Regulation of AE2-mediated Cl\(^-\) Transport by Intracellular or by Extracellular pH Requires Highly Conserved Amino Acid Residues of the AE2 NH\(_2\)-terminal Cytoplasmic Domain

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ABSTRACT We reported recently that regulation by intracellular pH (pH\(_i\)) of the murine Cl\(^-\)/HCO\(_3\)- exchanger AE2 requires amino acid residues 310–347 of the polypeptide’s NH\(_2\)-terminal cytoplasmic domain. We have now identified individual amino acid residues within this region whose integrity is required for regulation of AE2 by pH. \(^{36}\)Cl\(^-\) efflux from AE2-expressing *Xenopus* oocytes was monitored during variation of extracellular pH (pH\(_e\)) with unclamped or clamped pH\(_i\), or during variation of pH\(_i\) at constant pH\(_e\). Wild-type AE2-mediated \(^{36}\)Cl\(^-\) efflux was profoundly inhibited by acid pH\(_i\), with a value of pH\(_{i(50)}\) = 6.87 ± 0.05, and was stimulated up to 10-fold by the intracellular alkalinization produced by bath removal of the preequilibrated weak acid, butyrate. Systematic hexa-alanine [(A)\(_6\)]

blocks substitutions between aa 312–347 identified the greatest acid shift in pH\(_{i(50)}\) value, ~0.8 pH units in the mutant (A)\(_6\)342–347, but only a modest acid-shift in the mutant (A)\(_6\)336–341. Two of the six (A)\(_6\) mutants retained normal pH\(_i\) sensitivity of \(^{36}\)Cl\(^-\) efflux, whereas the (A)\(_6\) mutants 318–323, 336–341, and 342–347 were not stimulated by intracellular alkalinization. We further evaluated the highly conserved region between aa 336–347 by alanine scan and other mutagenesis of single residues. Significant changes in AE2 sensitivity to pH\(_i\) and to pH\(_e\) were found independently and in concert. The E346A mutation acid-shifted the pH\(_{i(50)}\) value to the same extent whether pH\(_i\) was unclamped or held constant during variation of pH\(_e\). Alanine substitution of the corresponding glutamate residues in the cytoplasmic domains of related AE anion exchanger polypeptides confirmed the general importance of these residues in regulation of anion exchange by pH. Conserved, individual amino acid residues of the AE2 cytoplasmic domain contribute to independent regulation of anion exchange activity by pH\(_i\) as well as pH\(_e\).

KEY WORDS: Cl\(^-\)/HCO\(_3\)\(^-\) exchange • weak acids • *Xenopus* oocytes • isotopic flux • pH-sensitive microelectrodes

INTRODUCTION

The SLC4 bicarbonate transporter gene superfamily includes the AE gene family of Na\(^+\)-independent Cl\(^-\)/HCO\(_3\)- exchangers AE1, AE2, and AE3. These anion exchangers contribute to the regulation of cell pH\(_i\), cell volume, tonicity, and intracellular pH\(_i\) homeostasis in vertebrate eukaryotic cells (Alper, 1994; Bevensee et al., 2000; Alper et al., 2002). All AE polypeptides share a highly conserved hydrophobic, polytopic, COOH-terminal transmembrane domain of >500 amino acids (aa), * with a short COOH-terminal cytoplasmic tail capable of binding carbonic anhydrase II (Vince and Reithmeier, 2000; Sterling et al., 2002b). This transmembrane domain is preceded by a less extensively conserved hydrophilic NH\(_2\)-terminal cytoplasmic domain of 400–700 aa (Alper, 1994). The COOH-terminal transmembrane domain suffices to mediate anion exchange in the absence of nearly the entire NH\(_2\)-terminal cytoplasmic domain (Grinstein et al., 1978; Kopito et al., 1989). Although the cytoplasmic NH\(_2\)-terminal domain of AE1 is important for its binding to multiple cytoskeletal proteins, glycolytic enzymes, and hemoglobin (Zhang et al., 2000), the functions of the cytoplasmic NH\(_2\)-terminal domains of AE2 and AE3 remain less extensively investigated.

The polypeptide products of the various AE genes differ in their acute regulation by pH. Native AE1-mediated Cl\(^-\)/HCO\(_3\)- exchange in erythrocytes (Funder and Wieth, 1976) and heterologous AE1-mediated Cl\(^-\)/Cl\(^-\) exchange in *Xenopus* oocytes (Humphreys et al., 1994; Zhang et al., 1996) displays a broad pH versus activity profile, consistent with its primary role in facilitating CO\(_2\)/HCO\(_3\)\(^-\) exchange between the respiring tissues and lungs (Jennings, 1992). In contrast, nonerythroid Na\(^+\)-independent Cl\(^-\)/HCO\(_3\)- exchange in many tissue culture cells is sensitively regulated by changes in pH\(_i\) (Reinertsen et al., 1988), consistent with its proposed
role in recovery from alkaline loads. Similarly, the recombinant nonerythroid anion exchanger AE2 is highly sensitive to changes in pH when expressed in tissue culture cells (Lee et al., 1991; Jiang et al., 1994) or in Xenopus oocytes (Humphreys et al., 1994; Zhang et al., 1996; Stewart et al., 2001). In contrast, recombinant AE3 expressed in 293 cells has been reported to be insensitive to changes in pH (Sterling and Casey, 1999).

