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Mutation of His 834 in human anion exchanger 1 affects substrate binding

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ABSTRACT

Anion exchanger 1 (AE1 or band 3) is responsible for Cl^-–HCO_3^- exchange on erythrocyte membrane. Previously, we showed that band 3 is fixed in an inward-facing conformation by specific modification of His 834 with DEPC, resulting in a strong inhibition of its anion transport activity. To clarify the physiological role of His 834, we evaluated the sulfate transport activities of various band 3 mutants: different mutants at His 834 and alanine mutants of peripheral residues around 834 (Lys 829–Phe 836) in yeast cell membranes. The K_m values of the His 834 mutants were 4–10 times higher than that of the wild type, while their V_{max} values were barely lower than that of wild type. Meanwhile, the K_m values of the peripheral alanine mutants were only slightly increased. These data suggest that His 834 is critically important for the efficient binding of sulfate anion, but not for the conformational change induced by substrate binding.

1. Introduction

Anion transporter band 3 (AE1) exchanges Cl^- for HCO_3^- across red blood cell membrane [1,2]. Human band 3 is divided into cytosolic and transmembrane domains. The C-terminal transmembrane domain (Gly 361–Val 911), which is thought to penetrate the membrane 12–14 times, has an anion transport function [3]. Many efforts using site-directed mutagenesis, chemical modification, and so on suggest that some lysine [4–6], arginine [7], glutamic acid [8], and histidine [9–11] residues of band 3 are essential amino acids for the transport activity.

The importance of histidine in anion transport has been clarified. The anion transport rate varied with the intracellular pH. This pH dependence suggests that intracellularly located histidine participates in anion transport [9–11]. By introducing a series of mutations at histidine residues, and Muller-Berger et al. [12] found that four of six histidines were involved in Cl^- flux through mouse band 3. Chemical modification of band 3 with DEPC, which specifically reacts with histidine, leads to the almost complete inhibition of anion transport and inhibition of Cl^- ion binding [10,13]. Mass spectrometric analysis of tryptic fragments of band 3 revealed that only a single histidine residue, His 834, was completely modified with DEPC under the conditions [9]. His 834 is predicted to exist on the intracellular side of the membrane. In recent cysteine-scanning works, the anion transport activity of a cysteine mutant at His 834 was drastically decreased [14]. Thus, His 834 is critically involved in anion transport.

Anion transport of band 3 was explained by a ping-pong model [15]. In this model, when a single anion binds to the binding site from an extracellular side, its outward-facing conformational changes to an inward-facing conformation and vice-versa. As a result, one molecule of the substrate anion is transferred across the membrane every time the conformational changes. These consecutive conformational changes are evident from the chemical modification of band 3. Stilbene compounds (DNDS, DIDS, and H_2DIDS), anion transport inhibitors, bind to band 3 from an extracellular side. The extracellular binding of DNDS to band 3, by which the band becomes fixed in the outward-facing form, hides His 834 from the intracellular side and so protects His 834 from DEPC modification. Conversely, the modification of His 834 with DEPC, by which band 3 becomes fixed in the inward-facing form, hides the extracellular reaction site of H_2DIDS from the cell surface and so abolishes H_2DIDS binding. These results suggest that His 834 changes its location along with the conformational change during the anion-exchange process. This finding, in light of His 834's importance in anion transport, suggests that His 834 contributes to the conformational change. However, there is a

Abbreviations: DEPC, diethyl pyrocarbonate; DIDS, 4,4-diisothiocyanostilbene-2,2-disulfonic acid; DNDS, 4,4-dinitrostilbene-2,2-disulfonic acid; ER, endoplasmic reticulum; GPA, glycophorin A; H_2DIDS, 4,4-diisothiocyanodihydrostilbene-2,2-disulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; TM, transmembrane spanning portion

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possibility that the conformation fixation by DEPC is simply caused by steric hindrance due to a DEPC adduct.

To rule out this possibility and to clarify the physiological role of His 834, in the present study we kinetically investigated the sulfate anion transport activity of mutants at His 834 as well as that of alanine mutants around His 834. For this purpose, the C-terminal transmembrane domain of band 3 was expressed in yeast and integrated into the plasma membrane. In our yeast system, both K_m and V_{max} values well correspond to those measured by using erythrocyte membrane [7]. Therefore, we consider that our results sufficiently reflect the kinetic properties of human band 3 although it is expressed on yeast membrane. Here we show that His 834 is important for substrate binding rather than for conformational change.

