyses, and because of their instability, it has been difficult to identify the histidine residues modified by DEPC within protein molecules (*22*). However, our LC/ESI-MS peptide mapping method (*18*) is mild, rapid, sensitive, and quantitative and was successfully applied to the analysis of the unstable DEPC−histidine adducts. As shown in Table 1 and Figure 1, two histidine residues (His547 and His834) among the six residues were modified with DEPC by 39% and 83%, respectively. Our previous study indicated that a histidine residue involved in the inward/outward conformational change relevant to anion exchange was modified with DEPC in a pH- and DNDS-dependent manner (*7*). The modification of His547 with DEPC, however, was neither pH dependent nor DNDS sensitive (Figures 2 and 3), and in addition, only a minor fraction (39%) of His547 was modified with DEPC. Thus, we conclude that the DEPC-susceptible histidine residue essential for the inward/outward conformational change is His834 and not His547.

From the results of site-directed mutagenesis and DEPC modification experiments (*14*, *23*), Muller-Berger et al. suggested that each of the four histidines at positions 703, 734, 819, and 834 were important for anion-exchange activity. They also predicted that a hydrogen bond between His734 and Glu681 was involved in the band 3-mediated Cl^- exchange at low pH and that His734 was susceptible to DEPC modification. In our experimental conditions, however, His734 was not modified with DEPC (Table 2). Thus, the DEPC-susceptible histidine residue relevant to the inward/outward conformational change was not His734, but His734 might nevertheless have another functional role for anion exchange.

Important residues for anion exchange are concentrated in the region from TM12 to TM14. When Lys814 or Lys817 in loop 12−13 is mutated to Asp, the anion-exchange activities of mutant molecules are dramatically decreased (*14*). His834 is located in the N-terminal side of TM13. Cys mutants at and near His834 contributed to the considerable decrease in the anion-exchange activity (*24*). Lys851 in TM13 is a specific reactive site with H_2DIDS (*10*) and is also the binding site of PLP that is a substrate for the band 3-mediated anion exchange (*25*). A natural variant of band 3 of Pro868 to Leu in TM14 has higher band 3-mediated anion-exchange activity (*26*). Our recent study (*27*) on the SAO mutant indicated that the structural changes in TM1−5 were transmitted to the TM13−14 region, and we theorized that the transmitted conformational change at theTM13−14 region induced diminishing of the anion-exchange activity of the SAO band 3 molecule. The evidence described here carves in relief the importance of the region from TM12 to TM14 including His834 for the band 3-mediated anion exchange.

Considering our previous experiments (*7*, *9*, *13*) as well as those of Zhu et al. (*24*), we could conclude that His834 is located on the cytosolic surface of the cell membranes. Extracellular binding of DNDS to band 3 induces a conformational change in the intracellular portion of band 3 such that His834 is hidden from the cytosolic surface of the cell membrane. Conversely, when the DEPC modification takes place at the intracellular side of the membrane, the extracellular binding sites of the stilbene compound (Lys539 and Lys851) are hidden from the cell surface as a result of the inward/outward conformational change in band 3. These results constitute evidence in support of the concept that

