Transmembrane domain histidines contribute to regulation of AE2-mediated anion exchange by pH

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Stewart AK, Kurschat CE, Burns D, Banger N, Vaughan-Jones RD, Alper SL. Transmembrane domain histidines contribute to regulation of AE2-mediated anion exchange by pH. Am J Physiol Cell Physiol 292: C909–C918, 2007. First published September 27, 2006; doi:10.1152/ajpcell.00265.2006.—Activity of the AE2/SLC4A2 anion exchanger is modulated acutely by pH, influencing the transporter’s role in regulation of intracellular pH (pHi) and epithelial solute transport. In Xenopus oocytes, heterologous AE2-mediated Cl−/Cl− and Cl−/HCO3− exchange is inhibited by acid pHi or extracellular pH (pHo) (pHiH). We have investigated the importance of pH sensitivity of the eight histidine (His) residues within the AE2 COOH-terminal transmembrane domain (TMD). Wild-type mouse AE2-mediated Cl−/Cl− exchange, measured as DIDS-sensitive 36Cl− efflux from Xenopus oocytes, was experimentally altered by varying pH, at constant pHi, or varying pHi. Pretreatment of oocytes with the His modifier diethylpyrocarbamide (DEPC) reduced basal 36Cl− efflux at pHi 7.4 and acid shifted the pHiH vs. activity profile of wild-type AE2, suggesting that His residues might be involved in pH sensing. Single His mutants of AE2 were generated and expressed in oocytes. Although mutation of H1029 to Ala severely reduced transport and surface expression, other individual His mutants exhibited wild-type or near-wild-type levels of Cl− transport activity with retention of pHiH sensitivity. In contrast to the effects of DEPC on wild-type AE2, pHiH sensitivity was significantly alkaline shifted for mutants H1144Y and H1145A and the triple mutants H846F/H849/H1145A and H846F/H849/H1160A. Although all functional mutants retained sensitivity to pHiH, pHiH sensitivity was enhanced for AE2 H1145A. The simultaneous mutation of five or more His residues, however, greatly decreased basal AE2 activity, consistent with the inhibitory effects of DEPC modification. The results show that multiple TMD His residues contribute to basal AE2 activity and its sensitivity to pHiH and pHiH.

pH regulation; histidine residues; Cl−/HCO3− exchange

THE SLC4 BICARBONATE TRANSPORTER gene superfamily includes the anion exchanger gene family of Na+−independent Cl−/HCO3− exchangers, AE1, AE2, and AE3. In mammalian cells, these anion exchangers are involved in the control of intracellular Cl−, pH, and cell volume (2). The anion exchanger polypeptides share a highly conserved COOH-terminal transmembrane domain (TMD) of ∼500 amino acids (aa) with a short COOH-terminal cytoplasmic tail. The TMD is preceded by a less extensively conserved ∼400-aa (AE1) to ∼700-aa (AE2 and AE3) NH2-terminal cytoplasmic tail (1). The COOH-terminal TMD can mediate anion exchange in the absence of the NH2-terminal cytoplasmic domain (12, 21, 37), but the physiological regulation of AE2 transport activity by pH requires the NH2-terminal cytoplasmic domain.

The anion exchanger polypeptides are expressed in tissue- and cell-specific patterns and differ in their acute response to a change of pH. Structural elements contributing to this pH sensitivity have been localized to the COOH TMD and NH2-terminal cytoplasmic domain (37, 46). AE1-mediated Cl−/HCO3− and Cl−/Cl− exchange in erythrocytes (10) and Xenopus oocytes exhibits a broad pH vs. activity profile (17, 46). In contrast, Na+−independent Cl−/HCO3− exchange in nonerythroid cells in culture is often characterized by strong pH sensitivity (24, 35) and is most likely mediated by AE2 and AE3 gene products and/or by polypeptides of the SLC26 gene family. A high sensitivity to intracellular pH (pHiH) and/or extracellular pH (pHiH) has been observed for recombinant AE2 and AE3 expressed in tissue culture cells (19, 23) and Xenopus oocytes (37, 46). AE2+/− mice are achlorhydric and fail to survive weaning (11). AE3+/− mice display enhanced susceptibility to pharmacologically induced seizures (14).

Structure-function studies have localized to the AE2 TMD a putative “pH sensor” that confers sensitivity of the anion transporter to changes in pHiH and pHi (37, 46). The pHi at which AE2 activity is half-maximal [pHiH(50)] is ∼6.9. Transcation of the NH2-terminal cytoplasmic domain acidic acid shifts pHiH by −0.7 pH unit and abolishes pHiH sensitivity (37). We have defined two noncontiguous regions of the NH2-terminal cytoplasmic domain, the highly conserved 336–360 and the less well conserved aa 391–410, in which mutations alter or abolish pH sensitivity (39). However, residues of the AE2 TMD whose mutation alters AE2 pH sensitivity have not been reported, and the molecular identity of the pH sensor(s) in the AE2 COOH-terminal TMD remains unknown.