2. Materials and methods

2.1. Materials

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

2.2. Expression of band 3 on the yeast plasma membrane

Band 3 expression vector pYES2-FHSHS, which contains GAL1 promoter, and glycophorin A (GPA) co-expression vector pGAD-C1 were prepared as described previously [7]. The mutants of His 834 (H834A, H834E, H834Q, H834R, H834F, and H834I) and peripheral alanine mutants around His 834 (K829A, T830A, W831A, R832A, M833A, L835A, and F836A) of residues 361–911 of band 3 were constructed by established PCR methods [16,17]. Using the pYES2-FHSHS vector, Flag-tag, His-tag, Strep-tag, His-tag, and Flag-tag sequences were added to N-terminuses of their mutants. Mutations were confirmed by DNA sequence analysis. For anion transport studies, the pYES2-FHSHS vector harboring each band 3 mutation was transfected into *Saccharomyces cerevisiae* strain BY4743 (MATa/α ura3/ura3 leu2/leu2 his3/his3 lys2/LYS2 met15/MET15). To increase the band 3 expression level on the plasma membrane, vector pGAD-C1, which contains DNA encoding Δ68–70 GPA, was co-transfected [18]. The transformants 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, 2% (w/v) galactose was added to the cultured medium to induce the expression of band 3. The cells were collected at 6 h after the galactose addition.

2.3. Protease accessibility assay

Using a protease accessibility assay, we measured the proportion of band 3 that transferred to the yeast plasma membrane surface [7,19]. 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. The proteins were precipitated with trichloroacetic acid (TCA) and acetone, and then were immunoblotted with anti-Flag and anti-Vma2p antibodies. To investigate chymotrypsin's effect on intracellular protein, we examined the cleavage of Vma2p. Vma2p is a cytoplasmic protein on the vacuolar membrane [20]. The gel images were visualized with a phosphorimager (FLA2000; Fuji, Tokyo, Japan). Quantitation was performed using MacBAS software (Fuji). The cleavage of the band 3 protein was complete under these conditions because longer chymotrypsin treatment did not increase the amount of the cleaved 22-kDa fragment (data not shown).

2.4. Sulfate transport assay

A sulfate transport assay was carried out as described previously [7]. In brief, the cell suspension was 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. 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) (final 10 µM) was added to each tube. There are two endogenous sulfate transporters in *S. cerevisiae* [21]. Therefore, we measured $^{35}SO_4^{2-}$ incorporation in the presence or absence of DIDS and calculated the difference for the band-3-specific $^{35}SO_4^{2-}$ incorporation. After pre-incubation for 90 min at 30 °C, the assay was started by adding 5 to 160 mM Na_2SO_4 (final concentration) and 7.5 µCi of $^{35}SO_4^{2-}$ (Amersham Biosciences, Piscataway, NJ) to the cell mixture. After 5 min of incubation at 30 °C at pH 6.0, the cells were rapidly trapped on a glass filter and washed three times with 1 ml of distilled water. The cells were resuspended in Clear-sol I solution (Nacalai Tesque, Kyoto, Japan), and then radioactivity was measured by scintillation counting (Liquid Scintillation Counter LSC-5100; Aloka, Tokyo, Japan). Protease accessibility and sulfate transport were assayed using cells of the same culture in each independent experiment.

