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The Functional Role of Arginine 901 at the C-Terminus of the Human Anion Transporter Band 3 Protein

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To determine which arginine residues are responsible for band 3-mediated anion transport, we analyzed hydroxyphenylglyoxal (HPG)-modified band 3 protein in native erythrocyte membranes. HPG-modification leads to inhibition of the transport of phosphoenolpyruvate, a substrate for band 3–mediated transport. We analyzed the HPG-modified membranes by reverse phase-HPLC, and determined that arginine 901 was modified by HPG. To determine the role of Arg 901 in the conformational change induced by anion exchange, we analyzed HPG-modification of the membranes when 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) or diethypyrocarbonate (DEPC) was present. DNDS and DEPC fix band 3 in the outward and inward conformations, respectively. HPG-modification was unaffected in the presence of DEPC but decreased in the presence of DNDS. In addition to that, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), which specifically reacts with the outward conformation of band 3, did not react with HPG-modified membranes. Furthermore, we expressed a band 3 mutant in which Arg 901 was replaced by alanine (R901A) on yeast membranes. The kinetic parameters indicated that the R901A mutation affected the rate of conformational change of the band 3 protein. From these results, we conclude that the most C-terminal arginine, Arg 901, has a functional role in the conformational change that is necessary for anion transport.

Key words: anion transporter, band 3 protein, peptide mapping, membrane protein.

Abbreviations: BSA, bovine serum albumin; $C_{12}E_8$, octaethylene glycol monododecyl ether; DEPC, diethypyrocarbonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; PEP, phosphoenolpyruvate; PG, phenylglyoxal; HPG, hydroxyphenylglyoxal; RP-HPLC, reverse phase-high performance liquid chromatography; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; TFA, trifluoroacetic acid; TM, transmembrane-spanning portion; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

Band 3 acts as an inorganic anion transporter in the human erythrocyte membrane. The protein consists of 911 amino acids, and its 40-kDa N-terminal domain functions as an attachment site for cytoskelton proteins and has a role in maintaining the shape of erythrocytes. The membrane is penetrated 12–14 times by the transmembrane-spanning portion (TM) of the 55-kDa C-terminal domain, which carries out erythrocyte anion transport (*1*, *2*). Kinetic studies of anion transport revealed that anion exchange is mediated a one-by-one electroneutral process in both directions, as indicated by the "ping-pong" model (*3*). The model suggests that transport is regulated through 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 (*4*). Many previous studies have shown that lysine, glutamate and histidine residues are essential for anion transport (*5–9*). The location of these essential residues was examined using a chemical modification approach. Lys 430 (*10*), Lys 539 (*11*, *12*), Lys 590 (*12*), Glu 681 (*13*), His 834 (*14*), and Lys 861 (*11*, *15*) were shown to be sites where chemical modification resulted in transport inhibition. In particular, His 834 participates in the conformational change between the outward facing and inward facing forms of band 3, which leads to the transfer of a single substrate anion across the membrane (*5*, *14*).

Other studies have shown that arginine residues also have important roles in the anion exchange process. Wieth *et al*. examined the pH dependence of chloride exchange (*7*, *16*). Under alkaline conditions, anion binding and translocation depend on the integrity and the degree of protonation of groups with an apparent p*K* value of 12 (*7*, *16*). Moreover, using chemically modification, Weith *et al*. and Zaki showed that phenylglyoxal (*7*, *17*) and 1,2-cyclohexadione (*18*), respectively, which modify arginines, inhibit anion transport. They also suggested that one or two of the arginine residues modified were involved

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in band 3–mediated anion transport. These results indicated that arginine residues play a significant role in anion transport, however, these arginines were not identified.

In the present study, we have determined that Arg 901 can be modified with hydroxyphenylglyoxal (HPG), one of the PG derivatives that inhibit the anion transport by band 3 (*19*). Arg 901 is the most C-terminal arginine in the band 3 membrane domain (Gly 361–Val 911). We also examined the reactivity of band 3 to HPG when DIDS or DEPC was present. These reagents immobilize band 3 in the outward and inward conformations, respectively (*5*, *14*, *20*, *21*). Furthermore, to examine the role of Arg 901, we compared the kinetic parameters of anion transport using wild type and R901A mutant band 3 expressed in yeast membranes. Based on the results, we discuss the role of arginine 901 in the structure/function relationships in band 3–mediated anion exchange.

MATERIALS AND METHODS

Materials—TPCK-trypsin (sequence grade) and *N*-glycosidase were purchased from Roche Diagnostics (Mannleira, Germany). TFA (trifluoroacetic acid), HPG (*p*-hydroxy phenylglyoxal) 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 an 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 (*22*). Resealed ghosts were prepared according to the method of Kuma *et al*. (*23*). In brief, erythrocytes were washed with 165 mM KCl, and then lysed at 4°C in 40 volumes of 4 mM $MgSO_4$ and 1.2 mM acetic acid. Two min after lysis, ammonium acetate was added to a final concentration of 150 mM. Ghosts were collected by centrifugation and re-suspended in 7.5 ml of resealing buffer (200 mM sucrose, 30 mM ammonium acetate, and 10 mM sodium phosphate, pH 6 or pH 7.4). Suspensions were incubated for 30–90 min at 37°C, diluted with resealing buffer (pH 7.4), and then centrifuged for 10 min at 28,000 × *g*. Each pellet was diluted with the resealing buffer (protein concentration, 2 mg/ml). Resealing was confirmed by the trypsin susceptibility of 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 then the mixture was subjected to SDS-PAGE. The resealed ghosts used in the present experiments comprised more than 95% sealed right-side-out membranes.

Treatment of Ghosts with HPG—One milliliter aliquots of unsealed and resealed ghosts (1 mg/ml) were treated with 0–100 mM HPG for 1 h at 37°C and pH 7.4. Pre-incubation with DNDS or DEPC at each concentration was carried out at 37°C or 4°C for 30 min before HPG treatment. These HPG-treated ghosts were washed at 4°C three times with 6 volumes of resealing buffer (200 mM sucrose, 30 mM ammonium acetate, and 10 mM sodium phosphate, pH 7.4), and then washed three times with 5 mM $NaHCO_3$. The prepared ghosts were stored at 4°C and used on the same day.

Measurement of Phosphoenolpyruvate Transport Rate—The phosphoenolpyruvate (PEP) transport rate was determined by measuring the amount of internal PEP in resealed ghosts. The amounts of PEP were determined according to the method of Matuyama *et al*. (*24*). In brief, after pre-incubation at 37°C for 5 min, resealed ghosts were incubated with 30 mM PEP for 0, 15, 30 and 60 min at 37°C. Three hundred μl of each sample fraction was washed three times by centrifugation (6,000 × *g* × 1 min) with washing buffer (200 mM sucrose, 30 mM ammonium acetate, and 5 mM HEPES, pH 7.4) containing 100 μM DNDS, which prevents leaking of the PEP from resealed ghosts. The ghosts were de-proteinized with 0.6 M perchloric acid and then extracts were subjected to PEP assaying.