Zhang et al. (1996) compared regulation by pH of anion influx mediated by AE1 and AE2 in Xenopus oocytes, and localized to the AE2 transmembrane domain a “pH sensor site” which conferred increased pH sensitivity of anion transport. These structure-function studies demonstrated that the extracellular proton sensitivity (pH$_{50}$ value) of AE2-mediated $^{36}$Cl$^{-}$ influx ($\sim$7.0 under conditions of unclamped pH) was acid-shifted 0.7 pH units by truncation of the cytoplasmic NH$_2$-terminal 510 aa. This result suggested a role for the cytoplasmic NH$_2$-terminal domain in regulation of AE2 transport by changing pH, and localized between aa 99 and 510 of the 705 aa AE2 NH$_2$-terminal cytoplasmic domain, a “pH modifier site” that modulated the pH$_{50}$ value of the transmembrane domain.

More recently, we demonstrated that variation of pH$_o$ regulates AE2 activity in efflux assays and requires the integrity of two noncontiguous regions in the NH$_2$-terminal cytoplasmic domain, encompassing aa 328–347 and aa 391–510 (Stewart et al., 2001). We further showed by varying pH$_i$ at constant pH$_o$, that removal of the NH$_2$-terminal 310 aa greatly reduced AE2 regulation by pH$_i$, but was without effect on AE2 regulation by varying pH$_o$. In the present work we have defined individual amino acid residues within the NH$_2$-terminal cytoplasmic domain whose mutation alters the regulation of AE2-mediated Cl$^{-}$ transport activity by changing pH$_i$, with constant or minimally changing pH$_o$, and by varying pH$_i$ at constant pH$_o$. We demonstrate that a delimited, highly conserved region of the AE2 NH$_2$-terminal cytoplasmic domain is involved in AE2 regulation by both pH$_i$ and pH$_o$. We further demonstrate that some of these conserved residues in corresponding regions of related anion exchanger polypeptides contribute similarly to their functional regulation by pH$_i$.

**MATERIALS AND METHODS**

**Reagents**

Na$^{36}$Cl was purchased from ICN. Other chemical reagents were of analytical grade and obtained from Sigma-Aldrich, Calbiochem, or Fluka. Restriction enzymes and T4 DNA ligase were from New England BioLabs, Inc. Taq DNA polymerase and dNTPs were from Promega.

**Solutions**

ND-96 medium consisted of (in mM): 96 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, and 2.5 sodium pyruvate, pH 7.40. Flux media lacked sodium pyruvate. pH values of 7.0, 8.0, and 8.5 in room air flux media were achieved with 5 mM HEPES. 5 mM Mes was used was used for room air flux media of pH values 5.0 and 6.0. In Cl$^{-}$-free solutions, NaCl was replaced isosmotically with 96 mM sodium isethionate, and equimolar K, Ca, and Mg gluconate were substituted for the corresponding Cl$^{-}$ salts. CO$_2$/HCO$_3^-$-buffered solutions of pH 7.4 were saturated with 5% CO$_2$/95% air at room temperature for $\sim$1 h and differed from Cl$^{-}$-free ND-96 in replacement of 24 mM sodium isethionate with 24 mM NaHCO$_3$.$^-$, pH of CO$_2$/HCO$_3^-$-buffered solutions was verified before each experiment. Addition to flux media of the weak acid salt sodium butyrate was in equimolar substitution for NaCl.

**Mutant AE2 cDNAs**

Murine AE2 encoded in plasmid pAX (Zhang et al., 1996) was used as template for PCR. The AE2 hexa-Ala bloc substitution mutants (A)$_{312-317}$, (A)$_{318-325}$, (A)$_{324-329}$, (A)$_{330-335}$, (A)$_{336-341}$, and (A)$_{342-347}$ were constructed by a four primer PCR method as described (Zhang et al., 1996; Chernova et al., 1997a,b; Stewart et al., 2001). Single point mutations were constructed by the same method to generate single residue nonsense mutants. Oligonucleotide primers were obtained from Bio-synthesis; primer sequences are available upon request. Integrity of PCR products and ligation junctions was confirmed by DNA sequencing of both strands.

**cRNA Expression in Xenopus Oocytes**

Mature female Xenopus (NASCO) were maintained and subjected to partial ovariectomy as described (Humphreys et al., 1994). Stage VI oocytes were manually defolliculated following incubation of ovarian fragments with 2 mg/ml collagenase A (Boehringer) for 60 min in ND-96 solution containing 50 ng/ml gentamycin and 2.5 mM sodium pyruvate. Oocytes were injected on the same day with cRNA or with 50 nl H$_2$O. Capped cRNA was transcribed from linearized cDNA templates with the T7 MEGA-script kit (Ambion), and resuspended in diethylpyrocarbonate-treated water. RNA integrity was confirmed by agarose gel electrophoresis in formaldehyde, and RNA concentration was estimated by A$_{260}$. Injected oocytes were then maintained for 2-4 d at 19°C.

**$^{36}$Cl$^{-}$ Efflux Measurements**

Individual oocytes in Cl$^{-}$-free ND-96 were injected with 50 nl of 130 mM Na$^{36}$Cl (10,000–12,000 cpm). After a 3–10 min recovery period, the efflux assay was initiated by transfer of individual oocytes to 6 ml borosilicate glass tubes, each containing 1 ml efflux solution. At intervals of 3 min, 0.95 ml of this efflux solution was removed for scintillation counting and replaced with an equal volume of fresh efflux solution. After completion of the assay with a final efflux period in the presence of the anion transport inhibitor 4,4'-di-isothiocyanato-stilbene-2,2'-disulfonic acid (DIDS; 200 µM), each oocyte was lysed in 100 µl of 2% SDS. Samples were counted for 3–5 min such that the magnitude of 2SD was <5% of the sample mean.