3. Results

3.1. Expression of various mutants of band 3 on yeast membrane

We expressed the wild type and mutants at His 834 (H834A, H834E, H834Q, H834R, H834F, and H834I) and alanine mutants around His 834 (K829A, T830A, W831A, R832A, M833A, L835A, and F836A) of the band 3 C-terminal domain (residues 361–911) on the yeast plasma membrane. The yeast expressing wild type as well as each mutant protein proliferated to a similar extent (data not shown). To distinguish the band 3 expressed on the plasma membrane from that on the endoplasmic reticulum (ER) membrane, we measured the amount of band 3 on the plasma membrane by a protease accessibility assay [19]. Band 3 expressed on the plasma membrane is completely cleaved by extracellular chymotrypsin between transmembrane spanning portion (TM) 5 and TM6, which are predicted in a 14-time membrane spanning model. Thereby, the cleavage of band 3 on the plasma membrane produces a 22-kDa N-terminal fragment. We measured the density of the 22-kDa N-terminal fragment bands. It was also confirmed that intracellular Vma2p was not affected by the extracellular chymotrypsin treatment (lower panel of Figs. 1A, B). We estimated that the proportion of band 3 on the plasma membrane in wild-type cells was about 10%, which was not significantly different between the wild-type cell and all mutant cells (upper panel of Fig. 1A, B). This indicates that those mutations do not affect band 3 trafficking to the plasma membrane. By comparing the band density between the band 3 expressed in the yeast and the band 3 purified from erythrocyte membrane, the amount of band 3 in the yeast was calculated. The amount of mutant band 3 expressed on the yeast membrane was estimated to be about 0.04 mg for the 1 l culture cells.

3.2. Sulfate transport of band 3 mutants

To examine the anion transport activity of band 3 mutants of His 834 on the yeast membrane, we measured $^{35}SO_4^{2-}$ incorporation. Fig. 2A shows the band-3-specific incorporation of sulfate ion. Using these data, we plotted the $[S/V]$–$[S]$ graph as shown in Fig. 2C. The coefficient of determination (r^2) ranged from 0.980 to 0.906, suggesting that the linearity of the plots was fairly good. S is the total sulfate concentration. V is the sulfate influx velocity obtained from the sulfate influx at each sulfate concentration. This influx was linear for throughout the period of data collection, which was more