Preparation of Lysylendopeptidase Fragments of Band 3—To remove the NH_2-teminal 40-kDa domain of band 3, ghosts (1 mg/ml of protein) were pretreated with a low concentration of lysylendopeptidase (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. The ghosts (1 mg of protein in membranes) were treated with *N*-glycosidase F (2 U) in 200 μl of 20 mM phosphate buffer (pH 7.2) for 24 h at room temperature, and then washed three times with 5 mM $NaHCO_3$. The washed membranes were dissolved in 0.1 M Tris-HCl (pH 9) buffer containing 0.5% $C_{12}E_8$ in a total volume of 200 μl. Two hundred microliters of the protein solution (120 μg) was digested with 4 μg lysylendopeptidase in 0.1 M Tris-HCl (pH 9) containing 0.5% $C_{12}E_8$ for 2 h at 37°C.

HPLC Separation of Peptide Fragments—The peptides produced by lysylendopeptidase were directly applied to a HPLC system after centrifugation (13,000 × *g* × 10 min). Each sample (50 μl; 30 μg) was loaded onto a C_{18} RP-HPLC column (4.6 ID × 150 mm, Shiseido Capcel Pack™; ESA Biosciences, Inc., Chelmsford, MA). The peptides were separated by HPLC using a gradient of an aqueous solution (solvent A) and 2:1 (v/v) isopropanol/acetonitrile (solvent B), each containing 20 mM NH_4CO_3 (pH 9.0), at a flow rate of 400 μl/min. For determination of the HPG-binding site, the mobile phase composition was maintained at 1% B for 10 min, and then increased from 1% to 100% over 150 min and maintained at 100% for 40 min. For measurement of HPG-modification, the mobile phase composition was maintained at 1% B for 10 min, and then increased from 1% to 6% over 30 min and maintained at 6% for 10 min. The eluted peptides were monitored by the absorbance at 214 nm and 340 nm. We collected the peptide fractions using the reference peaks, and fractions were lyophilized.

Detection of DIDS-Modification—White ghosts were or were not pretreated with 12.5 mM HPG at pH 7.4 and 37°C for 1 h. The ghosts were treated at pH 7.4 and 37°C for 90 min with 10 μM DIDS in the dark. For detection of the DIDS-modified peptides, we used an RP-HPLC separation system under acidic conditions with TFA according to Abe *et al*. (*12*). In brief, the ghosts were dissolved in 0.1 M Tris-HCl (pH 9) buffer containing 0.5% $C_{12}E_8$ and then digested with 2 μg/ml TPCK-trypsin. After centrifugation (20,000 × *g* × 10 min), the digested peptides were directly applied to the RP-HPLC system. A sample was loaded onto a C_{18} RP-HPLC column (4.6 ID × 250 mm, Cosmosil® $5C_{18}$-AR-II; Nacalai Tesque, Inc., Kyoto, Japan). The peptides were separated by HPLC using a gradient of an aqueous solution (solvent A) and 2:1 (v/v) isopropanol/acetonitrile (solvent B), each containing 0.1% (v/v) TFA, at a flow rate of 1 ml/min. The mobile phase composition

was maintained at 5% (v/v) B for 5 min, and then increased from 5% (v/v) to 85% (v/v) over 60 min, and from 85% (v/v) to 100% (v/v) over 20 min. The eluted peptides were monitored by the fluorescence at 414 nm.

Amino Acid Sequencing and Mass Spectrometry—The peptides were analyzed with a gas phase sequencer (Applied Biosystems, model 492, Foster City, CA). The phenylthiohydantions (PTHs) were identified with an Applied Biosystems 140C PTH analyzer on line system. MALDI-TOF mass spectrometric analyses were performed using a Voyager RP spectrometer (Applied Biosystems). The acceleration voltage was set to 20 kV. Data were acquired in the positive linear mode of operation. Spectra were externally calibrated with calibration kits I and II (Applied Biosystems). The collected peptides were dissolved in 2:1 (v/v) isopropanol/acetonitrile and 0.1% TFA. The peptide solutions (0.5 μl) were mixed with 0.5 μl of matrix solutions (saturated sinapinic acid and α-cyano-4-hydroxycinnamic acid in 0.1% TFA and 50% acetonitrile in an aqueous solution, respectively). The theoretical mass of each peptide was calculated using the protein prospector on the World Wide Web (http://prospector.ucsf.edu/).

Construction of an Expression Vector—Band 3 expression vector pYES-FHSHS was prepared by modification of the pYES vector (Invitrogen Co., Carlsbad, CA), which contains the Ura3 and GAL1 promotor. Using the pYES-FHSHF vector, we could add the Flag-tag, His-tag, Strep-Tag, His-tag, and Frag-tag (FHSHF) sequences to band 3 at the N-terminal. DNA fragments encoding amino acids 361–911 of band 3 were amplified by PCR. The band 3 fragments were digested with *Bam*HI and *Eco*RI, and then ligated into pYES-FHSHS. The R901A mutant constructs were obtained by the previously described method involving overlap extension (*25*). For co-expression of glycophorin A, we used vector pGAD-C1 containing the LEU2 and ADH1 promotor (*26*). DNA fragments encoding the amino acids of glycophorin A were amplified by PCR, and we prepared the DNA construct of Δ68–70 GPA, in which the amino acids from Phe68 to Glu70 were removed using the mega-primer method (*27*). The Δ68–70 GPA fragment was digested with *Bam*HI and *Eco*RI, and then ligated into pGAD-C1 after removal of the GAL4 activation domain (AD). The entire coding sequence of each vector was verified by DNA sequencing.