Experimental data were plotted as ln (percentage cpm remaining in the oocyte) versus time. $^{36}$Cl$^{-}$ efflux rate constants were measured from linear fits to data from the last three time points sampled for each experimental condition. All single time point values for $^{36}$Cl$^{-}$ efflux from AE2 cRNA-injected oocytes into chloride medium exceeded 150 cpm. Efflux cpm values for water-injected oocytes (40–90 cpm) were indistinguishable from those for AE2 cRNA-injected oocytes in the presence of DIDS, and both less than threefold higher than machine background values (typically 20 cpm, peak 30 cpm). Within each experiment,
water-injected and AE2 cRNA–injected oocytes from the same frog were subjected to parallel measurements. On each experimental day, activity of tested mutant AE2 polypeptides was compared with wt AE2 activity at pH 7.4. Each AE2 mutant was tested in oocytes from at least three frogs.

Individual oocytes maintained in Cl−-free solution at pHo 7.4 were exposed sequentially to (Cl−-containing) ND-96 at pH 5.0, 6.0, 7.0, 8.0, and 8.5, then to solution of pH 8.5 in the presence of DIDS. During this variation of bath pH between pH 5.0 and 8.5, oocyte pH varied between extreme values of ~7.1 and ~7.6, but usually to a lower degree (Zhang et al., 1996; Stewart et al., 2001).

Rate constants measured at each pHo value for wt AE2 and for the tested AE2 mutants in each individual experiment were fit (Sigma Plot) to the following first-order logistic sigmoid equation:

\[
\frac{v}{V_{\text{max}}} = \frac{(V_{\text{max}} \times 10^{-k})/(10^{-k} + 10^{-x})}{d},
\]

where \(v\) = AE2-mediated Cl− efflux rate constant, \(V_{\text{max}}\) = the maximum AE2-mediated Cl− efflux rate constant, \(x\) = pHo at which rate constant was measured, \(K = \text{pH}_{50}\), the pHo at which \(v\) is half-maximal, and \(d = 0\) in all cases except that of the AE2 mutant (At)342–347, for which level of significance was \(P < 0.05\).

Measurement of Oocyte pHo

Oocyte pHo was measured during bath superfusion using pH microelectrodes as described previously (Romero et al., 1997; Stewart et al., 2001). Oocyte pHo was measured during bath superfusion using BCECF ratio fluorimetry, also as described previously (Zhang et al., 1996).

RESULTS

\textbf{AE2-mediated Cl− Transport Is Sensitive to Changing pHo when pHi Is Clamped}

We have shown previously that AE2 function in Xenopus oocytes is highly sensitive to changes in pHo, within the physiological range when pHo is held constant, using introduction and removal of the permeant weak acid butyrate that is neither inhibitor nor substrate of AE2 (Stewart et al., 2001). Our earlier studies had demonstrated the high sensitivity of AE2 function to changes in pHo (Zhang et al., 1996; Stewart et al., 2001), but under conditions in which near-steady-state pHi was unclamped and changed in parallel with pHo. Although the range of pHo change in these experiments far exceeded the range of unclamped pHo change, the ability of changing pHo alone to regulate AE2 function at constant pHi remained in question. We therefore devised conditions in which variation of pHo in tandem with variation of bath butyrate concentration might effectively clamp pHo during changes in pHo.

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Fig. 1 A depicts microelectrode measurements of changing pHo in a representative AE2-expressing oocyte subjected to sequential 15 min exposures to varying pHo, matching the 36Cl− efflux protocol. Exposure of a different AE2-expressing oocyte (Fig. 1 B) to the same pattern of pHo change while simultaneously varying bath butyrate concentration almost completely damped the corresponding changes in pHo (Fig. 1 C). Fig. 1 C summarizes the pHo versus pHi relationship in the absence (top slope = 0.02) and presence of adjusted butyrate concentrations (bottom slope = 0.002). Only in the absence of changing [butyrate] did oocyte pHo change during an increase in pHo from 5.0 to 8.5 (\(P = 0.03\), Student’s unpaired \(t\) test). Parallel measurement of oocyte pHo by BCECF ratio fluorimetry in the absence (\(n = 4–8\)) or presence of step changes in graded extracellular [butyrate] (\(n = 6–12\)) similarly showed that butyrate damped pHo change in response to changing pHo, (unpublished data). Thus, simultaneous variation of bath butyrate concentration and pHo allowed evaluation of AE2 regulation by changing pHo while pHi remained nearly constant (nominal “pHi clamp”).

Fig. 1, D–F, show traces of 36Cl− efflux from oocytes expressing wild-type (wt) AE2 and maintained under pHi clamp conditions during three protocols of pHo change: sequentially from 5.0 to 8.5 (1D), sequentially
from 8.5 to 5.0 (1E), and nonsequentially (1F). As shown in Fig. 1 G, the dependence of AE2 activity on pH was essentially indistinguishable for these three pH clamp protocols, and none of the three differed significantly from the pH dependence of AE2 activity measured in the absence of pH clamp (Fig. 1 H). These experiments confirm that changing pH can regulate AE2-mediated Cl⁻ transport even when pH remains essentially unchanged. Moreover, imposition of pH clamp conditions does not alter the apparent pH dependence of AE2 activity.