With their imidazole side chain pKa of ∼6.0 as the free amino acid, AE2 histidine (His) residues are strong a priori candidate pH sensors within the TMD (29). In contrast to the established role of His residues in conferring pH sensitivity on various ion channels (5, 6), the potential role of TMD His residues in the regulation of acid/base transporters is less well understood. For example, TMD His residues are not essential for cation transport through the ubiquitous eukaryotic Na+/H+ exchanger NHE1, but their mutation alters the inhibitory potency of amiloride (42). In contrast, mutation of a single His residue in the prokaryotic Sul2 Na+/H+ exchanger of S. pombe impairs proton translocation (43). Juxtamembranous His residues of the NHE3 COOH-terminal cytoplasmic domain
have also been implicated in sustaining basal transport activity and its regulation by pH (4), but a role for His residues in the TMD has not been reported. Four of the five TMD His residues in mouse AE1 are essential for basal transport activity (13, 32), and, although all five are conserved among the eight His residues of the mouse AE2 TMD, the influence of those His residues on AE2 pH sensitivity has not been studied.

In the present work, we have investigated the role of TMD His residues in AE2-mediated Cl⁻/anion exchange and its regulation by pH, and pH. Through functional assay of AE2 polypeptides into which we have introduced mutations of individual and multiple His residues, we have determined their contributions to basal anion flux and polypeptide accumulation and to the pH sensitivity of AE2-mediated anion transport.

MATERIALS AND METHODS

Materials. Na⁺[36]Cl was purchased from ICN (Irvine, CA). The other chemical reagents, which were of analytic grade, were obtained from Sigma (St. Louis, MO), Calbiochem (San Diego, CA), or Fluka (Milwaukee, WI). Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs (Beverly, MA), and Tag DNA polymerase and dNTPs from Promega (Madison, WI) or Invitrogen (Paisley, UK).

Construction of AE2 His mutants. Murine AE2a encoded in plasmid pΔK (46) was used as template for PCR. AE2a His substitution mutants were constructed by a four-primer PCR method as described elsewhere (15, 46). Integrity of PCR products and ligation junctions was confirmed by DNA sequencing of both strands. The AE2 mutant H846A/H849A constructed by the same method was cut with StuI and BsrGI and then ligated into the appropriate AE2 His mutant backbone (H1144Y, H1145A, or H1160A) to generate three-His mutants. The five- or six-His mutants were constructed from the appropriate three- or four-His mutant templates by four-primer PCR or ligation of StuI/BsrGI insert fragments containing the desired His mutations into the similarly restricted three-His mutant backbone. The wild-type AE2a-green fluorescent protein (GFP) fusion protein with a COOH-terminal GFP insert was constructed by the same method and confirmed by sequencing. Each AE2 mutant was then generated by ligation of complementary restriction fragments. Oligonucleotide primers were obtained from Biosynthesis (Woodlands, TX); primer sequences are available on request.

cRNA expression in Xenopus oocytes. Mature female Xenopus (Xenopus One, Madison, WI) were maintained and subjected to partial ovarianectomy as described (46). Stage V–VI oocytes were manually defolliculated after incubation of ovarian fragments with 2 mg/ml collagenase A (Boehringer Mannheim, Indianapolis, IN) for 60 min in ND-96 solution containing 50 ng/ml gentamicin and 2.5 mM sodium pyruvate. Oocytes were injected on the same day with cRNA or water in a volume of 50 nl. Capped cRNA was transcribed from linearized cDNA templates with the T7 MEGAscript kit (Ambion, Paisley, UK).

EXPERIMENTAL PROCEDURES

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ized $^{36}$Cl$^-$ efflux rate constants for wild-type and mutant AE2 polypeptides).

**Immunoblot analysis of mouse AE2 polypeptide in Xenopus oocytes.** Ten oocytes previously injected with a single cRNA were suspended at 4°C in oocyte lysis buffer (10 µl/oocyte) containing 1% Triton X-100, Complete protease inhibitor (Roche Diagnostics, Indianapolis, IN), 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA. After the sample was shaken vigorously for 30 min at 4°C, the extract was centrifuged for 10 min at 4°C in a microcentrifuge. Clarified total oocyte lysate was fractionated by SDS-PAGE (8% gels), transferred to nitrocellulose, developed with affinity-purified rabbit polyclonal antibody to mouse AE2 COOH-terminal aa 1224 – 1323, and imaged with a laser scanning confocal microscope (model MRC1024, Bio-Rad). Representative sections imaged at uniform laser intensity and filter settings were compiled in Microsoft PowerPoint.