Expression of Band 3 on the Yeast Plasma Membrane—For anion transport studies, the pYES-FHSHS vector containing the DNA encoding wild type or R901A band 3 was co-transfected into *Saccharomyces cerevisiae* strain BY4743 (MATa/α ura3/ura3 leu2/leu2 his3/his3 lys2/LYS2 met15/MET15) with vector pGAD-C1 containing the DNA encoding Δ68–70 GPA. The transformants were selected by pre-culture at 30°C for 20 h in the leucine and uracil minus medium containing 0.67% yeast nitrogen base, 1% succinate, 0.6% NaOH, 2% raffinose, and 0.08% amino acid mix. After the pre-cultured cells had been diluted to 1/50 with the leucine and uracil minus medium, the cells were cultured for 30°C until the optical density at 600 nm (OD_{600}) became 0.7 (about 4 h). To induce band 3 expression, 2% (w/v) galactose, final concentration, was added to the culture medium, and then the cells were cultured at 30°C for 6 h. We measured the proportion of band 3 that moved to the yeast plasma membrane surface using a protease accessibility assay (*28*). In brief, 1 ml of the cultured medium containing cells after band 3 induction was centrifuged by $1,000 \times g$ for 10 min. After the centrifugation, we added 5 μg chymotrypsin and 1 ml of 0.1 M Tris-HCl (pH 8.0) containing 1.2 M sorbitol to the precipitated cells, and then incubated them at 4°C for 1 h. After washing three times with 0.1 M Tris-HCl (pH 8.0) containing 1.2 M sorbitol and 2 mM PMSF, the chymotrypsin-treated cells were incubated with 1% 2-mercaptoethanol and 240 mM NaOH, final concentrations. Finally, we precipitated the proteins using TCA and acetone, and then analyzed them by SDS-PAGE. The proteins were detected using ECL-stained immunoblots with anti-Flag antibodies for band 3 and anti-Vma2p antibodies for Vma2p. The gel images were visualized using a phosphorimager (FLA2000; Fuji, Tokyo, Japan). Quantitation was performed using MacBAS software (Fuji).

Sulfate Transport Assay—Aside from checking the expression level of band 3, we prepared cells for the sulfate transport assay from the same culture. After induction of band 3, the cultured cells were concentrated by centrifugation ($1,000 \times g \times 10$ min), and then resuspended at OD_{600} = 10 in 5 mM HEPES buffer (pH 6.0) containing 200 mM sucrose, 25 mM gluconate, 25 mM citrate and 1 mM Na_2SO_4. To 200 μl samples of the cell suspension, either 1 μl of water or 1 μl of 2 mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) was added to give a final concentration of 100 μM in each tube. After incubation for 10 min at 30°C, the assay was started by adding 10 μl of a 5 to 160 mM Na_2SO_4, solution, each containing 7.5 μCi/ml of $S^{35}O_4^{2-}$ (Amersham Biosciences Corp., Piscataway, NJ), to the cell mixture. The influx of $S^{35}O_4^{2-}$ was usually measured after 5 min at 30°C for triplicate samples. The cells were washed rapidly three times by adding 1 ml of distilled water using a glass filter. The cells were collected and resuspended in Clear-sol I solution (Nacalai) and then the radioactivity was measured by scintillation counting (Liquid Scintillation Counter LSC-5100; Aloka, CO., LTD., Tokyo, Japan).

Analytical Procedures—Sodium dodecyl sulfate–polyacrylamide gel electrophoresis for protein and peptide analyses was performed according to the methods of Laemmli (*29*), and Kawano and Hamasaki (*30*). Protein concentrations were determined by the method of Lowry *et al*. (*31*), using BSA as the standard.

RESULTS

HPG Inhibited the Band 3–Mediated Anion Transport—It is known that Arg residue(s) within the band 3 molecule are involved in band 3–mediated anion transport (*7*, *9*). The anion transport activity is specifically inhibited by treating red blood cells with phenylglyoxal (*7*, *16*, *17*). In this study, we treated erythrocyte membranes (ghosts) with HPG, a derivative of phenylglyoxal, and then examined the phosphoenolpyruvate (PEP) transport in resealed ghosts treated with HPG (*19*). PEP is a substrate for the band 3–mediated anion transport system (*24*). As shown in Fig. 1, the band 3–mediated PEP transport was inhibited on treatment of resealed ghosts with HPG in an HPG-dependent manner (Fig. 1). The Ki of HPG calculated from Fig. 1 was 12.5 mM, which was 5-fold higher than that of PG (*32*). The binding constant of PG at pH 8 was 5-fold

greater than that of HPG (*19*). This is consistent with the results of previous PG and HPG experiments (*19*, *32*).

Identification of HPG-Modified Arginines—To identify the HPG-susceptible arginines, white ghosts were incubated with 12.5 mM HPG at pH 7.4 and 37°C for 1 h. Membranes treated with HPG were solubilized with 0.5% $C_{12}E_8$ and then digested with lysylendopeptidase. Solubilized and digested membrane fragments were separated by RP-HPLC. Arginine residues modified with HPG show 340-nm absorbance under weak alkaline conditions (*33*). Thus, each HPLC-fraction was monitored at 340 nm. Figure 2A shows a typical elution pattern on monitoring at 340 nm. The major peak (retention time, 35 min) indicated by an arrow in Fig. 2A must contain arginine residues modified with HPG. The peak fraction was subjected to further analysis by amino acid sequencing and MALDI-TOF mass spectrometry. The amino acid sequence from the N-terminus of the peak was ATFDEEEGR, and the molecular masses were 2,203.6 and 2,335.5 (Fig. 2B). This indicated that the fraction comprising the major peak corresponded to the sequence from Ala 893 to Val 911 of band 3 (ATFDEEEGR901….), and that Arg 901 was the arginine modified with HPG. The two molecular masses were 2,202.1 and 2,334.2, respectively.

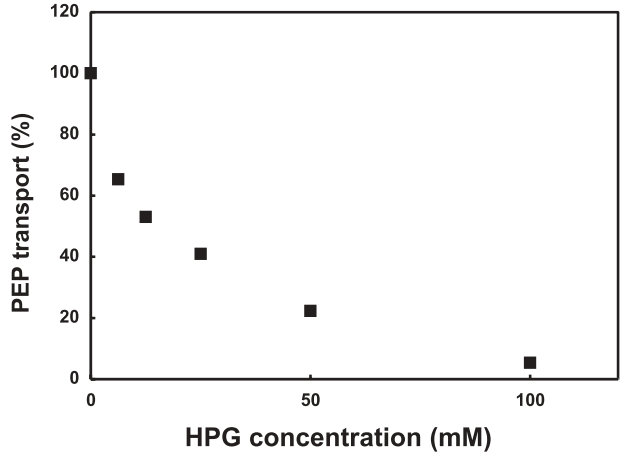

Fig. 1. **Inhibition of transport activity by HPG-modification.** Resealed ghosts were pretreated at pH 7.4 and 37°C for 1 h in the presence or absence of 6.25, 12.5, 25, 50, or 100 mM HPG. The phosphoenolpyruvate (PEP) transport rate was determined by measuring the amount of internal PEP in the resealed ghosts.