**Regulation of AE2 NH₂-terminal Hexa-alanine Substitution Mutants by Varying Bath pH**

We have shown previously that the region encompassing amino acids 310–347 within the NH₂-terminal cytoplasmic domain of the murine AE2 anion exchanger is required for normal regulation of AE2-mediated Cl⁻ transport by pH, and by pH (Stewart et al., 2001). We studied this region in greater detail through the systematic substitution of hexa-alanine blocs into consecutive six-amino acid stretches of AE2, as illustrated in the schematic of Fig. 2 A. Fig. 2 B presents the ³⁶Cl⁻ efflux rate constants measured at pH 7.4 exhibited by oocytes expressing AE2 hexa-alanine bloc substitutions. With the sole exception of (A)₃₁₈–₃₂₃, all mutants exhibited ³⁶Cl⁻ efflux activity statistically indistinguishable from that of wild-type AE2. The low ³⁶Cl⁻ efflux activity of the (A)₃₁₈–₃₂₃ mutant at pH 7.4 precluded subsequent evaluation of its regulation by pH, (Fig. 2 E), but sufficed to evaluate its regulation by pH (Fig. 2 G).

To define those portions of the region encompassing residues 312–347 with importance for AE2 regulation by pH, we measured ³⁶Cl⁻ efflux activity of the hexa-Ala bloc mutants under conditions of varying pH, at unclamped pH. Fig. 2 C shows a representative ³⁶Cl⁻ efflux trace from three AE2-expressing oocytes and from one water-injected oocyte as a function of sequentially increasing pH. Wild-type AE2–mediated ³⁶Cl⁻ efflux was minimal at low pH, increased at higher pH values, and was inhibited by 200 μM DIDS added at the final pH of 8.5. Whereas AE2 (A)₃₃₀–₃₃₅ and 3₄₂–₃₄₇ exhibited a pattern of ³⁶Cl⁻ efflux similar to that of wild-type AE2, (A)₃₄₂–₃₄₇ showed reduced inhibition of ³⁶Cl⁻ efflux activity at lower pH compared with wild-type AE2. Fig. 2 D profiles ³⁶Cl⁻ efflux activity (normalized as described in MATERIALS AND METHODS) as a function of pH for wild-type AE2 and two representative hexa-Ala bloc mutants, (A)₃₃₀–₃₃₅ and (A)₃₄₂–₃₄₇. The pHₐ value at which the rate constant for wild-type AE2–mediated ³⁶Cl⁻ efflux was half-maximal [pHₐ(50)] was 6.87 ± 0.05 (n = 3; Fig. 2 D and E). The hexa-Ala bloc mutant (A)₃₃₀–₃₃₅ exhibited pHₐ dependence indistinguishable from that of wild-type AE2, whereas the pHₐ(50) value of the AE2 mutant (A)₃₄₂–₃₄₇ was shifted to a more acidic pH value of 6.11 ± 0.07 (n = 12, P < 0.05). ² Fig. 2 E summarizes the pHₐ(50) values measured as shown in Fig. 2 C and 2 D for all hexa-Ala bloc mutants. The data suggest that amino acid residues 342–347 are particularly important for regulation of AE2 by pH. The hexa-Ala bloc mutant (A)₃₃₆–₃₄₇ displayed a pHₐ(50) value only marginally acid shifted from that of wild-type AE2, as assessed by all pairs Tukey-Kramer analysis. Nonetheless, the high degree of conservation among these amino acid residues (see below) warranted their inclusion among those investigated in greater detail.

**Regulation of AE2 NH₂-terminal Hexa-alanine Substitution Mutants by Varying pH at Constant pH**

We next investigated regulation of the same set of AE2 mutants by changing pH at constant pH, using the weak acid butyrate as described previously (Stewart et
Fig. 2 F shows a representative efflux trace in which \(^{36}\text{Cl}^-\) efflux activities of wild-type AE2 and of AE2 (A)\(^{63}30–335\) are reduced at low pH \(i\) and subsequently stimulated when pH \(i\) is elevated (by butyrate removal). In contrast, AE2 (A)\(^{63}42–347\)-mediated \(^{36}\text{Cl}^-\) efflux activity was insensitive to changes in pH \(i\) imposed by butyrate addition and removal. Fig. 2 G summarizes similar experiments for all hexa-Ala bloc mutants, expressed as fold stimulation of \(^{36}\text{Cl}^-\) efflux rate constant after butyrate removal. The data reveal the importance of two discrete regions encompassing aa 318–323 and 336–347 for wild-type regulation of AE2-mediated Cl\(^-\) transport by varying pH \(i\). Hexa-Ala substitutions in these regions reduced AE2 stimulation by butyrate removal from the wild-type 10-fold to below 1.5-fold (P < 0.002, Student’s unpaired t test). In contrast, the mutants (A)\(^{63}324–329\) and (A)\(^{63}330–335\) retained wild-type stimulation. The (A)\(^{63}312–317\) mutant retained significant, though reduced, sensitivity to pH \(i\) elevation, showing 3.5-fold stimulation by butyrate removal (P < 0.002).