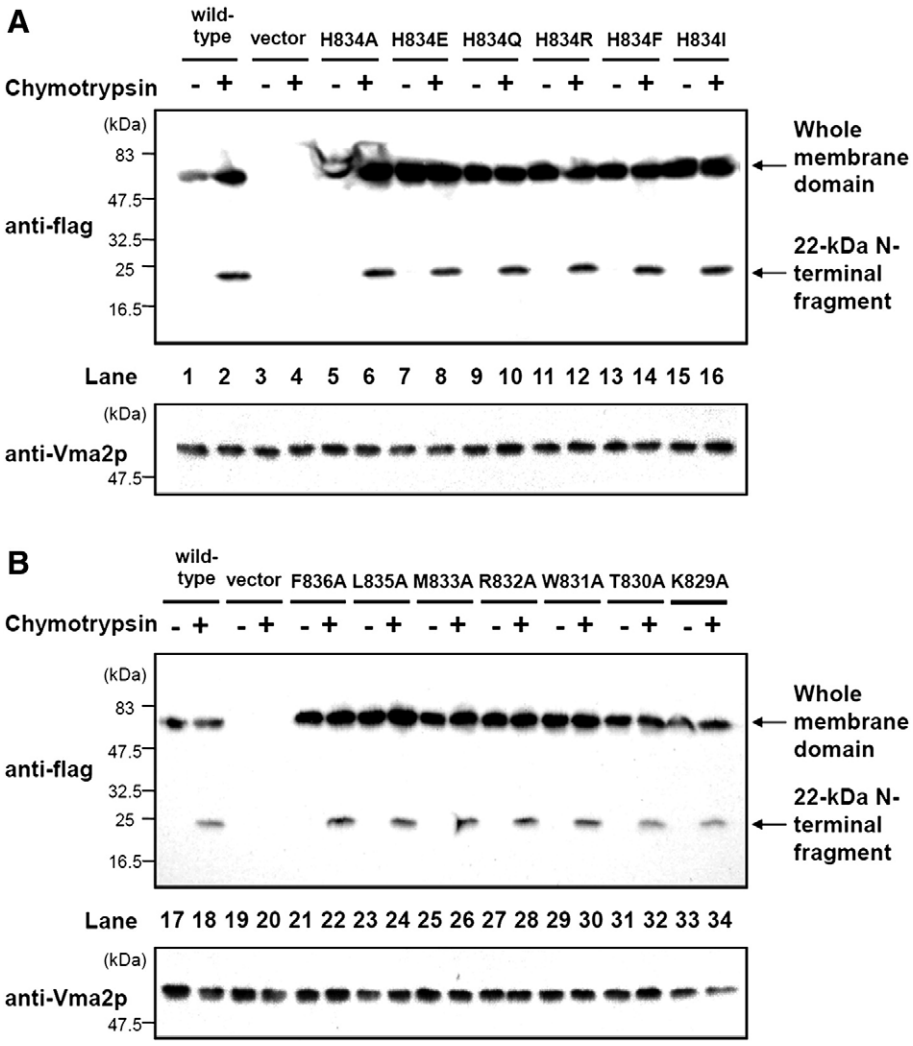

Fig. 1. Expression levels of various mutants of band 3. Wild type and various mutants of band 3 were expressed in yeast. The mutants at His 834 and alanine mutants around His 834 are shown in A and B, respectively. Expression levels of band 3 and Vma2p were estimated using Western blotting with anti-Flag and anti-Vma2p antibodies. The figure shows the expression of the whole membrane domain of band 3 (odd-numbered lanes) and the production of the N-terminal fragment of the membrane domain after the chymotrypsin treatment of the wild-type and various mutant cells (even-numbered lanes; see Section 2).

than 5 min (data not shown). We calculated the K_m and V_{max} values using the $[S/V]$–$[S]$ graphs. The level of cell surface expression did not vary significantly between the mutants. Sulfate incorporation was adjusted by the amount of each mutant in cell surface. Thus, the difference of V_{max} values is due to the intrinsic activity of the transporter but not to the difference of cell surface expression. In fact, the calculated values for the wild type and mutants are summarized in Table 1. The K_m (23.7 ± 5.9 mM) and V_{max} (5.5 ± 0.6 μmol/min per mg band 3 protein) values of the wild type were essentially the same as the values previously estimated for band 3 in red blood cells (23 mM and 5.8 μmol/min per mg band 3 protein [22]). We compared the kinetic parameters of the mutants. The V_{max} values of the His 834 mutants were almost the same as that of the wild type. On the other hand, the K_m values of H834A, H834E, H834Q, H834F, and H834I markedly increased, to 218.6 ± 49.5, 213.1 ± 69.5, 181.5 ± 47.9, 202.7 ± 36.6, and 233.3 ± 21.4 mM, respectively. The K_m values of H834R moderately increased to 83.9 ± 26.6 mM. These results were summarized as follows; 1) the K_m values of His mutants except H834R were markedly increased, 2) that of H834R was moderately increased, and 3) V_{max} values of all His mutants were not markedly different. These large increases in K_m for the His 834 mutant suggest that His 834 plays a very important role in anion binding.

A series of peripheral alanine mutants around His 834 also had V_{max} values similar to that of the wild type (Fig. 2B, 2D, Table 1). The K_m values of K829A, T830A, W831A, R832A, M833A, L835A, and F836A increased to 44.5 ± 8.5, 38.4 ± 12.9, 41.2 ± 4.7, 32.2 ± 2.0, 63.7 ± 16.9, 27.5 ± 7.4 and 30.9 ± 1.3 mM, respectively. Thus, the increases in K_m of the peripheral mutants were at most moderate compared to those of the His mutants.

4. Discussion

The significance of His 834 was reported by mutation [12,14] and chemical reagent studies [9,10]. Muller-Berger et al. reported that H852Q mutation in mouse band 3 (corresponding to His 834 in human band 3) led to complete inhibition of Cl^- transport using a *Xenopus* oocyte expression system. Mutation of the DIDS-binding site, Lys 558 (K558N), restored the transport activity of His 852 mutation. Therefore, they suggested that Lys 558 and His 852 were allosterically linked to the transfer site. Furthermore, Zhu et al. examined the effect of transport activity in the region of Phe806–Cys885 using cysteine scanning [14]. In their experiment, the transport activity of H834C mutant was almost completely inhibited. Taken together with our previous studies, His834 is critical for the anion transport activity.