**Statistical analysis.** Values are means ± SE. Values for individual mutants and multiple groups of mutants were compared with that of wild-type AE2 by Dunnett’s two-way ANOVA.

**RESULTS**

**DEPC inhibits AE2-mediated anion transport.** We previously showed that the AE2 COOH-terminal TMD mediates anion exchange in the absence of nearly the entire NH2-terminal cytoplasmic domain (37). The AE2 TMD includes eight His residues, only three of which are not conserved in the closely related but pH-insensitive AE1 TMD. We tested the importance of His residues in basal AE2-mediated $^{36}$Cl$^-$ efflux by exposing the oocytes to the moderately specific His-modifying agent DEPC (Fig. 1A). Basal rates of $^{36}$Cl$^-$ efflux from individual oocytes expressing wild-type AE2 were measured at pH 7.4 (period a) and exposed for 12 min to 5 mM DEPC at pH$_b$ 6.0 (period b); then DEPC was washed out at pH 7.4 in the absence of Cl$^-$ (period c) and Cl$^-$ was restored (period d). The assay was terminated with addition of the anion transport inhibitor DIDS (200 µM; period e). In the absence of DEPC, reduction of pH$_b$ from 7.4 to 6.0 decreased wild-type AE2-mediated $^{36}$Cl$^-$ efflux to 10–15% of the control rate, with restoration of efflux to ~85% of the initial rate on return to pH$_b$ 7.4. In the presence of 5 mM DEPC at pH 6.0, AE2-mediated $^{36}$Cl$^-$ efflux was inhibited by ~98% and recovered to only ~20% of the initial rate after pH$_b$ was restored to 7.4 (Fig. 1B).

Erythroid AE1-mediated anion transport was earlier shown to be inhibited by DEPC partially at pH$_b$ 6.0 and maximally at pH$_b$ 7.4, effects attributed to bath pH-induced changes in red cell pH$_i$ (24). We therefore compared the pH and concentration dependence of DEPC inhibition of Cl$^-$ transport mediated by AE2. AE2-mediated Cl$^-$ transport was completely inhibited by 0.5 or 5.0 mM DEPC at pH$_b$ 6.0, whereas oocytes treated with 0.5 mM DEPC at pH$_b$ 7.4 retained ~20% of residual activity (Fig. 1B). The greater inhibition at pH$_b$ 6.0 likely reflects combined inhibitory effects of acidic pH$_b$ and DEPC. Recovery of AE2-mediated Cl$^-$ transport on DEPC washout was independent of pH$_b$ during DEPC exposure but was proportional to DEPC concentration. Recovery of AE2 function was

![Diethylpyrocarbonate (DEPC) modulates regulation of anion exchanger (AE2)-mediated Cl$^-$ transport. A: time course of $^{36}$Cl$^-$ efflux from 2 representative oocytes expressing wild-type AE2 and sequentially exposed to pH$_b$ 7.4 (a) and then pH$_b$ 6.0 in the presence or absence of 5.0 mM DEPC (b) followed by return to pH$_b$ 7.4 in the absence (c) and then the presence of Cl$^-$ (d) and, finally, addition of 200 µM DIDS (e). B: normalized rate constants (±SE) of AE2-mediated $^{36}$Cl$^-$ efflux during exposure to DEPC at the indicated concentrations at pH$_b$ 6.0 or pH$_b$ 7.4 (black bars, period b in A) and after DEPC removal and subsequent recovery at pH$_b$ 7.4 (gray bars, period d in A); (n) oocytes from two or more frogs. C: regulation by pH$_b$ of $^{36}$Cl$^-$ efflux from oocytes expressing AE2 after pretreatment without (filled circles) or with (open circles) 5 mM DEPC. Normalized rate constants are means ± SE; curves were fit to data as described in MATERIALS AND METHODS. D: pH$_{50}$ values of (n) oocytes expressing wild-type AE2 pretreated with or without 5 mM DEPC, as calculated from fits of normalized $^{36}$Cl$^-$ efflux rate constant vs. pH$_b$ as in B (mean ± SE). *P < 0.005.**

