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Arg 901 in the AE1 C-terminal tail is involved in conformational change but not in substrate binding

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ABSTRACT

In our previous paper, we demonstrated that Arg 901 in the C-terminal tail of human AE1 (band 3, anion exchanger 1) had a functional role in conformational change during anion exchange. To further examine how Arg 901 is involved in conformational change, we expressed various Arg 901 mutants and alanine mutants of the C-terminal tail (from Leu 886 to Val 911) on the plasma membrane of *Saccharomyces cerevisiae* and evaluated the kinetic parameters of sulfate ion transport. As a result, Vmax decreased as the hydrophobicities of the 901st and peripheral hydrophilic residues increased, indicating that the hydrophobicity of the C-terminal residue is involved in the conformational change. We also found the alkali and protease resistance of the C-terminal region after Arg 901 modification with hydroxyphenylglyoxal (HPG) or phenylglyoxal (PG), a hydrophobic reagent. These results suggested that the increased hydrophobicity of the C-terminal region around Arg 901 leads to inefficient conformational change by the newly produced hydrophobic interaction.

1. Introduction

Anion exchanger 1 (AE1 or band 3) facilitates the electro-neutral exchange of Cl^- for HCO_3^- across the plasma membrane as part of the process of CO_2 transport in the blood [1,2]. Human AE1 consists of 911 amino acids and has two structurally and functionally distinct domains [3]: a 40-kDa cytoplasmic domain (1–360) and a 55-kDa membrane domain (361–911). The cytoplasmic domain interacts with cytoskeleton proteins and maintains an erythrocyte shape. The 55-kDa membrane domain penetrates the membrane 12–14 times and is responsible for anion exchange activity. Recently, the three-dimensional structure of the outward-facing conformation of the membrane domain was solved at 7.5 Å by electron microscopy [4,5]. However, the relationship between structure and function was still unsolved.

Kinetic studies showed that one-by-one exchange mediates the anion exchange of AE1, as the "ping-pong" model indicates [6]. This model suggests that a single substrate anion is transported through a single conformational change between the inward-facing and outward-facing conformations. Furthermore, studies of chemical modification and mutation suggested that some Lys, Glu, and His residues are important for anion exchange [7–13].

Some studies suggested that arginine is also an important residue for anion exchange. Wieth et al. [14,15] showed that chloride exchange depended on the protonation of residue with an apparent pK value of 12. In addition, anion transport was inhibited by arginine-specific reagents such as phenylglyoxal (PG) [16], 1,2-cyclohexanedione [17], and hydroxyphenylglyoxal (HPG) [18]. Our previous protein chemical study revealed that HPG specifically modified Arg 901, which is the most C-terminal arginine in the membrane domain [19]. The HPG modification prevented the reaction of 4,4-diisothiocyanostilbene-2,2-disulfonic acid (DIDS), which binds preferentially to the outward-facing conformation and thus inhibits conformational change [7,20,21]. These findings suggested that the modification of Arg 901 by HPG fixed AE1 to the inward-facing conformation. Moreover, the Vmax value of R901A, by which Arg 901 was mutated to alanine, decreased whereas the Km value did not change. Therefore, we concluded that Arg 901 played an important role in conformational change rather than in

Abbreviations: AE1, anion exchanger 1; $C_{12}E_8$, octaethyleneglycol monododecyl ether; DEPC, diethylpyrocarbonate; DIDS, 4,4-diisothiocyanostilbene-2,2-disulfonic acid; GPA, glycophorin A; HPG, hydroxyphenylglyoxal; PG, phenylglyoxal; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; TM, transmembrane spanning portion

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anion binding. However, it was not clear how most C-terminal Arg is involved in conformational change.

In the present study, Arg 901 was mutated into various amino acids and its peripheral residues into alanine. We then evaluated the kinetic parameters of these mutants. In addition, we also examined the alkali and protease sensitivity of the C-terminal region by hydrophobic modification with HPG or PG. The results demonstrated that the increased hydrophobicity of the C-terminal tail in AE1 leads to more rigid structure and thus inefficient conformational change.

2. Materials and methods

2.1. Materials

Anti-Vma2p antibody was purchased from Molecular Probes, Inc. (Eugene, OR). The BY4743 strain was purchased from Invitrogen (Carlsbad, CA). Other chemical reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Expression of AE1 on the yeast plasma membrane

The membrane domain (Gly 361~Val 911) of AE1 mutants, which contain Arg 901 mutants (R901K, R901E, R901A, R901M, R901C, R901I) and alanine mutants of peripheral residues in the C-terminal region (L886A, D887A, D889A, D890A, K892A, T894A, F895A, D896A, E897A, E898A, E899A, G900A, D902A, E903A, Y904A, D905A, E906A, V907A, M909A, P910A, V911A) were constructed [22,23] and expressed on the yeast membrane according to the previous papers [11,19]. In belief, The pYES2-FHSHS and pGAD-C1 vectors co-transfected into *Saccharomyces cerevisiae* strain BY4743 were cultured in leucine/uracil minus medium containing 0.67% yeast nitrogen base, 1% succinate, 0.6% NaOH, 2% raffinose, and 0.08% amino acid mix. When the optical density at 600 nm became 0.7, 0.5–2% (w/v) galactose was added to the cultured medium to induce AE1 expression. The yeasts were collected after 6 h. Yeasts of the same culture were separated for protease accessibility and sulfate transport assays in each independent experiment.