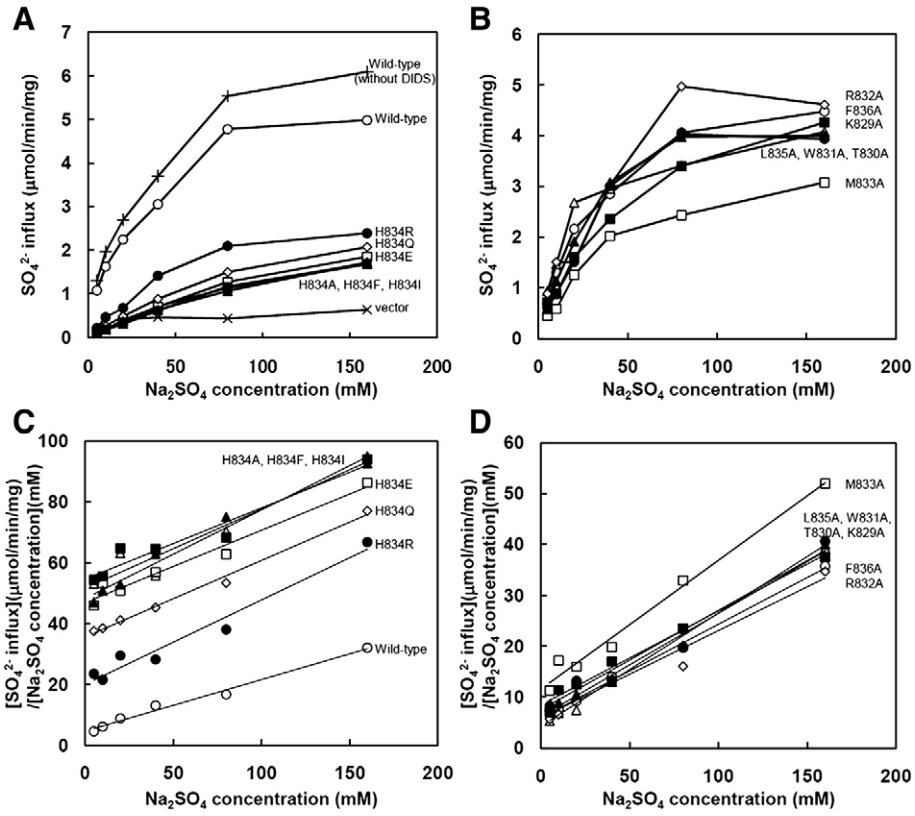

Fig. 2. Sulfate incorporation in band-3-expressing yeast cells against total sulfate concentration. The cells expressing mutants at His 834 (A) and alanine mutants around His 834 (B) were pretreated with or without 10 μM DIDS. The cells were incubated in 5 to 160 mM Na_2SO_4 containing 7.5 μCi of $^{35}SO_4^{2-}$ for 5 min at 30 °C, and sulfate incorporation was measured with a scintillation counter. The difference between the values obtained with and without DIDS was estimated as the band-3-specific incorporation. The $[S/V]$–$[S]$ plot graphs are shown for the cells expressing mutants at His 834 (C) and alanine mutants around His 834 (D). The r^2's, which were calculated from the $[S/V]$–$[S]$ plot, were 0.995–0.906. In A and C, wild-type-expressing cells, cells transfected with the empty vector, H834A-expressing cells, H834E-expressing cells, H834Q-expressing cells, H834R-expressing cells, H834F-expressing cells, H834I-expressing cells and wild-type-expressing cells without DIDS are shown by the open circle, cross, open triangle, open square, open diamond, closed circle, closed triangle, closed square and plus, respectively. In B and D, K829A-expressing cells, T830A-expressing cells, W831A-expressing cells, R832A-expressing cells, M833A-expressing cells, L835A-expressing cells and F836A-expressing cells are shown by closed square, closed triangle, closed circle, open diamond, open square, open triangle and open circle, respectively.

However, His 834's physiological role and its molecular mechanism largely remain to be solved. For example, (1) what amino acid properties are important at amino acid position 834?; (2) where is the most important position around this region?; and (3) which is His 834 more important for, substrate binding or conformational change? (The location of His 834 and its peripheral residues are shown in Fig. 3). To answer these questions, in the present study we expressed various mutants of His 834 on yeast membrane and evaluated their kinetic parameters.

Table 1
Kinetic parameters of the wild type and various mutants.