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**Fig. 1.** Diethylpyrocarbonate (DEPC) modulates regulation of anion exchanger (AE2)-mediated Cl$^-$ transport. A: time course of $^{36}$Cl$^-$ efflux from 2 representative oocytes expressing wild-type AE2 and sequentially exposed to pH$_b$ 7.4 (a) and then pH$_b$ 6.0 in the presence or absence of 5.0 mM DEPC (b) followed by return to pH$_b$ 7.4 in the absence (c) and then the presence of Cl$^-$ (d) and, finally, addition of 200 µM DIDS (e). B: normalized rate constants (±SE) of AE2-mediated $^{36}$Cl$^-$ efflux during exposure to DEPC at the indicated concentrations at pH$_b$ 6.0 or pH$_b$ 7.4 (black bars, period b in A) and after DEPC removal and subsequent recovery at pH$_b$ 7.4 (gray bars, period d in A); (n) oocytes from two or more frogs. C: regulation by pH$_b$ of $^{36}$Cl$^-$ efflux from oocytes expressing AE2 after pretreatment without (filled circles) or with (open circles) 5 mM DEPC. Normalized rate constants are means ± SE; curves were fit to data as described in MATERIALS AND METHODS. D: pH$_{50}$ values of (n) oocytes expressing wild-type AE2 pretreated with or without 5 mM DEPC, as calculated from fits of normalized $^{36}$Cl$^-$ efflux rate constant vs. pH$_b$ as in B (mean ± SE). *P < 0.005.
75–80% after exposure to 0.5 mM DEPC but only 10–20% after exposure to 5 mM DEPC. We next tested whether hydroxylamine, a reagent that removes N-carbethoxy groups from DEPC-modified His, could reverse the effects of DEPC modification of AE2. However, the persistent inhibition after removal of 5 mM DEPC was not reversed by subsequent 12-min exposure of oocytes to 10 or 50 mM hydroxylamine (n = 12 and 4, respectively; data not shown).

DEPC acid-shifts pHo sensitivity of AE2-mediated anion transport. Oocytes expressing wild-type AE2 were pretreated with 5 mM DEPC for 10 min at pHo 6.0 before assay of pHo-dependent 36Cl⁻ efflux (see MATERIALS AND METHODS). The normalized efflux data revealed an apparently acid-shifted pHo sensitivity of anion transport (Fig. 1C). In contrast to the control wild-type AE2 pHo(50) of 6.9 ± 0.05, DEPC-treated AE2 exhibited a pHo(50) of 5.9 ± 0.22 (P < 0.005; Fig. 1D).

As these data suggest a possible role for TMD His residues in setting the pHo sensitivity of AE2, we studied AE2 mutants with individual amino acid substitutions in each of the eight His residues of the TMD, as well as in eight multiple-His mutants of AE2.

TMD His residues are important for basal levels of AE2-mediated Cl⁻ transport. The schematic diagram in Fig. 2A shows the locations of the eight His residues within the AE2 TMD, mapped according to the topographical models of AE1 proposed by Zhu et al. (47) and Fujinaga et al. (9). Figure 2B shows that AE2 polypeptide levels in oocytes expressing wild-type or mutant cRNAs did not consistently correlate with basal transport activity at pHo 7.4 (Fig. 2C). In particular, AE2 mutants H1029A and H1060A showed similar low levels of 36Cl⁻ efflux, despite very different levels of protein accumulation. However, the six other single-His mutants displayed sufficient 36Cl⁻ efflux activity to allow analysis of their sensitivities to pHo and pHt. The low activity of AE2 mutant H1060A contrasted with the near-wild-type activity of mutant H1060E. The low activity of AE2 H1144A contrasted with the ~50% wild-type activity of mutant H1144Y, substituting of the Tyr residue present in the corresponding position of the relatively pH-insensitive AE1 (Fig. 2C).

Oocyte surface expression of low-activity AE2 mutants was determined with the corresponding COOH-terminal GFP fusion proteins (Fig. 2D), since wild-type AE2-GFP exhibited 36Cl⁻ efflux rate constants comparable to that of native wild-type AE2 (not shown). The low expression and activity of AE2 H1029A were corroborated by low GFP fluorescence inside the oocyte and at or near the oocyte surface. The low activity of AE2 H1060A, despite high polypeptide expression, was accompanied by substantial expression at or near the oocyte surface. The low activity of AE2 H1060A, despite high polypeptide expression, was accompanied by substantial expression at or near the oocyte surface. Thus, whereas His modification at AE2 H1060 impairs polypeptide accumulation, His modification at AE2 H1060 can modify functional activity at or near the oocyte surface. By this criterion, at least some His residues are important for basal levels of AE2-mediated Cl⁻ transport.