2.3. Protease accessibility assay

The protease accessibility assay was used to measure AE1 expression on the plasma membrane [19,24]. In brief, we treated the collected cells with 0.1 M Tris–HCl (pH 8.0) containing 1.2 M sorbitol and 5 mg/ml chymotrypsin at 4 °C for 1 h. The chymotrypsin-treated cells were washed three times with 0.1 M Tris–HCl (pH 8.0) containing 1.2 M sorbitol and 2 mM phenylmethanesulfonyl fluoride (PMSF) and were incubated with 1% 2-mercaptoethanol and 240 mM NaOH at 4 °C for 1 h. The proteins were precipitated using trichloroacetic acid (TCA) and acetone, and then were immunoblotted with anti-Flag antibodies and anti-Vma2p antibodies. The gel images were visualized using a phosphorimager (FLA2000; Fuji, Tokyo, Japan). Quantitation was performed using MacBAS software (Fuji). By comparing the band density between the AE1 expressed in the yeast and the AE1 purified from the erythrocyte membrane, the absolute amount of AE1 expressed in the yeast was calculated. And we measured the density of the 22-kDa N-terminal fragment bands, which were produced by the cleavage of extracellular chymotrypsin. From the density of 22-kDa fragments, we estimated the AE1 expression on the plasma membrane. The AE1 cleavage was complete under these conditions because longer chymotrypsin treatment did not increase the amount of the cleaved 22-kDa fragments (data not shown).

2.4. Sulfate transport assay

A sulfate transport assay was carried out as described previously [11,19]. In brief, the collected cells were concentrated by centrifugation (1000×*g*, 10 min), and then its OD_{600} was re-adjusted to 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. Then, 0 or 10 μM DIDS (final concentration) was added to the cells. As described in the previous paper, about 25% of the sulfate uptake remained after DIDS treatment [11]. This remaining uptake was not included in the kinetic parameter calculation as DIDS-insensitive sulfate uptake. After pre-incubation for 90 min at 30 °C, the assay was started by adding from 5 to 160 mM Na_2SO_4 (final concentration) containing 7.5 μCi of $^{35}SO_4^{2-}$ (Amersham Biosciences, Piscataway, NJ). After 5 min incubation at 30 °C and pH 6.0, the cells were trapped rapidly on a glass filter and washed three times with distilled water. The cells were resuspended in Clear-sol I solution (Nacalai Tesque, Kyoto, Japan), and then the radioactivity was measured by scintillation counting (Liquid Scintillation Counter LSC-5100; Aloka, Tokyo, Japan). The influx in wild type AE1 expressing cell was about 5 μmol/min per mg AE1 at an extracellular concentration of 100 mM Na_2SO_4. This value was calculated using amount of plasma membrane expressed AE1 (40.5 μg per 1 l culture in wild type cell). In this case, the total radioactivity on the filter after 5 min was 1640DPM, which was enough to measure. The difference in $^{35}SO_4^{2-}$ incorporation in the absence or presence of DIDS was calculated as AE1-specific $^{35}SO_4^{2-}$ incorporation [11,19].

2.5. Assay for evaluation of alkali and protease resistance in the C-terminal region

Unsealed ghosts from human erythrocytes were prepared as described previously [25]. One-milliliter aliquots of unsealed ghosts (1 mg/ml) were treated with 0, 50 mM HPG or 10 mM PG for 1 h at 37 °C and pH 7.4 in standard buffer (5 mM HEPES (pH7.4) containing 200 mM sucrose, 25 mM gluconate, 25 mM citrate, and 1 mM Na_2SO_4). After being washed three times with the standard buffer, the ghosts were incubated with 50 mM NaOH at 4 °C for 30 min. After centrifugation at 20,000 *g* for 30 min at 4 °C, the precipitated ghosts were re-dissolved into 0.1 M Tris–HCl (pH 8.0). The NaOH treated ghosts were divided into halves. A half of the ghosts were incubated with 15 μg/ml trypsin at 37 °C for 30 min. After centrifugation again at 20,000×*g* for 30 min at 4 °C, the supernatant, which contained digested peptides, was recovered. On the other hands, another half of NaOH treated ghost were solubilized in a total volume of 1 ml of 0.1 M Tris–HCl (pH 8) buffer containing 0.1% octaethyleneglycol monododecyl ether ($C_{12}E_8$). The solubilized membrane was digested with 4 μg/ml trypsin for 2 h at 37 °C. Liquid chromatography/electron electrospray ionization mass spectrometry (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, USA). The digested peptides were separated by HPLC using a gradient of H_2O (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid at a flow rate of 400 nl/min. The mobile phase composition was held at 3% solvent B for 5 min, and then ramped from 5% to 100% in 60 min and 85–100% in 20 min. For separation of peptides in LC/ESI-MS, a C_{18} reversed-phase HPLC column was used (L-column Micro; 0.075×50 mm; Chemicals Evaluation and Research Institute, Japan). The column effluent from the HPLC separation was directed into the ESI source. The alkali and protease resistance was calculated by the formula; [(the peak area of total peptide)−(the peak area of the alkali and protease release peptide)]/(the peak area of total peptide). Data were acquired and analyzed using LCQ version 2.0 software.