The concentration-dependence of HPG-modification at Arg 901 is shown in Fig. 3. The modified and unmodified peptide peaks were traced at 210-nm absorbance (dashed line), and the HPG-modified peptide peak was traced at 340-nm absorbance (solid line). The elution patterns indicated that the HPG-modified peptides (retention time, 35 min; the peaks indicated by arrows) were eluted after the unmodified peaks (retention time, 34 min). As shown in Fig. 3, the HPG-modified peaks increased depending with the HPG concentration. Simultaneously, the unmodified peaks decreased as the HPG concentration increased (Fig. 3). Since each peak was single, HPG-modification was calculated as the intensity ratio between unmodified and modified peaks. The HPG-modification at Arg 901 corresponded quantitatively with the inhibition ratio for PEP transport, as shown in Fig. 4. HPG-modification and PEP transport inhibition are completely consistent. Therefore, we concluded that HPG-modification of Arg 901 caused inhibition of anion transport.

HPG-Modification in the Presence of DNDS or DEPC—Stilbene compounds such as DIDS, DNDS and H_2DIDS inhibit anion transport activity, and bind preferentially to and fix band 3 in the outward facing conformation (*11*, *20*, *21*). DEPC-modification also inhibits anion transport across the erythrocyte membrane, however, DEPC-modification leads to decreased H_2DIDS modification of the erythrocyte membrane and DEPC-modification of the erythrocyte membrane decreases in the presence of DNDS in a concentration-dependent manner (*5*). These findings indicated that DEPC modification fixes band 3 in the

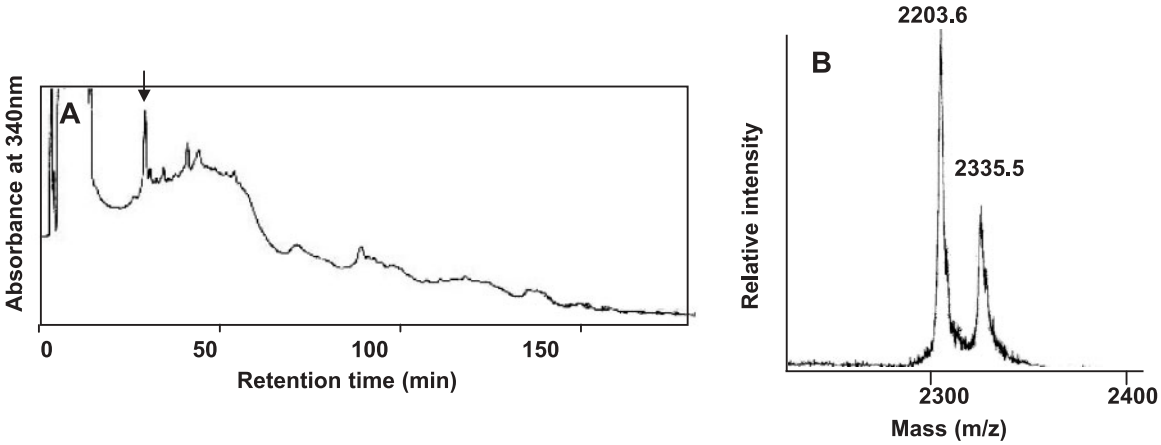

Fig. 2. **HPG-modified peptide identification.** (A) RP-HPLC profiles of the peptide fragments obtained from HPG-modified-membranes with detection at 340 nm. White ghosts were treated at pH 7.4 and 37°C for 1 h with 12.5 mM HPG. The ghosts were dissolved in 0.1 M Tris-HCl (pH 9) buffer containing 0.5% (v/v) $C_{12}E_8$ and digested with 2 μg/ml lysylendopeptidase. The digested peptides were separated by RP-HPLC using a gradient of 20 mM ammonium carbonate and 2:1 (v/v) isopropanol/acetonitrile at a flow rate of 400 μl/min. The indicated peak at 35 min contains the HPG-modified peptides. (B) MALDI-TOF MS of the indicated peak fraction. The two molecular masses were 2,202.1 and 2,334.2, respectively. The mass peaks indicate that the fraction contains the peptide comprising Ala 893 to Val 911 of band 3 and the peptide modified by HPG. The peptide contains Arg 901.

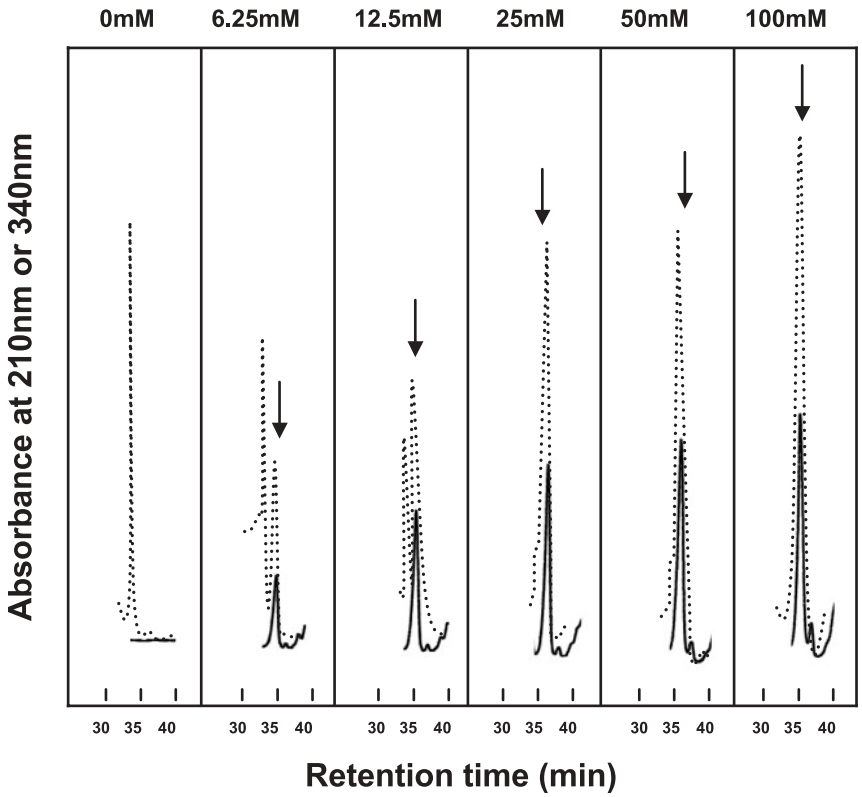

Fig. 3. **HPG concentration-dependence of the HPG-modified and unmodified peptides with the RP-HPLC system.** Resealed ghosts were treated at pH 7.4 and 37°C for 1 h in the presence or absence of 6.25, 12.5, 25, 50, or 100 mM HPG. The solid lines indicate the HPLC profile on monitoring at 340-nm absorbance and the dashed lines the HPLC profile on monitoring at 210-nm absorbance. The arrow indicates the peak of the HPG-modified peptide. The HPG-modified peak increased depending upon the HPG concentration.