Band 3	K_m (mM)	V_{max} (μmol/min/mg)	r^2
Wild type	23.7 ± 5.9[a]	5.5 ± 0.6	0.971 ± 0.018
H834A	218.6 ± 49.5**	4.0 ± 0.7	0.953 ± 0.030
H834E	213.1 ± 69.5**	4.5 ± 1.0	0.958 ± 0.033
H834Q	181.5 ± 47.9**	4.6 ± 0.8	0.933 ± 0.058
H834R	83.9 ± 26.6*	4.3 ± 0.7	0.980 ± 0.015
H834F	202.7 ± 36.6**	4.4 ± 1.1	0.939 ± 0.040
H834I	233.3 ± 21.4**	4.3 ± 0.4	0.906 ± 0.030
K829A	44.5 ± 8.5	5.5 ± 0.8	0.984 ± 0.004
T830A	38.4 ± 12.9	5.3 ± 0.7	0.976 ± 0.014
W831A	41.2 ± 4.7	5.0 ± 0.4	0.970 ± 0.005
R832A	32.2 ± 2.0	6.2 ± 0.3	0.967 ± 0.021
M833A	63.7 ± 16.9	4.7 ± 0.7	0.941 ± 0.064
L835A	27.5 ± 7.4	4.9 ± 0.5	0.979 ± 0.018
F836A	30.9 ± 1.3	5.1 ± 0.3	0.995 ± 0.002

[a] Kinetic parameters of the wild type and various mutants are expressed as means ± S.E. obtained for four or six experiments. r^2 is the coefficient of determination. When compared to value of wild-type, the markedly and moderately changed values are marked with ** and *, respectively.

First, to learn what properties of amino acid are important at this position, we prepared hydrophobic (H834I and H834F), acidic (H834E) and basic (H834R) mutants. The K_m values of all of these mutants except H834R were increased by about 8- to 10-fold (Table 1). H834R increased the K_m by only 4-fold, suggesting that the positive charge of arginine partially compensates for that of histidine. Previous studies suggest that the protonation of the histidine residue is involved in the anion transport. For instance, the phosphoenolpyruvate transport rate increased under acidic conditions [11]. Therefore, we expect that the positive charge at position 834 is important for anion binding. Band 3 transports anions by the ping-pong mechanism. The bound anion in the intracellular side has to be released to the extracellular side according to the conformational change. Therefore, tight anion binding due to the electrostatic interaction may rather interrupt the anion release. Therefore, His, a weaker base than Arg, may be suitable for the anion transport or for the anion binding and release under physiological condition. In addition to its weak basic nature, the structure of histidine itself could be important.

Next, to evaluate the importance of the position, we measured the kinetic parameters of alanine mutants of peripheral residues of His 834 (Lys 829–Phe 836). The increases in the K_m values of these mutants were at most moderate (1.7- to 2.7-fold) compared to those of all the His 834 mutants, including H834A, suggesting that position 834 is most important in this region (Lys 829–Phe 836). For further confirmation, histidine can replace the peripheral amino acids of Lys