The active His substitution mutants of AE2 allowed a test of the hypothesis that inhibition of AE2 by DEPC might be mediated entirely, or in large part, by modification of a single His residue of the AE2 TMD. As shown in Fig. 3A, anion transport by wild-type AE2 and AE2 H1160A was nearly completely inhibited by exposure to 5 mM DEPC at pHo 6.0, with only ~20% recovery after DEPC removal at pHo 7.4. (Initial DEPC exposure at pHo 7.4 produced similar degrees of

Fig. 2. Individual His mutations within AE2 transmembrane domain (TMD) reveals key His residues involved in Cl⁻ transport. A: putative locations of the 8 His residues of the AE2 TMD; topography proposed by Zhu et al. (47) based on Cys accessibility mutagenesis (46). AE2 His residues in boldface are nonconserved in AE1. B: oocyte expression of wild-type and mutant AE2 polypeptides as detected by immunoblot of Triton X-100 lysates. C: 36Cl⁻ efflux rate constants measured at pHo 7.4 in oocytes expressing wild-type AE2 or TMD His mutants (means ± SE). *P < 0.05. D: representative confocal fluorescence images of cryosections from oocytes previously injected with water or with cRNA encoding WT or mutant AE2-GP-fusion protein. Scale bar, 180 μm.
inhibition and recovery, as shown for AE2 H1160A in Supplemental Fig. 1C). In contrast, the AE2 mutant H1144Y was the only tested polypeptide that showed substantially increased post-DEPC recovery at pHo 7.4 (Fig. 3B). This enhanced recovery was also evident after DEPC exposure at pHo 7.4 and in the setting of a triple-His mutant (Fig. 3C; see Supplemental Fig. 1B). These data thus suggest that the nonconserved H1144 of AE2 is an important participant in irreversible AE2 inhibition by DEPC, although modification of this single-His residue does not mediate the full effect of DEPC.

Extracellular Zn2⁺ (1 mM), which targets His and other residues, inhibited wild-type AE2 by ~45% but was without effect on wild-type AE1. This selective AE2 inhibition also appeared to be mediated in part by H1144, since the AE2 effect on wild-type AE1. This selective AE2 inhibition also summarizes pHo(50) values measured for several AE2 His substitution mutants. In addition to AE2 H1144Y, the mutant AE2 H1160A also exhibited a modestly alkaline-shifted pHo(50) (as was the triple mutant H846A/H849A/H1144Y; see Supplemental Fig. 3). These data reinforce the importance of H1144 in regulating AE2 activity.

His residues of the AE2 TMD are involved in regulation of AE2 by pH. We showed previously that His residues of the AE2 NH2-terminal cytoplasmic domain are important for pH sensitivity (39). Figure 4A profiles normalized 36Cl⁻ efflux activity as a function of pHo for wild-type AE2 and the H1144Y mutant. The alkaline-shifted pHo(50) for H1144Y (7.15 ± 0.08, n = 15) was significantly different from that of wild-type AE2 (6.82 ± 0.04, n = 62. P < 0.005). Figure 4B summarizes pHo(50) values measured for several AE2 His substitution mutants. In addition to AE2 H1144Y, the mutant H1145A also exhibited a modestly alkaline-shifted pHo(50) (P < 0.005). The apparently alkaline-shifted pHo(50) values of AE2 mutants H846A and H849A did not reach statistical significance (Fig. 4B).

We next tested the hypothesis that individual His residues of the TMD influence the pH sensitivity of AE2. Bath addition and subsequent removal of the weak acid butyrate was used to decrease and then increase oocyte pH (37). Figure 4C shows the 36Cl⁻ efflux time course of oocytes expressing wild-type AE2 or the AE2 mutants H846A, H1029A, and H1145A. The mutants were inhibited at low pH (in the presence of butyrate) and subsequently stimulated (on butyrate removal) by a rise of pH. Figure 4D shows that all active His mutants retained pH sensitivity. Notably, the AE2 mutant H1145A exhibited a uniquely enhanced inhibition by acidic pH (P < 0.005).

We previously demonstrated that double mutations of selected residues in the AE2 NH2-terminal cytoplasmic domain can have effects on the pH sensitivity of AE2 that are different from the effects of their component single-mutant counterparts (39). We therefore tested the effect of mutating multiple-His residues in the TMD, as schematized in Fig. 5A. Figure 5B shows that mutant polypeptides containing three or fewer His substitutions retained 36Cl⁻ efflux activity at pHo 7.4 sufficient for analysis of pH sensitivity (mutants His2, His3a, His3b, and His3c). In contrast, mutants containing five or more His substitutions exhibited very low anion transport activity, which precluded assessment of pH sensitivity (mutants His5a, His5b, His6a, and His6b). Among the multiple-His mutants that exhibited sufficient basal activity, the double mutant H846A/H849A (His2; Fig. 5C) displayed a near-wild-type pHo dependence (P = 0.07), whereas the triple mutants H846A/H849A/H1145A (His3b) and H846A/H849A/H1160A (His3c) exhibited alkaline-shifted pHo(50) values of 7.29 ± 0.09 and 7.30 ± 0.09, respectively (each n = 22, P < 0.02 compared with wild-type AE2). Figure 5D summarizes the pHo(50) values derived from experiments similar to those shown in Fig. 5C. AE2 residues H846 and H849 are likely to have contributed to the alkaline-shifted pHo(50) of mutant His3c, inasmuch as the pHo(50) of mutant H1160A did not differ from the wild-type value (Fig. 4B). Multiple-His mutations also affected sensitivity to pH. Thus inhibition by low pH was enhanced in AE2 double mutants His3b and His3c (P < 0.02), although the mutants His3a and His2 retained wild-type sensitivity (see Supplemental Fig. 2C).