3. Results

3.1. Expression of AE1 mutants

Arg 901 mutants (R901K, R901E, R901A, R901M, R901C, and, R901I) and alanine mutants in the C-terminal region (Leu 886-Val

911 except for A888, A891, A893, and A908, which are originally alanine) were expressed on the yeast plasma membrane (Figs. 1 and 2). Mutants expressing on the plasma membrane were quantified using the protease accessibility assay as described previously [11,19]. We measured the density of the 22-kDa N-terminal fragment bands, which were produced by the cleavage of extracellular chymotrypsin. Although chymotrypsin is large molecule, however some of large molecules were able to pass through yeast cell wall [26]. Since yeast vacuolar protein Vma2p was not digested by chymotrypsin (Fig. 1 and Supplement Table 1), chymotrypsin influenced on only plasma membrane band 3 [11]. We then estimated AE1 expression on the plasma membrane (Fig. 1). The proportions of Arg 901 mutants on the plasma membrane were nearly the same as those of the wild type, indicating that Arg 901 mutation does not affect trafficking to the plasma membrane (Supplement Table 1). The approximate amounts of AE1 mutants on the plasma membrane were calculated and estimated as 40.5 ± 16.3 μg per 1 l culture. In the case of the peripheral alanine mutants, the bands of the 22-kDa N-terminal fragments of D902A and P910A were not detected (Fig. 2B, D). This indicates that there was no traffic of D902A or P910A to the plasma membrane. Previously, removal of only the last five amino acids was enough to impair the proper trafficking of AE1 in HEK 293 cells [27]. Therefore, Asp 902 and Pro 910 are important residues for proper trafficking.

3.1.1. Kinetic parameters of sulfate transport of the C-terminal mutants

AE1-specific sulfate transport of Arg 901 mutants was measured by $^{35}SO_4^{2-}$ incorporation according to our previous papers [11,19]. The sulfate ion incorporation of mutants is shown in Fig. 3A. It was linear throughout the data collection period, which was more than 5 min (data not shown). In addition, sulfate incorporation was correctly adjusted by the amount of each mutant expressed on the plasma membrane. The amounts of sulfate ion incorporation in R901K and R901E were similar to that of the wild type. On the other hand, the amounts of sulfate ion incorporation in R901I, R901M, and R901C mutants were lower than that of the wild type. The [S/V]–[S] graph was plotted (Fig. 3B; S is the total sulfate concentration and V is the velocity of sulfate incorporation at each sulfate concentration). We then calculated the Vmax and Km values (Table 1). Vmax values were determined by the reciprocal number of intercepts on the vertical axis, and Km values were determined by the slopes in the [S/V]–[S] graph. The coefficient of determination (r^2) ranged from 0.976 to 0.941 on the [S/V]–[S] graph, indicating that these kinetic parameters are obtained by plots having good linearity. In addition, Km (23.7 ± 5.9 mM) and Vmax (5.5 ± 0.6 μmol/min per mg AE1) values in the wild type were the same as the previous values estimated by sulfate transport of AE1 in yeast cells (24.5 ± 2.5 mM and 5.7 ± 0.9 μmol/min per mg AE1, respectively; [19]) and in red blood cells (23 mM and 5.8 μmol/min per mg AE1 [28]).

Although the Km values for sulfate transport of AE1 mutants slightly changed, however their values are similar to that of wild type AE1 in comparison with those of His 834 mutants in the previous study [11]. Since the Km values of His 834 mutants increased about 5–10-fold, thus we concluded that His 834 affects on substrate binding [11]. Therefore, the similar Km values mean that the Arg 901 mutation had little effect on the anion affinity to the binding site in AE1. In contrast, the Vmax values of mutants for sulfate transport differed from that of the wild type. The Vmax values of R901M, R901C, and R901I, in which Arg 901 was replaced with hydrophobic residues, decreased to 2.3 ± 0.4, 2.1 ± 0.2, and 1.7 ± 0.2 μmol/min per mg AE1, respectively. Their hydrophobic mutations significantly affected on the sulfate influx (Fig. 3A). This shows that the mutation of Arg 901 to hydrophobic residue leads to the impairment of the sulfate ion transport rate. Based on the ping-pong model, the rate of anion transport depends on the rate of conformational change [11,19]. This indicates that the impairment of Vmax value means inefficient conformational change. Moreover, we plotted the Vmax values of AE1 mutants against ΔG as the hydrophobicity index, which is the transfer energy of amino acids from organic solvent to water (Fig. 3C) [29,30]. The plot was quite linear. This indicated that the conformational changes in AE1 were affected by hydrophobicity at the 901st residue.