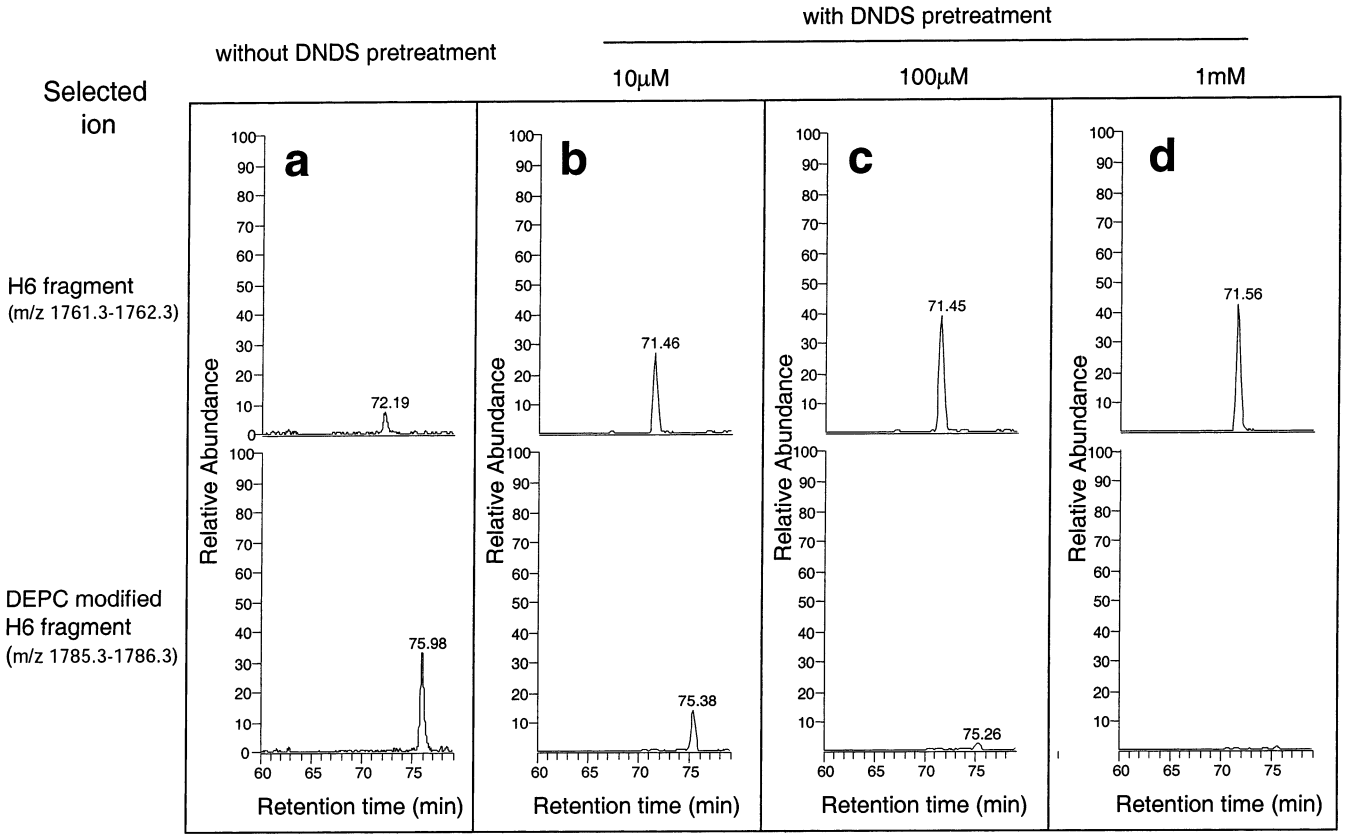

FIGURE 3: DNDS concentration dependence of DEPC modifications at His834. The membranes were treated with 0.5 mM DEPC at pH 7.4 in the absence and presence of 0.01, 0.1, and 1 mM DNDS. The membranes were dissolved in 0.1 M Tris-HCl (pH 8) buffer containing 0.1% $C_{12}E_8$ and digested by trypsin. The tryptic peptides in the solubilized membrane were analyzed by the LC/MS system. Selected ion chromatograms of DEPC-modified and unmodified H6 fragments (a) without or with (b) 10 μM, (c) 100 μM, and (d) 1 mM DNDS pretreatment were indicated. The DEPC modifications of each condition were 82.4%, 33.4%, 6.2%, and 0%, respectively.

inward and outward forms of band 3 are relevant to anion exchange. Further, they indicate that His834 plays a pivotal role in this inward/outward conformational change as an allosteric site. The cysteine scanning experiment of Zhu et al. (*24*) indicated that His834 was located on the cytosolic surface of the cell membranes, but only a limited fraction of the His834 to Cys mutant was modified with hydrophilic biotin maleimide, suggesting that, in their experimental conditions, the conformation of the His834 to Cys mutant takes the outward conformation in which the 834 site is hidden from the cell surface and inaccessible to hydrophilic biotin maleimide.