Do His residues of the AE2 TMD interact with other TMD residues to mediate sensitivity to DIDS and to pH? Muller-Berger et al. (32) suggested that mouse AE1 H752 (corresponding to AE2 H1060) is allosterically coupled to K558, part
Fig. 4. Mutagenesis of individual His residues within AE2 COOH-terminal TMD modulates regulation by pH. A: regulation by pHo of normalized 36Cl efflux from oocytes expressing wild-type AE2 or AE2 H1144Y. Values are means ± SE. B: pH_{s50} for His mutants of AE2 COOH-terminal TMD (means ± SE). C: time course of 36Cl efflux from representative individual oocytes expressing WT AE2 or AE2 mutants H846A, H1029A, or H1145A during the presence and subsequent removal of bath butyrate (40 mM) to change pH_i at constant pHo and, concluded by the addition of 200 μM DIDS. D: normalized rate constants of 36Cl efflux rate constants (±SE) in the presence of bath butyrate for oocytes expressing wild-type AE2 or mutants. Values are means ± SE of number of oocytes in parentheses. Shaded bars and * in B and D indicate significant difference (P < 0.0005) from wild-type AE2.

Fig. 5. Effect of mutagenesis of multiple His residues within the AE2 COOH-terminal TMD on AE2 regulation by pH. A: Location of the 7 individual AE2 TMD His residues mutated in combination (as indicated by x). B: 36Cl efflux rate constants measured at pHo 7.4 in (n) oocytes expressing wild-type AE2a or the indicated AE2 TMD multi-His mutants (mean ± SE). C: regulation by pHo of normalized 36Cl efflux from oocytes expressing wild-type AE2a or the mutants His2, His3a, or His3b. Values are means ± SE. D: pH_{s50} values for the indicated His mutants (means ± SE). Gray bars and * indicate significant difference from wt AE2 (P < 0.01).
of the AE1 DIDS-binding site corresponding to AE2 K838. We therefore tested DIDS sensitivity of the AE2 mutant H1060E. Figure 6A shows that wild-type AE2-mediated $^{36}\text{Cl}^{-}$ efflux was inhibited $\sim80-85\%$ by 200 $\mu$M DIDS in the presence or absence of extracellular $\text{Cl}^{-}$. In contrast, AE2 H1060E-mediated $^{36}\text{Cl}^{-}$ efflux was inhibited by only 20% ($n = 15, P < 0.05$) in the presence of extracellular $\text{Cl}^{-}$ (although, in the absence of $\text{Cl}^{-}$, inhibition resembled the wild-type phenotype). This finding is consistent with an influence of AE2 H1060 on the DIDS isothiocyanate covalent reaction site K838, reflected in an apparent decrease in inhibitory potency of DIDS.

Muller-Berger et al. (31) also observed acid-shifted pH sensitivity of $\text{Cl}^{-}$ efflux as a result of substitutions in mouse AE1 residues H752 and E699 (corresponding to mouse AE2 H1060 and E1007). This observation led them to propose that a hydrogen bond between the side chains of these two TMD residues is required for pH sensitivity.

We tested this hypothesis for AE2. The mutant H1060E retained wild-type $^{36}\text{Cl}^{-}$ efflux activity at pHo 7.4 (Fig. 6B) and wild-type pHs sensitivity (Fig. 6C), in contrast to the loss-of-function mutant H1060A (Fig. 3). Figure 6B also shows that the AE2 mutant E1007H exhibited loss of function (as did...