**Determination of Individual Amino Acid Residues Important for AE2 Regulation by pH**

The hexa-Ala bloc substitution mutants implicated AE2 aa 336–347 as important for regulation of AE2 activity by pH. To define the individual residues required for this regulation, we tested the functional properties of point mutants created as part of a modified alanine scan of this region (Fig. 3 A). Threonine 339, a weak candidate...
PKC site, was mutated to glutamate and (not depicted) to valine. Alanine 340 was mutated to glycine. Fig. 3 B shows that none of the mutants exhibited significant reduction of $^{36}\text{Cl}^-/\text{H}^{11002}$ efflux activity at pHo 7.4. Fig. 3 C compares representative $^{36}\text{Cl}^-$ efflux timecourse for three wild-type AE2 oocytes (bottom traces) and one H2O-injected oocyte (top trace) measured during stepwise increases in pHo (top bar). (D) Regulation by pHo of normalized $^{36}\text{Cl}^-$ efflux from oocytes expressing wild-type AE2 (filled circles), mutant AE2 (A)$_6$330–335 (open circles), and mutant AE2 (A)$_6$342–347 (filled inverted triangles). Values are means ± SEM; curves were fit to data as described in MATERIALS AND METHODS. (E) pHo(50) values exhibited by wild-type AE2 and the indicated AE2 (A)$_6$ mutants, calculated from fits of pHo versus $^{36}\text{Cl}^-$ efflux activity plots such as that in D for n individual oocytes (means ± SEM). Asterisk indicates P < 0.05. AE2 (A)$_6$318–323 Cl$^-$ efflux rate constant (B) was too low for measurement of an inhibitory pHo(50) value (n.d., not done). (F) Representative time course of $^{36}\text{Cl}^-$ efflux from oocytes expressing wild-type AE2 (closed circles), AE2 mutant (A)$_6$330–335 (open circles), and AE2 mutant (A)$_6$342–347 (filled inverted triangles) during elevation of pHi by removal of bath butyrate (40 mM) and subsequent inhibition by DIDS (200 μM). (G) Mean fold stimulation of Cl$^-$ efflux (± SEM) after bath butyrate removal from n oocytes expressing wild-type AE2 or the indicated AE2 (A)$_6$ mutants. Asterisk indicates P < 0.002.
$^{36}$Cl$^{-}$ efflux by butyrate removal was indistinguishable from wild-type level (black bars) in 4 of the 12 mutants, and was only slightly reduced for K344A. In contrast, 7 of the 12 alanine substitution mutants exhibited severely attenuated stimulation by butyrate removal (gray bars for W336, E338, R341, W342, I343, F345, and E347) (P $\leq$ 0.05, Student’s unpaired t test).

The AE2 mutants W336A, R341A, and E347A were concordant in their mutant phenotypes of regulation by pH$_{o}$ and by pH$_{i}$, whereas mutants R337A, T339E, T339V (not depicted), A340G, and K344A were concordant in their wild-type or near wild-type phenotypes. Four AE2 mutants, E338A, I343A, F345A, and W342A, were insensitive to changing pH$_{i}$ while maintaining wild-type or near wild-type pH$_{o}$ sensitivity (Fig. 3, D and F). AE2 E346A was the only mutant that displayed acid-shifted pH$_{o}$ sensitivity (Figs. 3 C and 2 D) together with wild-type pH$_{i}$ sensitivity (Fig. 3 E). Alanine substitution of the neighboring residue E347 yielded both acid-shifted pH$_{o}$ sensitivity and loss of pH$_{i}$ sensitivity. These data define several amino acid residues whose mutation can alter the wild-type regulation of AE2 activity by pH. The differential consequences for regulation by pH$_{o}$ and by pH$_{i}$ based on the point mutations studied further supports the hypothesis that AE2 activity is regulated independently by extracellular and by intracellular protons.

**The Effects of Missense Mutations on AE2 Sensitivity to Changing pH$_{o}$ Are Not Altered Under Conditions of pH$_{i}$ Clamp**

We compared the effect of the mutation E346A on pH$_{o}$ dependence of AE2 function in conditions of “unclamped” and “clamped” pH$_{i}$. The pH$_{o}$ versus Cl$^{-}$
Regulation of AE2-mediated Anion Exchange by pH

The regulation of AE2-mediated anion exchange by pH is critical for maintaining cellular homeostasis. Changes in extracellular pH (pHo) can alter the activity of AE2, a cytosolic anion exchanger that plays a key role in chloride (Cl\(^{-}\)) and bicarbonate (HCO\(_3^{-}\)) transport. The activity profile of AE2 is sensitive to pHo, with pHo(50) values for wild-type AE2 and mutant AE2 E346A differing under various conditions.

**Figure 4** illustrates the importance of AE2 residue E346 for pHo sensitivity at constant pHi. (A) pHo versus activity profile for oocytes expressing wild-type AE2 (filled circles) or AE2 E346A (open circles). (B) pHo versus activity profile for oocytes expressing wild-type AE2 (filled circles) or AE2 E346A (open circles), measured under the panel B conditions of nominal “pHi clamp.” (C) Comparison of pHo(50) values for wild-type AE2 and AE2 E346A. (D) Comparison of pHo(50) values for wild-type AE2 and AE2 E346A in conditions of nominal “pHi clamp.” Values in C and D are means ± SEM for n oocytes.