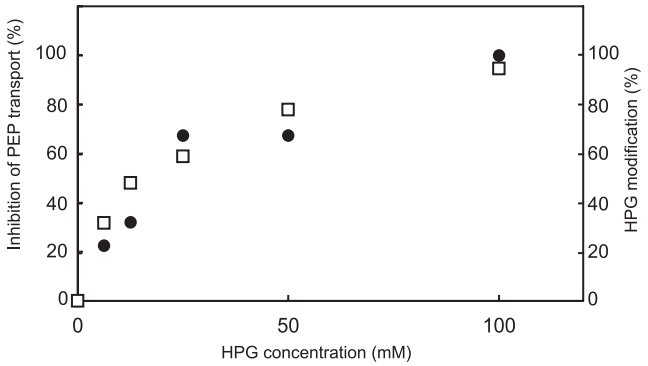

Fig. 4. **HPG-modification at Arg 901 corresponded quantitatively with the ratio of inhibition of PEP transport.** HPG-modification at Arg 901 was calculated from the peak intensity under each condition in Fig. 3 (closed circles). Inhibition of PEP transport activity at pH 7.4 (open squares) was estimated from Fig. 1. HPG-modification and PEP transport inhibition are completely correlated.

inward facing conformation (*5*, *14*). Peptide analysis showed that H_2DIDS modified Lys 539 and Lys 851 (*11*), and that only His 834 was modified with DEPC (*14*). Therefore, the reagent accessibility of these amino acids reflected the conformation change between inward facing and outward facing.

Thus, we attempted to determine the effect of DNDS or DEPC on HPG reactivity with Arg 901. HPG-modification of Arg 901 in the presence of 0–1 mM DNDS or 0–1 mM DEPC is shown in Fig. 5. It was monitored at 340 nm by RP-HPLC and we calculated HPG-modification as the peak intensity indicated by arrows. As the DNDS concentration increased, the peak intensity decreased (Fig. 5A, upper panel). In the presence of 1 mM DNDS, HPG-modification was 80% suppressed. On the other hand, even in the presence of 1 mM DEPC, the peak intensities of HPG-modification peaks did not change. Thus, DEPC had no effect on HPG-modification (Fig. 5A, lower panel). Furthermore, we examined the reactivity of DIDS with HPG pre-treated membranes. In band 3 in native membranes, DIDS modifies only Lys 539 (*11*, *12*). The DIDS-modified band 3 was digested with TPCK-trypsin and the modified peptide could be monitored by RP-HPLC using 430-nm fluorescence (*12*). Using this monitoring system, we attempted to detect the DIDS-modification in HPG untreated or pre-treated membranes. A typical elution pattern of the DIDS-modified peptides in HPG untreated membranes is shown in Fig. 6A. We obtained the indicated single peak at a retention time of 80 min. On the other hand, for the HPG pre-treated membranes, we could not detect such a peak (Fig. 6B). Therefore, HPG pretreatment prevents DIDS modification of Lys 539.

Kinetic Analysis of Anion Transport by the R901A Mutant in the Yeast Expression System—To further examine the role of Arg 901 in anion transport, we expressed the band 3 transmembrane domain (amino acids 361–911) as a

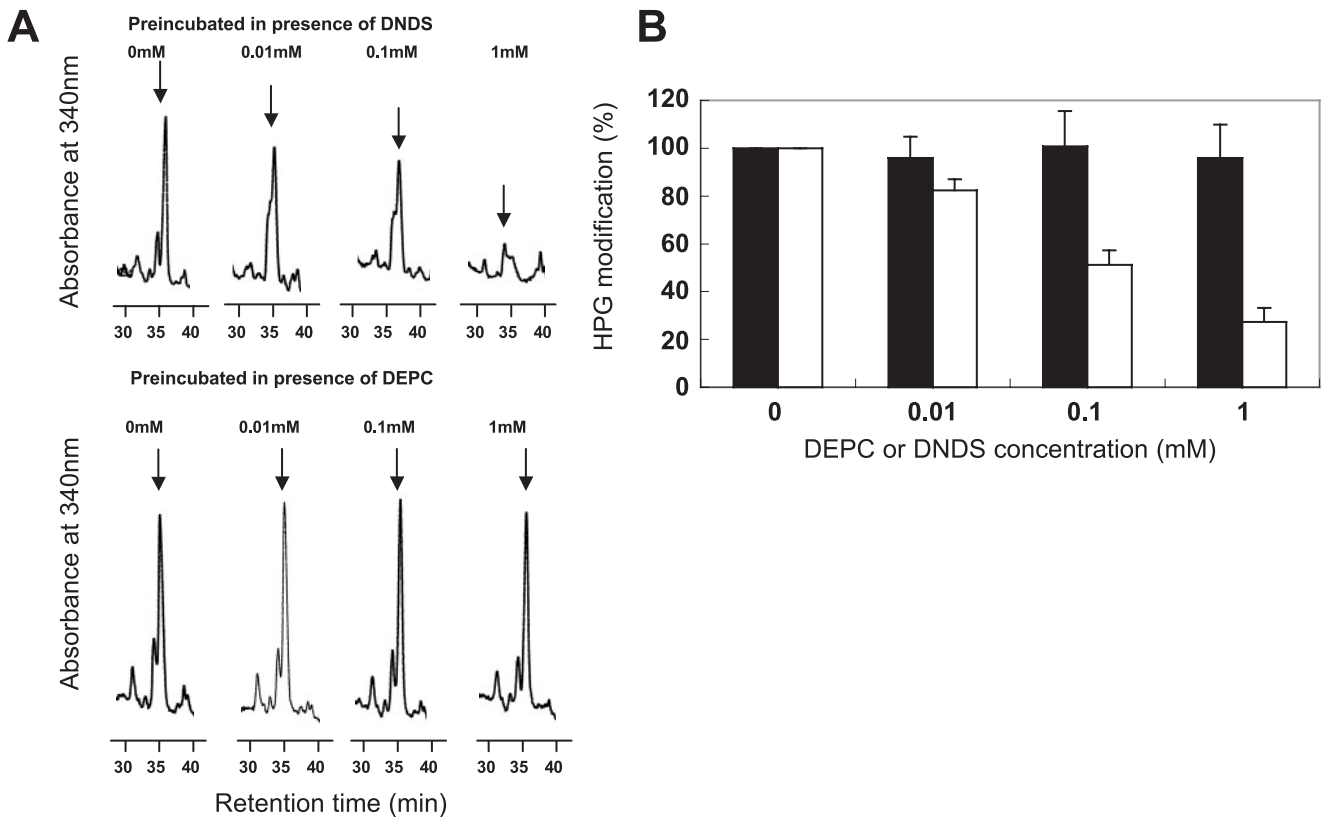

Fig. 5. **DNDS and DEPC concentration-dependence of HPG reactivity at Arg 901.** (A) RP-HPLC profiles for HPG-modified peptides under the conditions in the presence of DEPC or DNDS. White ghosts were pre-treated without or with 0.01, 0.1, 1 mM DNDS, or DEPC in the dark. HPG-modification, lysylendopeptidase digestion, and HPLC were performed under the conditions given in the Fig. 2 legend. The peaks indicated by arrows were derived from the HPG-modified peptide. The peak intensity decreased with increasing concentration of DNDS but not DEPC. (B) The HPG-modification at Arg 901 under each condition was calculated as the ratio of the peak intensities for the HPG-modified and unmodified peptides. The filled and open bars indicate HPG-modification in the presence of DEPC and DNDS, respectively. Values are expressed as means ± SE obtained for three experiments.