Furthermore, we evaluated the anion transport of a series of alanine mutants in the C-terminal region (Leu 886-Val 911). Their kinetic parameters were calculated in the same manner as in the Arg 901

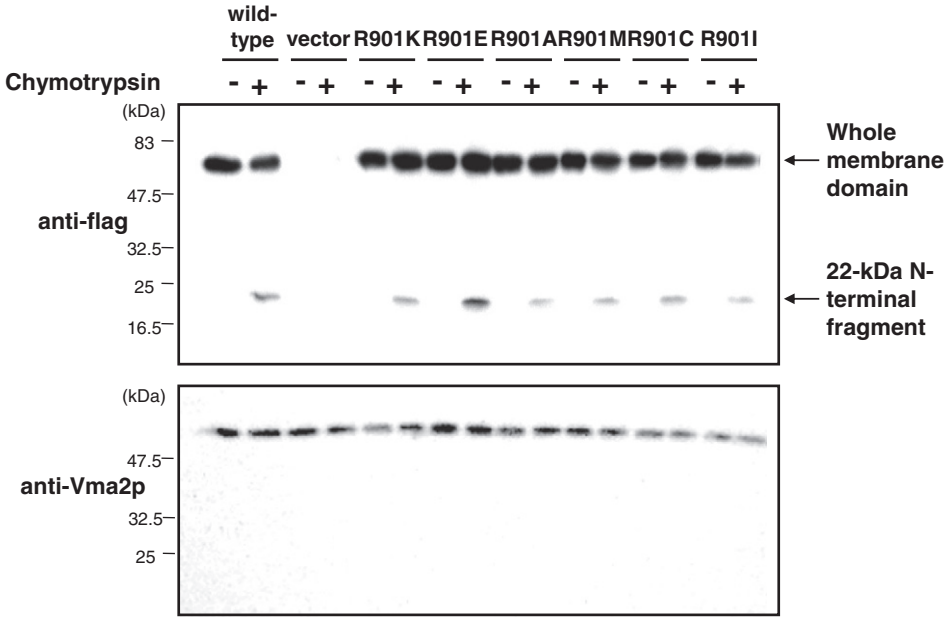

Fig. 1. Expression levels of Arg 901 mutants on the plasma membrane. Arg 901 mutants (R901K, R901E, R901A, R901M, R901C, and R901I), to which were added Flag-tag, were expressed in yeast. The yeast was treated without (−) or with 5 mg/ml chymotrypsin (+). The proteins extracted from the yeast were immunoblotted with anti-Flag antibodies. Whole-membrane domain and a 22-kDa N-terminal fragment of AE1 are indicated by arrows (upper panel). The proteins were also immunoblotted with anti-Vma2p antibodies (lower panel). An intracellular protein, Vma2p, was used as a protease-insensitive control.