Efflux activity profile in the absence of butyrate (Fig. 4 A) reveals respective pHo(50) values for wild-type and mutant AE2 of 6.76 ± 0.05 (n = 18) and 6.10 ± 0.07, n = 14, P < 0.05), similar to values presented in Fig. 3 E. Fig. 4 B shows that this difference between the pHo sensitivity of mutant and wild-type AE2 did not change when pHo was varied during conditions of “pHi clamp.” In these conditions, the difference between the pHo(50) values of wild-type AE2 (6.97 ± 0.06, n = 7), and of AE2 E346A (6.29 ± 0.13, n = 3) was maintained (P < 0.05). Fig. 4 C and D, summarize the pHo sensitivity data for wild-type AE2 and E346A and further confirm that AE2-mediated Cl\(^{-}\) transport can be regulated by changing pHo when pHi remains nearly constant. Moreover, at least one residue of the AE2 NH2-terminal intracellular domain among the several important for AE2 regulation by changing pHo is also important for regulation by changing pHo at near constant pHi.

**Comparison of Charge and Steric Effects at AE2 Residues 346 and 347**

Mutation of any one of the three glutamate residues among aa 336–347 of AE2 altered at least one aspect of AE2 regulation by pH. We therefore compared the consequences of substituting Ala in place of E346 and E347 with those of the charge-conserving Asp substitution (Fig. 5 A). Mutant transport activity at pH 7.4 was not significantly reduced from wild-type level (not depicted). The 36Cl\(^{-}\) efflux traces of Fig. 5 B reveal that, in contrast to wild-type AE2, neither mutant E346D nor E347D was sensitive to the intracellular alkalinization produced by butyrate removal. Fig. 5 C shows a similar pHi phenotype for the double mutant E346D/E347D and emphasizes its contrast with the wild-type pHi sensitivity of the mutant E346A. These data suggest that both structure and charge of the amino acid side chain at position 346, if not equally so at position 347, can be important in conferring wild-type regulation of AE2 by pH.

**Regulation of AE2-mediated Cl\(^{-}\)/HCO\(_3^{-}\} Exchange by pH**

The above mutagenesis experiments investigating structure-function relationships in the AE2 anion exchanger examined effects on nominal Cl\(^{-}\)/Cl\(^{-}\} exchange activity in room air. However, the experimental advantages offered by measurement of Cl\(^{-}\)/Cl\(^{-}\} exchange in *Xenopus* oocytes may not reflect the physiological function of Cl\(^{-}\)/HCO\(_3^{-}\} exchange by AE2 in intact vertebrates or chordates. We therefore investigated whether the findings of Fig. 3 concerning the influence of single amino acid residues on the regulation of AE2-mediated Cl\(^{-}\)/Cl\(^{-}\} exchange by pHi might also apply to AE2-mediated Cl\(^{-}\)/HCO\(_3^{-}\} exchange. Fig. 6 A shows representative 36Cl\(^{-}\} efflux traces of oocytes subjected to the butyrate removal assay in Cl\(^{-}\}-free solutions equilibrated with 5% CO\(_2\)/24 mM HCO\(_3^{-}\}, pH 7.40. Whereas addition and removal of 40 mM butyrate first reduced and then stimulated wild-type AE2–mediated 36Cl\(^{-}\} efflux, AE2 E347A–mediated Cl\(^{-}\)/HCO\(_3^{-}\} exchange was insensitive to butyrate-associated changes in pHi. These data, summarized in Fig. 6 B, suggest that at least some of the AE2 structure-function relationships derived from study of Cl\(^{-}\)/Cl\(^{-}\} exchange regulation by pHi in *Xenopus* oocytes apply equally to regulation of AE2-mediated Cl\(^{-}\)/HCO\(_3^{-}\} exchange. The result also suggests that pHi rather than [HCO\(_3^{-}\}] regulates AE2 activity in the butyrate removal assay.
The Amino Acid Residues Important for Regulation of AE2 by pHi and by pHo Are Conserved Among AE2-related Anion Transporters

Alignment of the AE2 region shown above to be important to AE2 regulation by pHo and pHi with corresponding regions of other SLC4 bicarbonate transporter polypeptides reveals absolute sequence conservation with the Na+/H+ independent chloride bicarbonate exchanger, AE3. This AE2 sequence also shows a high degree of sequence identity with Na+/H+ independent bicarbonate cotransporters, including several not known to transport chloride (Fig. 7). In contrast, no homologous region is present in the very divergent NH2-terminal putative cytoplasmic domain of BTR1 (Parker et al., 2001). The acute regulation by pH of most of these anion transporters has yet to be studied. However, the evident sequence conservation suggests that these residues may make similar contributions to regulation of these AE2-related anion transporters. We tested this hypothesis for regulation by pHi of the Na+/H+ independent anion exchangers, AE3 and AE1.

As shown in Fig. 8, B and C, cardiac AE3 (cAE3) can be stimulated by intracellular alkalinization, whereas AE1 is insensitive to this change in pHi. The cAE3 residues E150 and E151 (corresponding to AE2 E346 and E347) are important to regulation of Cl−/HCO3− exchange by pHi. (A) Representative time course of Cl−/HCO3− exchange in oocytes previously injected with water (closed squares) or with cRNA encoding wild-type AE2 (filled circles) or the AE2 mutant E347A (open circles). 36Cl− efflux into bath solution containing HCO3− as the only nominal permeant anion is measured first in the presence of butyrate, then after its removal and during subsequent inhibition by DIDS (200 μM). (B) Cl−/HCO3− exchange mediated by wild-type AE2 is stimulated by intracellular alkalinization, whereas Cl−/HCO3− exchange mediated by the mutant AE2 E347A is not stimulated. This phenotype resembles that for Cl−/Cl− exchange mediated by these polypeptides. Values are means ± SEM for n oocytes. Asterisk indicates P < 0.05 (Student’s unpaired t test).
E347) were then mutated to alanine, individually and in tandem. Fig. 8, D and E, show that stimulation of cAE3-mediated ⁸⁶Cl⁻ efflux by intracellular alkalinization is severely attenuated by each of these mutations. This suggests that pHᵢ regulates cAE3 in oocytes as suggested previously for brain AE3 (bAE3) in 293 cells (Lee et al., 1991), and that such regulation requires integrity of residues corresponding to those required by AE2.