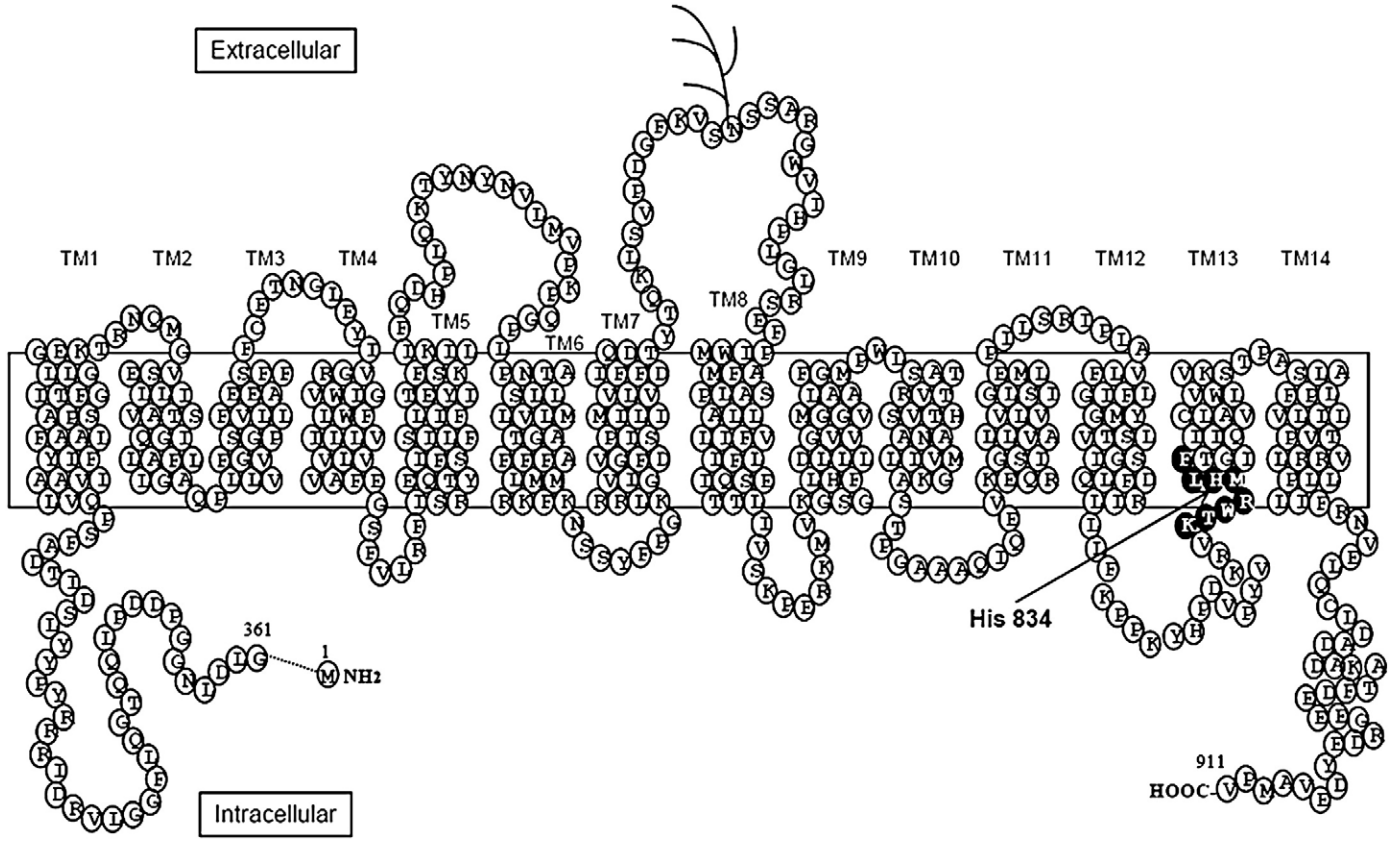

Fig. 3. Topology model of the transmembrane domain of band 3. The residues of Lys 829–Phe 836 are shown by closed black circles. His 834 is indicated by an arrow.

829-Phe836. Some of peripheral mutant transport activity (at Met833 and Phe836) differed between our and Zhu's studies. However, we used alanine mutants, and they used cysteine mutants. Therefore, these residues are thought as sensitive residues in transport activity. One possibility is that the SH group may interact with another residue and induce conformational change around His834.

Finally we consider the role of His 834 in the conformational change. We previously showed that modification with DEPC at His 834 fixed band 3 in the inward-facing conformation and inhibits anion transport completely [9]. We therefore have proposed that His 834 has an essential role in the conformational change between inward- and outward-facing conformations. However, chemical modification with DEPC produces a mono-carbetoxy group adduct on histidine. It may be possible that the freezing of the conformation by the DEPC modification of His 834 results simply from steric hindrance caused by the DEPC adduct. To avoid this possibility, we produced the His 834 mutants. Based on the ping-pong model, V_{max} should reflect conformational change and K_m should reflect the anion binding. In the present study, we demonstrated for the first time that the V_{max} values of the His 834 mutants were only slightly decreased (16%–24%) while the K_m increased markedly. Based on the ping-pong model, anion transport is performed through the conformational change. The rate of anion transport depends on the rate of conformational change. Therefore, this small decrease in V_{max} suggests that His 834 may hardly contribute to the conformational change. Thus, the unaltered V_{max} and increased K_m values of the His mutants strongly suggest that His834 is important for the anion binding. Our finding about His 834 may support the previous results that the mutation of the DIDS reactive lysine residue did not inhibit transport, but chemical modification did [6,23].

The expression of the C-terminal domain of band 3 on the yeast membrane allows us to evaluate not only the K_m but also the V_{max} (or specific activity). These two parameters are fundamental features of enzymes. In this study, we performed for the first time an intensive kinetic analysis of the His 834 mutants by taking advantage of this expression system. Here, we have revealed that the primary role of His 834 is in anion binding and not in conformational change. Hereafter, this expression system will be an important tool for further molecular and functional analyses of band 3.

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