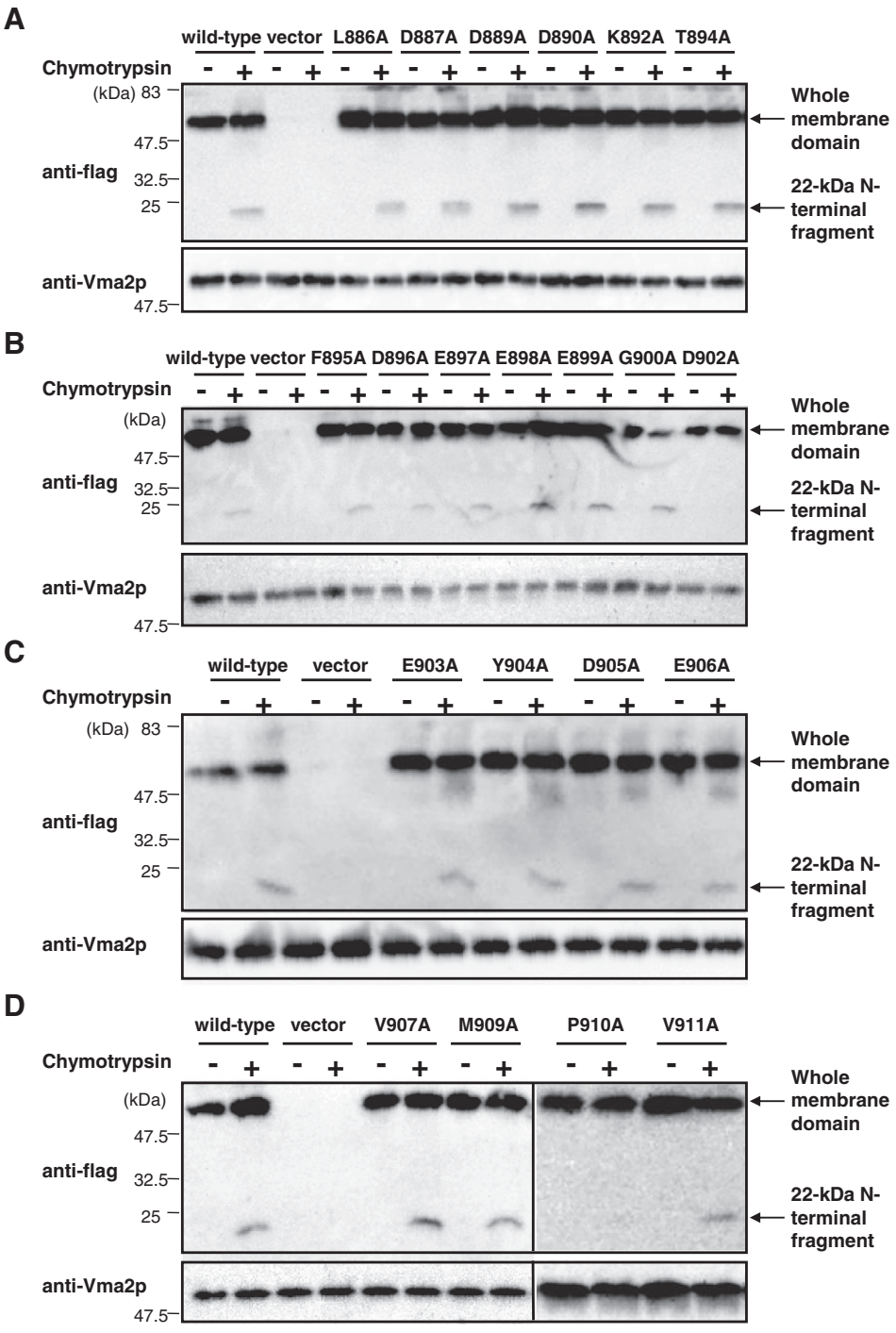

Fig. 2. Expression levels of alanine mutants of the C-terminal region on the plasma membrane. A series of alanine mutants of the C-terminal region were expressed in yeast. The yeast was treated without (−) or with 5 mg/ml chymotrypsin (+). The extracts were immunoblotted with anti-Flag (upper panel) and anti-Vma2p (lower panel) antibodies. Figure A shows the results for L886A, D887A, D889A, D890A, K892A, and T894A. Figure B shows the results for F895A, D896A, E897A, E898A, E899A, G900A, and D902A. Figure C shows the results for E903A, Y904A, D905A, and E906A. Figure D shows the results for V907A, M909A, P910A, and V911A.

mutant study (Table 2). Km values of their mutants not so changed comparing those of His 834 mutants [11]. The Vmax values of D887A, D889A, K892A, E897A, E898A, and R901A were decreased (3.2, 4.4, 3.2, 3.2, 2.8 and 2.7 μmol/min per mg AE1, respectively) relative to the other peripheral mutants. These mutants reduced hydrophilicity because hydrophilic residues were mutated to alanine. Thus, the alanine mutation of hydrophilic residues Asp 887, Asp 889, Lys 892, Glu 897, and Glu 898 also could contribute in a fashion similar to that of Arg 901.

3.1.2. Increase in alkali and protease resistance by HPG or PG modification of Arg 901

We examined the alkali and protease resistance of the C-terminal region after PG or HPG modification using erythrocyte unsealed ghosts. HPG, a hydrophobic reagent, modified Arg 901 specifically in AE1 [19]. HPG modification at Arg 901 inhibited AE1 anion transport because AE1 was fixed to inward-facing conformation [19]. PG also inhibited AE1 anion transport in a manner similar to HPG [18] and PG is more hydrophobic than HPG because it lacks a