The experimental results summarized above strongly suggest that Lys539 (in TM5), Glu681 (in TM8), His734 (in TM10), His834 (in TM13), and Lys851 (in TM14) are located in close proximity in the native band 3 and form the anion channel for the anion exchange. In addition, a sequence of G^{381}LVRD in the N-terminal region of the transmembrane domain of band 3 is essential for the anion exchange (*28*), and the N-terminal region of the membrane domain (from Gly361 to Ala408) interacts with the loop between TM13 and TM14 (*27*). Band 3 SAO contains a deletion of nine amino acids (Ala400−Ala408) at the cytoplasmic boundary of TM1 and does not transport anions (*29*). We therefore conclude that the anion-exchange channel of band 3 consists, at least, of TM1, TM5, TM8, TM10, TM13, TM14, and the N-terminal region of the membrane domain. The similar proposal for an "anion access channel" has been suggested by Passow et al. (*14*, *30*). TM10, TM13, and TM14 are

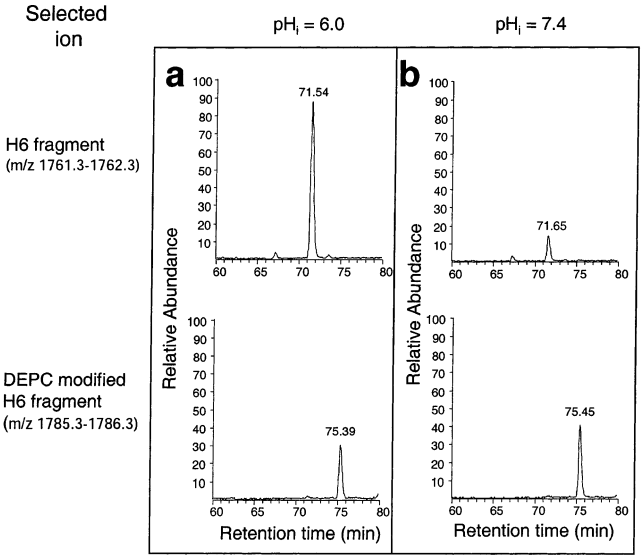

FIGURE 4: Influence of intracellular pH of the resealed ghost on DEPC modification at His834. Resealed ghosts at an intracellular pH of 6.0 or 7.4 were prepared as described in Experimantal Procedures. Each ghost was treated with 0.5 mM DEPC in media with an extracellular pH of 7.4. After unsealing, the ghost membranes were dissolved in 0.1 M Tris-HCl (pH 8) buffer containing 0.1% $C_{12}E_8$ and digested by trypsin. The tryptic peptides in the solubilized membrane were analyzed by the LC/MS system. Selected ion chromatograms of DEPC-modified and unmodified H6 fragments of (a) intracellular pH 6.0 and (b) pH 7.4 were indicated. The DEPC modifications at His834 were 26.1% and 73.1%, respectively.

unusual transmembrane segments and are classified as "category 2" (*31*−*33*). Transmembrane peptide portions classified as category 2 are flexible in the membrane lipid bilayer, because they do not directly contact with lipids in membranes (*31*−*33*). Considering our results (*7*, *10*, and this paper), we could speculate that the regions near Lys539/Lys851 and near His834 form the extracellular exit and the intracellular exit of the anion-exchange channel, respectively. The extracellular exit is open in the outward conformation of band 3 and closed in the inward conformation of band 3 while the intracellular exit is closed and open and vice versa. Such transmembrane flexibility must be maintained by the nature of transmembrane peptide portions belonging to category 2 such as TM10, TM13, and TM14.

In conclusion, the LC/ESI-MS peptide mapping procedure described in this paper is a mild, rapid, sensitive, and quantitative method, which we have successfully applied to the analysis of an unstable DEPC−histidine adduct. Using this method we have been able to identify the DEPC-susceptible histidine residue involved in band 3-mediated anion exchange as His834.

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