recombinant protein in the yeast plasma membrane, and then analyzed the kinetic parameters of band 3 containing the R901A mutation. Band 3 was incorporated into a plasmid (FHSHS-pYES2) that has the FHSHS sequence tag (this sequence contains the Flag-tag, His-tag, Strep-Tag, His-tag, and Frag-tag sequences) for detection and purification. The plasmid bearing band 3 cDNA was transfected into *Saccharomyces cerevisiae* and then co-transfected with the plasmid (pGAD-C1) bearing GPA cDNA. GPA is known to enhance the surface presentation of band 3 in *Xenopus* oocytes (*34*). In addition to this, we used GPA of which Phe 68–Glu 70 was deleted because the deletion mutant eliminates the enhancing effect of GPA on the anion transport activity of band 3 (*35*). After transformation of the co-plasmid, growth of cells, and induction of band 3 by galactose addition, we examined the expression in yeast. Figure 7A shows the results as to the expression of the wild type (lane 1), R901A (lane 5), and the empty vector, as a control (lane 3). The transmembrane domain of band 3 was expressed in all except the cells containing the empty vector (Fig. 7A, lanes 1 and 5). The levels of expression for the wild type and R901A were not very different.

Furthermore, we measured the proportion of band 3 that moved to the yeast plasma membrane surface using a protease accessibility assay (*28*). Band 3 has a single site susceptible to extracellular chymotrypsin cleavage, which is located between TM5 and TM6. Therefore, the expressed band 3 was treated with chymotrypsin. The 22-kDa N-terminal fragment generated on extracellular cleavage of band 3 was separated from the uncleaved band 3 located within the cells by SDS-PAGE. Using cytoplasmic protein Vma2p (*36*) as a control, we also confirmed that cellular proteins were not affected by extracellular chymotrypsin. Immunoblotting with anti-flag antibodies was performed to detect and estimate the relative proportions of 22-kDa (surface band 3) and intact band 3 (internal band 3) in the cells. Figure 7A shows the results of protease accessibility assaying of the wild type (lanes 1 and 2), R901A (lanes 5 and 6). On estimation of the density of bands on SDS-PAGE, the protease cleavage was found not to be different. This finding indicates that the R901A mutation does not affect band 3 surface presentation on the plasma membrane. From estimation of the density of the bands, each band 3 was expressed at the level of 0.04 mg (in 1 liter culture) on the plasma membrane surface (Fig. 7A). This was a sufficient amount to examine sulfate transport in the cells, as described below.

The sulfate transport by the wild type and R901 mutant were measured as the DIDS-sensitive sulfate uptake. After DIDS treatment, about 25% of the sulfate uptake remained (data not shown). This remaining uptake was not included in the kinetic parameter calculation as DIDS-insensitive

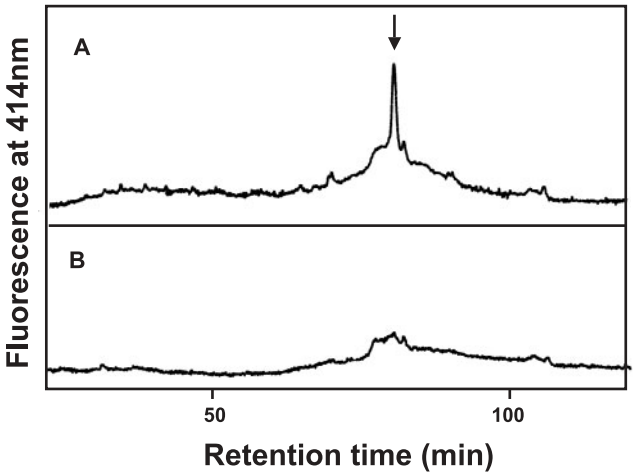

Fig. 6. **DIDS reactivity depends on HPG-modification.** A: Typical RP-HPLC profile of the DIDS-treated band 3 peptide. Ghosts were treated with 10 μM DIDS in the dark. The DIDS treated ghosts were digested with TPCK-trypsin. The digested peptides were separated by RP-HPLC using a gradient of an aqueous solution and 2:1 (v/v) isopropanol/acetonitrile, each containing 0.1% (v/v) TFA, at a flow rate of 1 ml/min. The DIDS-modified peptide was monitored at 414-nm fluorescence and was eluted at 80 min (indicated by an arrow). B: RP-HPLC profile of the HPG pre-treated and DIDS treated band 3 peptide. White ghosts were pre-treated with 12.5 mM HPG and then the HPG pre-treated ghosts were treated with 10 μM DIDS. After HPG treatment, we could not confirm the presence of DIDS-modified peak.

sulfate uptake. Figure 7B (upper) shows the DIDS-sensitive sulfate uptake by each cell type against the total sulfate concentration. DIDS-sensitive sulfate uptake was suppressed in the cells containing the empty vector. However, sulfate specifically incorporated into the band 3–expressing cells depended on the total sulfate concentration (Fig. 7B). On comparison of the sulfate incorporation between wild type- and R901A-expressing cells, the amount of incorporated sulfate for R901A was found to be small at each concentration (Fig. 7B). Therefore, R901A impairs sulfate uptake.

We further estimated the kinetic parameters of R901A and the wild type (Table 1) from the sulfate incorporation data. Using the DIDS-sensitive sulfate uptake and the amount of band 3 surface expression, we calculated the Km and Vmax of the wild type or R901A mutant band 3 on the yeast membrane. A [S/V]–[S] plot is shown in Fig. 7C (S means the total sulfate concentration and V means the sulfate influx velocity calculated from the sulfate influx at each sulfate concentration). According to the linearity of each plotted point, r^2 was 0.99–0.95 in the [S/V]–[S] graph in these experiments, therefore, the kinetic parameters are reliable (Fig. 7C). In addition to that, the K_m (24.5 ± 2.5 mM) and Vmax (5.7 ± 0.9 μmol/min/mg) values for the wild type were the same as the values previously estimated for band 3 in red blood cells (23 mM and 5.8 μmol/min/mg, respectively; Ref. *37*). Based on the reliablity of the measurements, we compared the kinetic parameters of the wild type with those of R901A. The results indicate that the R901A mutation had no effect on the K_m value (26.7 ± 1.2 mM), but that the V_{max} value (2.6 ± 0.2 μmol/min/mg) was 55% decreased.