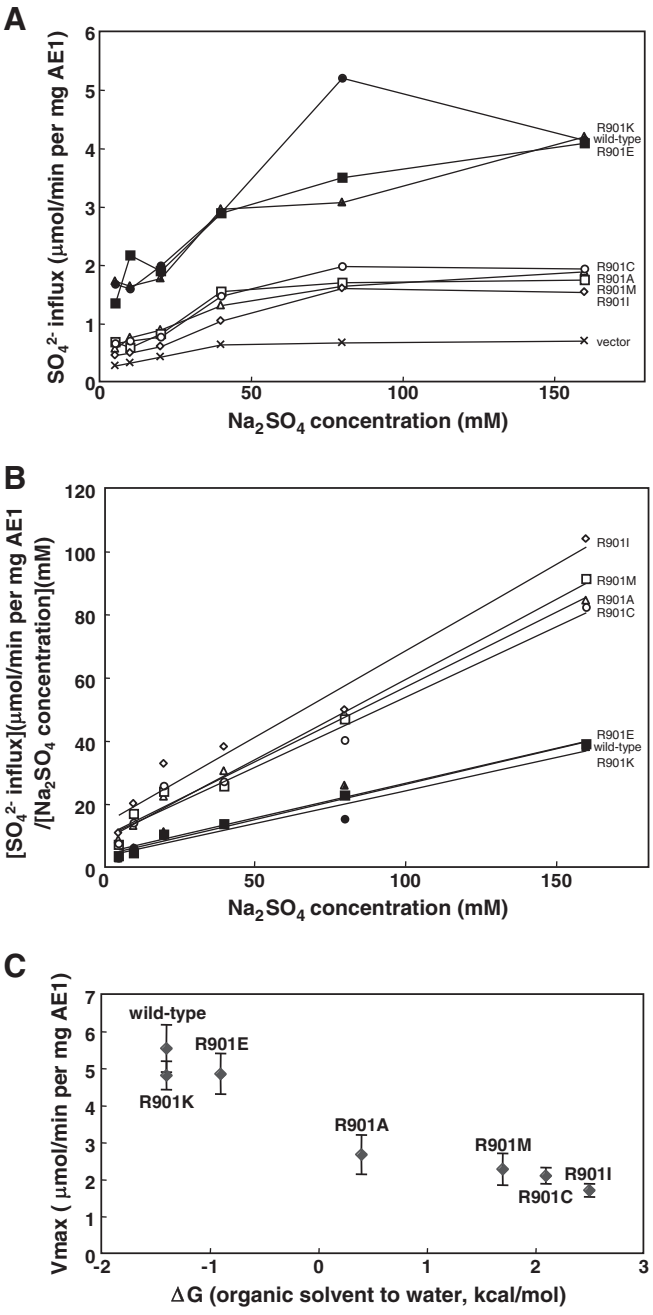

Fig. 3. AE1-specific sulfate incorporation of yeast cells expressing Arg 901 mutants. (A) Sulfate incorporation in AE1-expressing yeast cells against total sulfate concentration. The cells containing the empty vector (cross), the wild type expressing cells (closed circle), R901C-expressing cells (open circle), R901I-expressing cells (open diamond), R901K-expressing cells (closed triangle), R901E-expressing cells (closed square), R901A-expressing cells (open triangle), and R901M-expressing cells (open square) were pretreated with or without 10 μM DIDS. The cells were incubated in 5 to 160 mM Na_2SO_4 containing 7.5 μCi of $^{35}SO_4^{2-}$ for 5 min at 30 °C. $^{35}SO_4^{2-}$ in cells was measured by a scintillation counter. The difference in sulfate incorporated with and without DIDS was calculated as the AE1-specific incorporation. Sulfate incorporation per 1 mg of AE1 expressed only on the plasma membrane is shown. (B) The [S/V]-[S] plot graphs were calculated by the sulfate incorporation of each cell. The r^2's, which were calculated from the [S/V]–[S] plot, ranged from 0.976 to 0.941. (C) The correlation between Vmax values of Arg 901 mutants and the hydrophobicity of the 901st residue. Vmax values of Arg 901 mutants were plotted against the hydrophobicity index of amino acids of the 901st residue. The free-energy ΔG, which is the transfer of each amino acid from organic solvents to water, was used as the hydrophobicity index [29,30].

hydroxyl group. Previous our data indicated that the modification with HPG was saturated at 50 mM and that the other Arg residues were not modified with HPG [19]. And the Ki of PG in anion transport was 2.5 mM, which was five-fold lower than that of HPG [18]. Therefore, the ghost was modified by 50 mM HPG and 10 mM PG.

Table 1
Kinetic parameters of the Arg 901 mutants. Kinetic parameters of the wild type and Arg 901 mutants are expressed as means ± S.E. obtained from more than four experiments, respectively. N are the numbers of independent measurements.

Band 3	Km (mM)	Vmax (μmol/min per mg AE1)	r^2	N
Wild type	23.7 ± 5.9	5.5 ± 0.6	0.971 ± 0.018	6
R901K	21.5 ± 0.1	4.8 ± 0.4	0.961 ± 0.006	4
R901E	20.6 ± 4.0	4.8 ± 0.5	0.969 ± 0.026	4
R901A	23.7 ± 3.2	2.7 ± 0.5	0.976 ± 0.017	4
R901M	21.2 ± 5.7	2.3 ± 0.4	0.971 ± 0.018	4
R901C	19.4 ± 2.8	2.1 ± 0.2	0.941 ± 0.045	4
R901I	23.6 ± 2.5	1.7 ± 0.2	0.974 ± 0.008	4

Our previous reports suggested that, after alkali treatment at pH 13 (100 mM NaOH), AE1 was digested by trypsin and the digested C-terminal peptide containing Arg 901 released from the membrane. In contrast, after alkali treatment at pH 12 (10 mM NaOH), AE1 was not digested and the peptide remained in the membrane [31–33]. In the native membrane, as the NaOH concentration increased, the protease resistance decreased greatly. After 50 mM NaOH treatment, 80% of the C-terminal peptide was released ([25], Supplement Fig. 1). Therefore, in this study, to evaluate the alkali and protease resistance, we treated the unsealed ghosts with 50 mM NaOH after HPG or PG modification. The half of alkali-treated ghosts were solubilized by $C_{12}E_8$, and the solubilized proteins were completely digested by trypsin [10]. The solubilized and digested peptides were collected as total peptides. And another half of the alkali-treated ghost was directly incubated with trypsin at 4 °C for 30 min. After centrifugation, the supernatant fraction was collected as alkali and protease released peptides. These digested peptides were analyzed and quantified by LC/ESI-MS combined with reversed-phase HPLC. From unmodified ghosts, we found the C-terminal peptide (m/z 1101.5, amino acids 893–911; ATFDEEEGRDEYDEVAMPV), which was identified by MS/MS. In the HPG or PG treated ghost, we found mass peaks derived from the HPG and PG modified peptide (mz 1174.7

Table 2
Kinetic parameters of alanine mutants of C-terminal region.