The AE1 NH₂-terminal cytoplasmic domain shows partial conservation of the AE2 residues demonstrated above to be critical for normal regulation of AE2 by pHᵢ (Fig. 7), yet wild-type AE1-mediated Cl⁻ transport is pHᵢ insensitive. However, the AE1_cryo/AE2_memb chimera exhibits regulation not only by changing pHᵢ (Zhang et al., 1996) but also by changing pHᵢ (Fig. 9, B and C). In contrast, the converse chimera AE2_cryo/AE1_memb exhibited an AE1-like phenotype, whether in response to changing pHᵢ (Zhang et al., 1996) or to intracellular alkalinization by butyrate removal (Fig. 9 C). The NH₂-terminal AE2 truncation mutant Δₓ659 displayed similar lack of stimulation by intracellular alkalinization. Thus, stimulation by intracellular alkalinization requires defined residues from the NH₂-terminal cytoplasmic domain in combination with yet-to-be defined portions of the AE2 transmembrane domain. The pHᵢ sensitivity of the AE1_cryo/AE2_memb chimera thus allowed experimental test of the potential importance of the AE1 region depicted in Fig. 7 when present in the “permissive environment” of proximity to the AE2 transmembrane domain (Fig. 9 A). Fig. 9, B and C, show that introduction into the AE1_cryo/AE2_memb chimera of the double Ala substitution mutation E99A/E100A severely attenuated stimulation of Cl⁻ efflux by intracellular alkalinization (n = 14, P < 0.05).

**DISCUSSION**

Ion exchanger and cotransporter polypeptides are important contributors to cellular and compartmental pH homeostasis. This homeostasis requires that transporters of H⁺ and HCO₃⁻, as well as pH-sensitive transporters of other solutes, be subject to regulation by pHᵢ, and in some cases also by pHᵢ. Regulation by pHᵢ and by pHᵢ of several K⁺, Na⁺, and Ca²⁺ channels is partially understood at the molecular level in terms of titration by protons of critical amino acid residues, similar to classical models invoked for pH-regulatory transporters (Gunn et al., 1973; Milanick and Gunn, 1984). However, the mechanisms by which pH acutely regulates the activities of pH-regulatory ion exchangers and cotransporters remain for the most part obscure. Even regulation by pHᵢ of the Na⁺/H⁺ exchanger, NHE1, perhaps the most extensively studied among these transporters, is not yet understood at the level of individual amino acid residues. Similarly, the molecular mechanisms by which protons regulate activity of Cl⁻/HCO₃⁻ exchangers of the SLC4 gene family have until recently remained uninvestigated.

The current work has shown that AE2-mediated Cl⁻ transport is sensitive to changing pHᵢ under conditions of constant pHᵢ (Fig. 1), complementing our earlier demonstration that AE2 activity is also sensitive to changing pHᵢ at constant pHᵢ (Stewart et al., 2001). We have found two stretches of amino acid sequence in the middle of the AE2 NH₂-terminal cytoplasmic domain (aa 318–323 and aa 336–347) that are required for wild-type pHᵢ sensitivity of AE2-mediated anion exchange in Xenopus oocytes (Fig. 2). These two regions are present in all five NH₂-terminal variant AE2 polypeptide products arising from alternate promoter usage or splicing of the AE2 gene (Stuart-Tilley et al., 1998; Medina et al., 2000). Alanine scan mutagenesis of aa 336–347 has identified individual amino acid residues whose mutation modulates AE2 regulation by pHᵢ, or abolishes AE2 regulation by pHᵢ under the conditions studied (Fig. 3). The AE2

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**Table 7.** Amino acid sequence alignment of AE2 aa 336–347 with corresponding regions of other members of the SLC4 bicarbonate transporter superfamily. Asterisks mark conserved residues in which mutation to alanine alters regulation of AE2-mediated Cl⁻ transport by pHᵢ (butyrate removal method). Boldface marks conservation of those pHᵢ-related residues. E346 is marked by an asterisk to indicate change to a mutant pHᵢ phenotype when mutated to aspartate. BTR1 lacks this conserved region (alignment as presented in Parker et al., 2001). EMBL/GenBank/DDJB accession nos. for these sequences are: mAE2 (murine anion exchanger 2; J04036), mAE3 (murine anion exchanger 3; AAA40692), mNCBE (murine sodium-dependent chloride/bicarbonate exchanger; BAB17922), hNCBE1 (human sodium-dependent chloride/bicarbonate exchanger 1; AF069512), DmNDAE1 (D. melanogaster sodium-dependent anion exchanger; AF047468), hNBCe2 (human electrogenic sodium bicarbonate cotransporter 2; AF293537), hNBCc1 (human electrogenic sodium bicarbonate cotransporter 1; AF007216), rNBCn1 (rat electroneutral sodium bicarbonate cotransporter 1; AF070475), hAE4 (human anion exchanger 4; AF332961), hAE1 (human anion exchanger 1; CAA31128), mAE1 (murine anion exchanger 1; J02756), trAE1 (trout anion exchanger 1; Z50848), hBTR1 (human bicarbonate transporter-related protein 1; AF336127).