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**Fig. 6.** Test of a predicted intra-membrane interaction in regulation of AE2-mediated $\text{Cl}^{-}$ transport by pH. **A:** $^{36}\text{Cl}^{-}$ efflux rate constants measured at pHo 7.4 in oocytes expressing wild-type AE2a or H1060E. Subsequent inhibition by 200 $\mu$M DIDS was measured in the presence (light gray bar) and absence (dark gray bar) of extracellular $\text{Cl}^{-}$. **B:** $^{36}\text{Cl}^{-}$ efflux rate constants in the presence (black bars) and subsequent absence of bath butyrate (gray bars), reflecting regulation by pHs at constant pHo. **C:** $^{36}\text{Cl}^{-}$ efflux rate constants in the presence (black bars) and subsequent absence of bath butyrate (gray bars), reflecting regulation by pHs at constant pHo. **D:** Oocyte expression of wt and the indicated mutant AE2 polypeptides as detected by immunoblot of Triton X-100 lysates. **E:** Representative confocal fluorescence images of cryosections from oocytes previously injected with water or with cRNA encoding the indicated AE2-GFP fusion protein. Scale bar, 180 $\mu$m.
also the AE2 mutants E1007K and E1007C, not shown). The AE2 double mutant E1007H/H1106E, rather than rescuing wild-type function, was inactive (Fig. 6B) and not further stimulated by changes in pH, (Fig. 6C). These changes were not explained by reduced accumulation of mutant polypeptides (Fig. 6D) or by low surface expression of the inactive mutant E1007H (Fig. 6E). The corresponding AE1 single and double mutants are well expressed in oocytes (31), and the AE2 single mutants E1007Q, E1007K, and E1007D are also abundantly expressed in HEK 293 cells (36) and oocytes (unpublished data). Thus, although reduced surface expression of the H1060E/E1007H double mutant remains a possibility, these results are consistent with absence in the AE2 TMD of an interhelical functional interaction corresponding to that proposed between H752 and E699 of mouse AE1 (31).

**DISCUSSION**

The molecular mechanism underlying acute pH-dependent regulation of anion exchange by AE2/SLC4A2 involves the NH2-terminal (cytoplasmic) and COOH-terminal (transmembrane) domains of the protein (46). Individual amino acid residues of the cytoplasmic domain important for this regulation have been identified (38, 39), but the contribution of residues within the TMD has not previously been studied. The present work has examined the role of TMD His residues. The His-modifying agent DEPC, which likely modifies multiple His residues of AE2, has dual effects: attenuation of basal activity at pH 7.4 and acid shift of the pH-sensitive REMOVER interactivity. Mutagenesis also reveals that multiple His residues are required for basal activity and contribute to pH sensitivity of AE2. Mutation of individual TMD His residue H1029 prevents polypeptide accumulation and surface expression, whereas distinct substitutions at H1060 can preserve or severely attenuate transport activity, each with preserved surface expression. Mutation of other His residues, such as H1144 or H1145, alters the pH sensitivity of transport, whereas mutation of H1144 to Tyr reduces the inhibitory effects of DEPC or Zn2+. Simultaneous mutation of five or more His residues abolishes AE2 transport activity in oocytes.

Taken together, the data suggest that multiple His residues of the AE2 TMD contribute to the modulation of AE2 activity by pH.

**DEPC sensitivity of AE2-mediated anion exchange suggests involvement of His residues.** The His-modifying agent DEPC is commonly used to assess possible involvement of His residues in native or recombinant ion channel or transporter function and, in particular, their involvement in pH sensitivity. DEPC carboxyethylates the proton-titratable imidazole group of His in a pH-sensitive reaction (7, 29), and DEPC specificity for His residues within the TMD has not previously been studied. The His-modifying agent DEPC, which likely modifies multiple His residues of AE2, has dual effects: attenuation of basal activity at pH 7.4 and acid shift of the pH-sensitive REMOVER interactivity. Mutagenesis also reveals that multiple His residues are required for basal activity and contribute to pH sensitivity of AE2. Mutation of individual TMD His residue H1029 prevents polypeptide accumulation and surface expression, whereas distinct substitutions at H1060 can preserve or severely attenuate transport activity, each with preserved surface expression. Mutation of other His residues, such as H1144 or H1145, alters the pH sensitivity of transport, whereas mutation of H1144 to Tyr reduces the inhibitory effects of DEPC or Zn2+. Simultaneous mutation of five or more His residues abolishes AE2 transport activity in oocytes.

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Cys accessibility studies, to adopt a reentrant loop structure (Fig. 2A) (47). In this AE1-derived model, AE2 H1136 resides at the extracellular side of the lipid bilayer and H1160 at the cytosolic side of the lipid bilayer. The adjacent AE2 residues H1144 and H1145 are situated such that they might be accessible to either side of the permeability barrier. Interestingly, mutation of mouse AE1 K832 and K835 (corresponding to AE2 M1140 and K1143) partially or completely inhibited anion transport (32). The results together show the importance of this distal TMD region and its His residues in anion transport and its regulation. These TMD His residues may combine their influence with that of AE2 His residues of the NH2-terminal cytoplasmic domain shown previously to contribute to pH regulation of AE2. The latter include an important contribution by H360 and more limited roles of H314 and H317 (39). In contrast, NH2-terminal cytoplasmic domain residues H78, H79, H81, H82, H423, and H449–H451 can be mutated individually or in combination (in the case of H78, H79, H81, and H82) without impact on AE2 function or regulation by pHh or pHo (unpublished data).