Alanine mutants	Km (mM)	Vmax (μmol/min per mg AE1)	Hydrophobicity[b]
L886A	26.8[a]	4.3	Decrease
D887A	14.8	3.2	Increase
(A888)	-	-	-
D889A	23.9	4.4	Increase
D890A	30.6	5.7	Increase
(A891)	-	-	-
K892A	17.2	3.2	Increase
(A893)	-	-	-
T894A	31.6	8.0	Decrease
F895A	25.1	5.5	Decrease
D896A	28.0	5.8	Increase
E897A	18.1	3.2	Increase
E898A	16.8	2.8	Increase
E899A	26.1	4.9	Increase
G900A	24.8	5.4	Increase
R901A	23.7	2.7	Increase
D902A	-	-	-
E903A	29.1	5.5	Increase
Y904A	23.2	5.3	Increase
D905A	27.7	5.4	Increase
E906A	20.3	4.8	Increase
V907A	27.4	5.1	Decrease
(A908)	-	-	-
M909A	21.8	5.6	Decrease
P910A	-	-	-
V911A	27.5	5.4	Decrease

[a] Each kinetic parameter calculated by one independent experiment.
[b] Change in hydrophobicity by alanine mutations.

and 1165.8), respectively. The unmodified peak could not be seen in the analysis of the HPG or PG treated ghost. Therefore, Arg901 was modified with HPG or PG] PGLC/ESI-MS were modified with Arg901 completely under our modification condition. Fig. 4A shows selected ion chromatograms of the alkali and protease released peptides from the modified and unmodified membrane. And then we calculated the alkali and protease resistance using the eluted peak area of alkali and protease released peptides and total peptides (Fig. 4B). In the native membrane, after 50 mM NaOH and trypsin treatment, only 20% of the C-terminal peptide was remained in membrane. This was consistent with our previous and the reversed phase-HPLC results using resealed ghost ([25] and Supplement Fig. 1). On the other hand, 50% or 60% of the C-terminal peptide remained in the HPG or PG treated membrane, respectively. It indicates that the alkali and protease resistance of the C-terminal peptide increased in the HPG or PG modified membrane. Thus, we found that the hydrophobic modification of the 901st residue induced a more alkali and protease resistant structure in the C-terminus of AE1.

4. Discussion

To further clarify Arg 901's role, we expressed various AE1 mutants of the 901st residues, such as basic (R901K), acidic (R901E), and hydrophobic mutants (R901M, R901C, and R901I) on the yeast plasma membrane and evaluated the kinetic parameters. Vmax values of the hydrophobic mutants of Arg 901 decreased while the Km values of all mutants not so changed. Furthermore, we found a clear relationship between the Vmax values of sulfate transport in Arg 901 mutants and the hydrophobicity of the 901st residue in the mutants (Fig. 3C). Thus, we concluded that the hydrophobicity of the 901st residue was related to the anion exchange in AE1. The mutants to glutamate and lysine did not reduce the Vmax values, indicating that a positive or negative charge of the 901st residue did not influence anion transport. Under higher pH (>pH 12) conditions, Arg 901 residues are hydrophobic by the occurrence of de-protonation of the guanidino group of Arg. This idea may be a cause of the pK value 12 of chloride exchange in AE1 [15].

Furthermore, we evaluated the kinetic parameters of a series of alanine mutants in the C-terminal region (Leu 886–Val 911). Similar to the case with the R901A mutant, since the Km values of the alanine mutants were not so changed. Some mutations slightly affect on Km values (increase: T894A, decrease: D887A, K892A, E898A). Possibly, the corresponded residues may interact with substrate binding site (i.e. His 834, [11]) by alanine mutation and then affect on the substrate binding. On the other hand, the Vmax values of D887A,