Table 1. **Kinetic parameters of the wild type and R901A band 3 expressed on yeast plasma membranes.**

Band 3	K_m (mM)	V_{max} (μmol/min/mg)
Wild type	24.5 ± 2.5[a]	5.7 ± 0.9
R901A	26.7 ± 1.2	2.6 ± 0.2

[a]Values are expressed as means ± SE obtained for three experiments.

DISCUSSION

Band 3–mediated anion transport is inhibited by chemical modification reagents reactive with lysine, glutamate, histidine, and arginine residues (*5–9*). Although the susceptible lysine, glutamate, and histidine residues have already been determined (*10–15*), the arginine residues remained unidentified until now. In this study, we monitored the HPG-modified peptides by RP-HPLC and identified the band 3 peptide fragment comprising Ala 893 to Val 911, which contains only one arginine, Arg 901. Therefore, we determined that the HPG-modification occurred at Arg 901. We could not directly recover the peptide modified with HPG on MALDI-TOF mass spectroscopy and amino acid sequencing, because HPG-modified arginine is easily regenerated to arginine under these analytical conditions (*38*). However, we showed clearly that the inhibition of PEP transport activity was completely consistent with HPG-modification (Fig. 4). In addition to this, the mutation replacing Arg 901 with alanine impaired sulfate uptake. Therefore we confirmed that Arg 901 has a functional role in band 3–mediated anion transport activity.

Stilbene compounds inhibit band 3–mediated anion transport activity, and bind preferentially to and fix the outward face conformation, and peptide analysis showed H_2DIDS modified Lys 539 and Lys 851 (*11*, *20*, *21*). DEPC-modification leads to decreasing H_2DIDS modification of the membrane and DEPC-modification of the erythrocyte membrane decreased in the presence of DNDS in a concentration-dependent manner. These results indicated that DEPC-modification of His 834 fixes the inward face conformation (*5*, *14*). In this study, we found that HPG-modification at Arg 901 was also protected by DNDS binding (Fig. 5), and that DIDS-modification was prevented by HPG-modifications (Fig. 6). Thus, we can consider that Arg 901 is involved in the local conformational change between the outward facing and inward facing structures. Moreover, the R901A mutation did not affect the K_m value, but the V_{max} value decreased compared to in the wild type. The constant K_m value means that the mutation had no effect on the anion affinity to the anion binding site in the band 3 structure. However, the V_{max} value was decreased by the single mutation. The ping-pong model indicates that anion transport is regulated through a single conformational change in band 3 (*3*). This conformational change leads to the transfer of a single substrate anion across the membrane. The rate of anion transport depends on the rate of the conformational change that leads to the translocation of the bound anion. Therefore, we assume that the R901A mutation leads to impairment of the conformational change and thereby causes the decreased Vmax value. The chemical modification and mutagenesis results indicates that Arg 901 has a role in the conformational change necessary for anion transport.