Whereas DEPC treatment induces an acid shift in the pH of wild-type-AE2, mutation of individual His residues within the AE2 TMD can induce an alkaline shift of pH sensitivity. Thus the individual AE2 mutants H1144Y (in which Tyr is the corresponding AE1 residue) and H1145A each exhibited an increase in pH of (Fig. 4B). In addition, AE2 H1145A exhibited enhanced inhibition in response to a fall of pH (Fig. 4D). The altered regulatory properties of these mutants are consistent with accessibility of the proposed reentrant loop to extracellular and intracellular solute. Because the presence of Tyr in the AE1 position corresponding to AE2 H1144 does not suffice to confer AE2-like pHh sensitivity on AE1, but substitution of AE2 H1144 with Tyr does suffice for an alkaline-shifted pH of AE2, it is more likely that the absence of His, rather than the presence of Tyr, alters the AE2 pH-h sensitivity phenotype.

Although the AE2 triple-mutant His3c (Fig. 5A) showed an alkaline-shifted pH of the “component” mutants H1160A and His2 exhibited wild-type pH sensitivity. Thus individual mutation of H1144 and H1145 can alter pH sensitivity of AE2-mediated Cl− transport, whereas mutation of H1160 [corresponding to human AE1 DEPC target H834 (20)] alters pH sensitivity only in cooperation with Ala substitutions at H846 and H849. A similar result was shown for the enhanced pH sensitivity of the multi-His mutants His3c and His5a, in which some of the component single-His mutants had previously shown wild-type pH sensitivity. Multiple-His residues were also shown to comprise a Zn2+-binding site on the dopamine D2 receptor, whereas single-His mutations had no effect (26). The above results suggest the involvement of multiple AE2 His residues in control of pHh and pHi sensitivity of anion transport, although not in a way predicted by the acid-shifted pH of AE2 produced by DEPC treatment of AE2. The inability of hydroxylamine to reverse AE2 inhibition by DEPC suggests that the DEPC modification(s) responsible for inhibition was His imidazole ring cleavage following its bis-carboxethylation (25). The contrast between the DEPC-induced acid shift in AE2 pH of the His substitution-induced alkaline shift in pH of AE2 may represent the different consequences to pH sensitivity of Ala substitution and of imidazole ring cleavage. It is also possible that DEPC treatment or mutation of individual or multiple His residues could alter the oligomeric structure or the interprotomeric interface of AE2 (45, 48), with possible consequences to regulatory properties and/or interaction with regulatory proteins. Alternatively, the acid-shifted pH-sensitivity of DEPC-treated wild-type AE2 may reflect a contribution of DEPC modification of non-His residues such as Cys, Tyr, Trp, or Lys (27–29). Examples of pH sensitivity control by residues other than His include Lys and Leu in renal outer medullary K+ channels (8), glutamate in TRPV5 (44), and a combination of five non-His-charged residues in TASK2 (30).

Muller-Berger et al. (31) postulated that mouse AE1 sustains anion exchange at pH values inhibitory to AE2 function via hydrogen bonding between the protonated conserved AE1 residue H752 and (in a separate helix) the conserved E699, at or near the permeability barrier. However, the role of mouse AE1 E699 in anion translocation suggests that such postulated hydrogen bonding itself might be regulated during the transport cycle. Our test of this proposal in AE2 is consistent with the absence of functional interaction between the two corresponding residues, or with altered folding and/or stability of the double mutant AE2 polypeptide (Fig. 6). Muller-Berger et al. also noted rescue of the mouse AE1 loss-of-function mutant H852Q with the second site reversion mutation K558N. However, a corresponding interaction in AE2 could not be tested in the setting of near-normal function of the AE2 mutant H1160A (Fig. 2C).

In conclusion, we have found that some His residues of the AE2 COOH-terminal TMD are necessary for protein stability and maintenance of basal AE2 activity. We have also found that certain TMD His residues are important for the regulation of AE2 activity by pH. No single His residue, however, seems to function as a unique TMD pH sensor. Rather, the presence of multiple His residues within the TMD defines a range of pH values within which physiological regulation of anion transport may occur. Proton titration of the residues may, for example, be necessary for coordinating appropriate interaction between the TMD and pH-sensitive regions of the cytoplasmic NH2-terminal portion of the AE2 protein (37–39). The opposing shifts in pH of AE2 produced by DEPC treatment and by single- or multiple-His substitution mutations remain unexplained, but the possibility that DEPC exerts additional effects on non-His residues cannot be excluded. Thus, in contrast to some ion channels in which protonation/deprotonation of one or a few His residues suffices to mediate pH sensitivity, the present study highlights a more complex molecular anatomy governing the pH sensitivity of an acid/base transporter.

REFERENCES