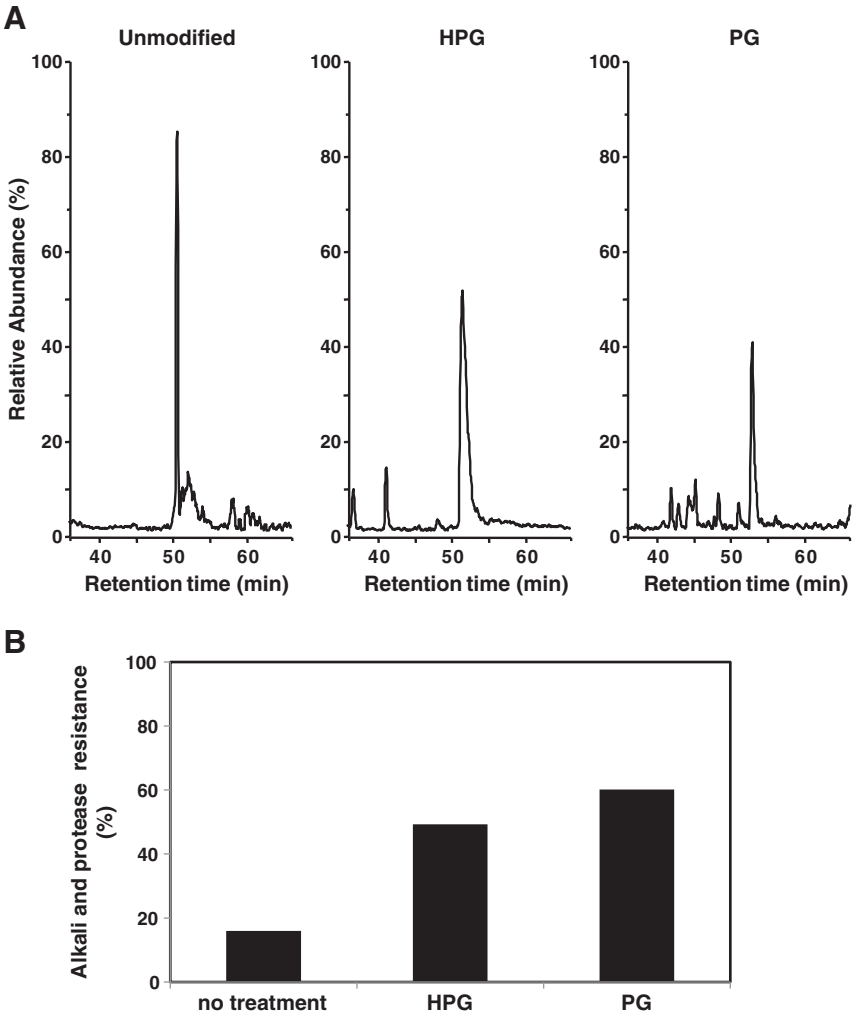

Fig. 4. Alkali and protease resistance of the C-terminal region after HPG or PG treatment. (A) Selected ion chromatograms from ESI-MS detection of the peaks eluted from HPLC separation of tryptic-digested C-terminal peptides. White ghosts were pretreated with 0, 50 mM HPG or 10 mM PG. The HPG or PG pre-treated ghosts were incubated with 50 mM NaOH and then were digested with 15 μg/ml trypsin at 37 °C and pH 8.0 for 30 min. Their peptides were separated by LS/ESI-MS using a gradient of an aqueous solution (solvent A) and acetonitrile (solvent B), at a flow rate of 400 nl/min. The chromatograph of unmodified (left), HPG-modified (center) and PG-modified C-terminal peptides (right) were generated by the molecular ions, m/z 1171.6, 1174.7 and 1165.8, respectively. (B) The alkali and protease resistance of the C-terminal region in unmodified (no treatment), HPG or PG modified membranes. Alkali and protease resistance was calculated by the peak area between alkali and protease released peptide and total peptide (see in the text).

K892A, E897A, and E898A mutants decreased (Table 2). Therefore, we claimed that the increased hydrophobicity resulting from the alanine mutation at the C-terminal region, especially the hydrophilic part of the Asp 887–Arg 901 region, caused the impairment of anion transport. The decrease of anion transport activity seemed to occur in every two or three residues. It may be involved in helical conformation of its C-terminal region. Vmax residue of Thr 894 mutant was increased. It may be caused by deletion of γ-methyl group, which was important for hydrophobic interaction in native AE1 structure.

HPG or PG modification increases the hydrophobicity of arginine because HPG or PG confers the phenyl group to arginine and eliminates the positive charge from arginine. When HPG modified Arg 901, changes from inward-facing to outward-facing conformation were inhibited, leading to the inhibition of anion transport [19]. The present study showed that the C-terminal region of HPG- or PG-modified AE1 was more alkali-resistant on the erythrocyte membrane than that of native AE1 (Fig. 4B). Furthermore, the more hydrophobic reagent, PG, was slightly more effective than HPG. Hamasaki suggested that alkali-sensitive structures, which have peptide-peptide interaction, are essential for the flexible conformational change involved in anion transport [33,34]. Thus, the change in the C-terminal region to the alkali-resistant structure by hydrophobic interaction caused low flexibility in the structure and then inefficient conformational change. In our previous and present mutant studies, we also revealed the decrease in Vmax values in a part of C-terminal hydrophobic mutants. Together with these results, we concluded that the additive hydrophobicity in the C-terminal tail in AE1 modulated the anion exchange involved in conformational change. The previous C-terminal truncation mutant study of AE1 suggested that 21 amino acids following Ala 891 were not needed anion exchange activity [35,36]. Thus, hydrophobic mutation or modification of C-terminal residues may produce new interaction in the AE1 structure, leading to a structure with little flexibility.

Our previous and present studies suggested that the increase in C-terminal hydrophobicity leads to a series of changes: hydrophobic interaction between the C-terminal and other regions, the flexible C-terminal region change to an alkali-resistant and low-flexibility structure, inefficient conformational change by the fixation of an inward-facing conformation, and the inhibition of anion transport. Our results will be valuable for understanding in greater detail the mechanism underlying the conformational change involved in AE1 anion transport when the structures are solved in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamem.2011.11.019.

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