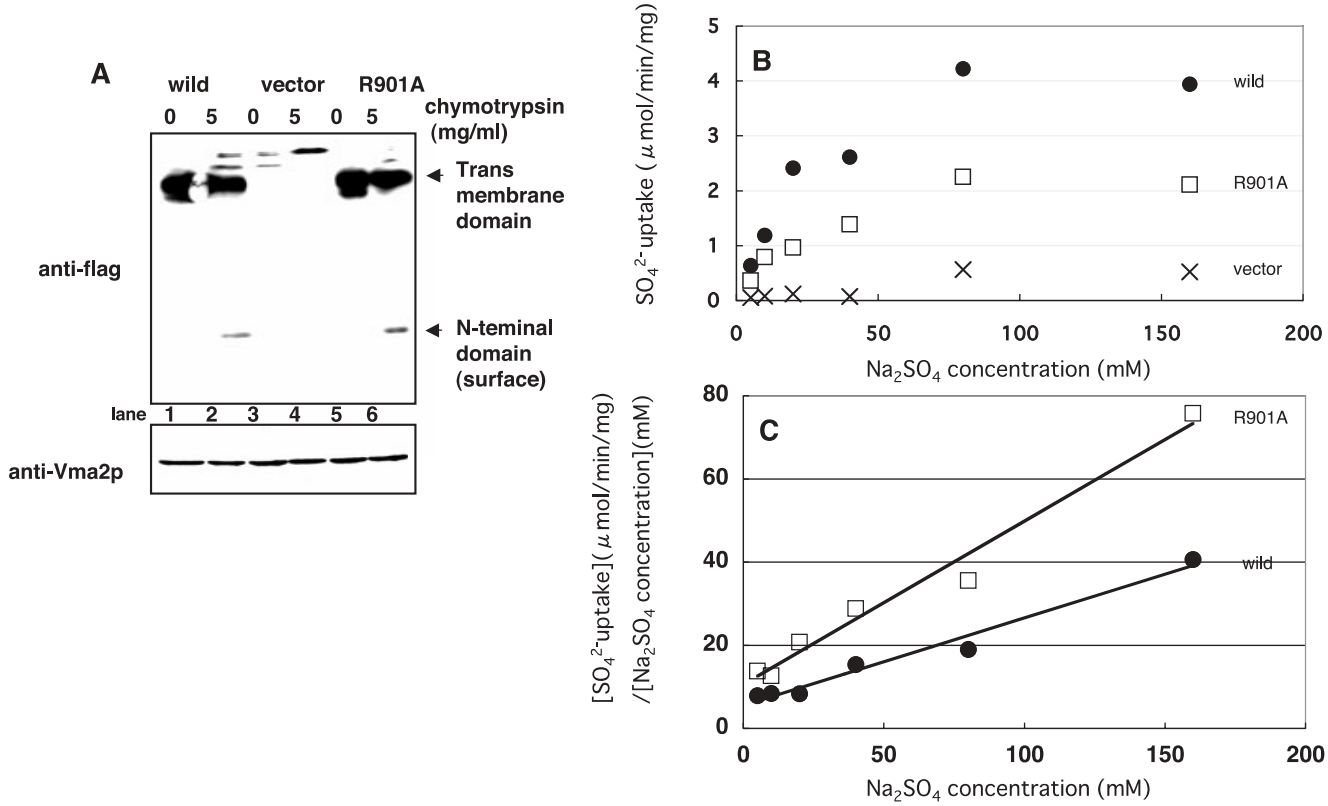

Fig. 7. **Expression of and sulfate uptake by the R901A mutant.** (A) Wild type and R901A mutant band 3 expression in yeast. The expression levels were estimated by Westernblotting with anti-flag antibodies. The total expression levels of R901A was the same as that of the wild type (lanes 1 and 5). The protease accessibility assay (see under "MATERIALS AND METHODS") showed the R901A mutation does not affect band 3 presentation on the plasma membrane (lanes 2 and 6). From estimation of the density of bands on SDS-PAGE, each band 3, 0.04 mg (in 1 liter culture), expressed on the plasma membrane surface. (B) Sulfate concentration–dependency of sulfate influx into band 3–expressing yeast cells. The cells containing the empty vector (crosses), the wild type-expressing cells (closed circles) and R901A-expressing cells (open squares) were grown for 6 h at 30°C. (C) The linearity of the [S/V]–[S] plot of sulfate influx. r^2 calculated from the [S/V]–[S] linear plot was 0.99–0.95. The [S/V]–[S] plot graphs were calculated with the sulfate influx by wild type–expressing cells (closed circles) and R901A-expressing cells (open squares).

DEPC-modification to His 834 on the intracellular side of TM 13 also induced the inward face conformation (*5*, *14*). Glu 681 on the intracellular side of TM 8 was also hidden on DNDS binding (*6*). The arginine and lysine residues concentrated around the TM 1 region, such as Arg 384, Arg 387, Arg 388, Arg 389, Arg 432, and Lys 430, were resistant to protease on SITS binding (*12*). These previous results indicated that these residues have a significant role in the conformational changes that occur during band 3–mediated anion exchange. Previous results suggested that subdomains are present within the transmembrane domain of band 3, and these subdomains comprise TM1-5, TM6-8, TM9-12 and TM13-14 (*39–41*). Incorporating these results, our proposed model of the conformational change in band 3 is shown in Fig. 8. We suggest that the subdomains TM1-5, TM6-8 and TM13-14 are mobile, and that the represented arginine, lysine, histidine, and glutamate residues are involved in the conformational change between the outward and inward facing conformations.

The C-terminal region around Arg 901 of band 3 on intact erythrocyte membranes was resistant to proteases. This region was only digested by protease after alkali treatment (pH 13) when band 3 is partial denatured (*42*, *43*). This indicated that the C-terminus of band 3 was involved in peptide–peptide interactions. Regions of this type (classified as "category 2") are shielded from the lipid bilayer by other membrane peptide portions and peptide–peptide interactions (*41*, *42*). These portions are more flexible than typical transmembrane segments that interact with lipids, and are important for transporter mechanisms induced by conformational changes (*44*). The arginine and lysine residues influenced by SITS binding (*12*), and other chemical inhibitor susceptible residues of band 3, Lys 539 (*11*, *12*), Lys 851 (*11*, *15*), Glu 681 (*13*), and His 834 (*14*), were also concentrated in category 2 peptide portions (*41*, *42*). In this report, we further show that Arg 901 is the HPG-modification site, located on the "category 2" peptide portion, and that this portion has a significant role in the conformational change involved in the anion transport mechanism.

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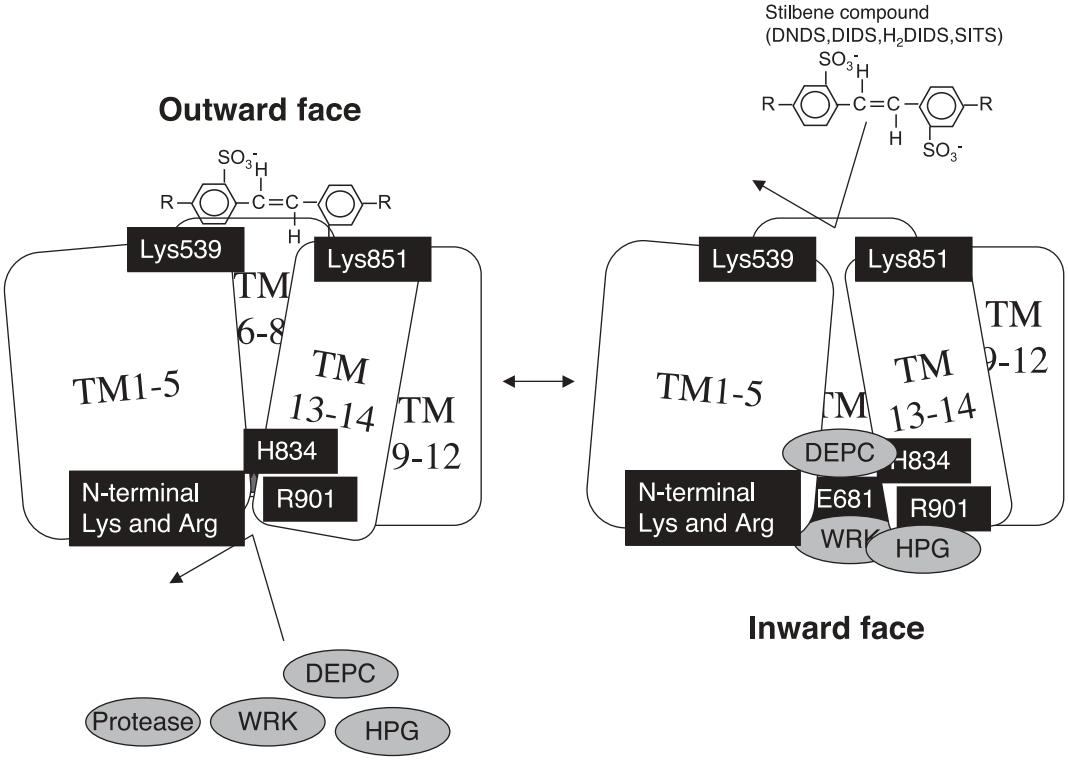

Fig. 8. **The proposed model of band 3 conformational changes.** Previous results indicate that the subdomains comprise TM1-5, TM6-8, TM9-12, and TM13-14, respectively. DNDS-binding prevented HPG-modification at Arg 901 and HPG-modification prevented DIDS modification (Figs. 5 and 6). DEPC-modification to His 834 also induced the inward face conformation. Glu 681 was also hidden on DNDS binding. The arginine and lysine residues in the N-terminal region of the 55-kDa domain, such as Arg 384, Arg 387, Arg 388, Arg 389, Arg 432, and Lys 430, were resistant to trypsin digestion on SITS binding. This evidence indicates that these residues have a significant role in the conformational changes that occur during the band 3–mediated anion exchange.

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