|    | mAE2 | mAE3 | mNCBE | hNCBE1 | DmNDAE1 | hNBCe2 | hNBCc1 | rNBCn1 | hAE4 | hAE1 | mAE1 | trAE1 | hBTR1 |
|----|------|------|-------|--------|---------|--------|--------|--------|------|------|------|-------|--------|
| A  | W     | R     | E      | T      | A       | R      | W      | I      | K    | F    | E    | Q     | T      |
| B  | E     | E     | T      | R      | W      | I      | K      | F      | E    | E    | E    | E     | E      |
| C  | T     | R     | W      | I      | K      | F      | E      | E      | E    | E    | E    | E     | E      |
| D  | R     | W     | I      | K      | F      | E      | E      | E      | E    | E    | E    | E     | E      |
| E  | W     | R     | E      | T      | A       | R      | W      | I      | K    | F    | E    | Q     | T      |

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**Figure 7.** Amino acid sequence alignment of AE2 aa 336–347 with corresponding regions of other members of the SLC4 bicarbonate transporter superfamily. Asterisks mark conserved residues in which mutation to alanine alters regulation of AE2-mediated Cl⁻ transport by pHᵢ (butyrate removal method). Boldface marks conservation of those pHᵢ-related residues. E346 is marked by an asterisk to indicate change to a mutant pHᵢ phenotype when mutated to aspartate. BTR1 lacks this conserved region (alignment as presented in Parker et al., 2001). EMBL/GenBank/DDJB accession nos. for these sequences are: mAE2 (murine anion exchanger 2; J04036), mAE3 (murine anion exchanger 3; AAA40692), mNCBE (murine sodium-dependent chloride/bicarbonate exchanger; BAB17922), hNCBE1 (human sodium-dependent chloride/bicarbonate exchanger 1; AF069512), DmNDAE1 (D. melanogaster sodium-dependent anion exchanger; AF047468), hNBCe2 (human electrogenic sodium bicarbonate cotransporter 2; AF293537), hNBCc1 (human electrogenic sodium bicarbonate cotransporter 1; AF007216), rNBCn1 (rat electroneutral sodium bicarbonate cotransporter 1; AF070475), hAE4 (human anion exchanger 4; AF332961), hAE1 (human anion exchanger 1; CAA31128), mAE1 (murine anion exchanger 1; J02756), trAE1 (trout anion exchanger 1; Z50848), hBTR1 (human bicarbonate transporter-related protein 1; AF336127).
amino acid residues required for wild-type regulation by pHi, and pHi are not identical (Fig. 3). Although mutations in some residues altered both modes of regulation, mutations in other residues altered selectively either the response to changing pHo or the response to changing pHi (Figs. 3–5). At least one structural requirement for regulation of AE2-mediated Cl\(^{-}/HCO_3^-\)/Cl\(^{-}/HCO_3^-\) exchange by pHo applies equally to pHo regulation of Cl\(^{-}/HCO_3^-\)/HCO_3^- exchange (Fig. 6). Regulation by pHo of Cl\(^{-}/Cl^-\)/Cl\(^{-}/Cl^-\) exchange mediated by the related polypeptide cAE3 (Fig. 8) and by the AE1cys/AE2memb chimera (Fig. 9) was severely attenuated by mutations corresponding to those which alter AE2 regulation.

Defined individual amino acid residues contribute to the independent regulation of AE2-mediated Cl\(^{-}\)/Cl\(^{-}\) transport by pHo, and by pHi. Earlier studies with large deletions and chimeras (Zhang et al., 1996) and with smaller NH2-terminal and internal deletions (Stewart et al., 2001) focused attention upon AE2 residues 312–347 as a region required for wild-type AE2 regulation by independent variation of pHo and of pHi. Subsequent hexa-Ala bloc substitution mutagenesis (Fig. 1) within this NH2-terminal region focused attention upon residues 336–347. Systematic alanine scan mutagenesis of this region (Fig. 3) then identified several individual amino acid residues important for AE2 regulation by pHo only, by pHi only, or for both. Individual mutation to alanine of the four residues W336, R341, E346, and E347 significantly shifted the AE2 pHo(50) to more acidic values. Uniquely among these four mutants, AE2 E346A retained wild-type pHi sensitivity. However, individual mutation to alanine of the seven residues W336, E338, R341, W342, I343, F345, and E347 eliminated AE2 regulation by pHi in the butyrate removal assay. Among these, E338A, W342A, I343A, and F345A retained wild-type or near wild-type pHo sensitivity. These distinct effects of individual mutations upon regulation of AE2 by pHo and by pHi, reinforce the hypothesis that AE2 independently senses and responds to protons on both sides of the plasma membrane.

**Contribution of Cytoplasmic Amino Acid Residues to AE2 Regulation by pHo**

Regulation by pHo of AE2-mediated anion transport, itself a pH-regulatory process in the physiological pres-
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ence of CO2/bicarbonate buffers, is intuitively reasonable across the normal range of pHi. The NH2-terminal cytoplasmic domain of AE2 is appropriately situated to contribute to such regulation. AE2’s function as an acid loader in physiological conditions suggests a rationale for inhibition of AE2-mediated anion transport not only by reduced pHi, but also by reduced pHo. AE2 itself mediates or contributes to the intracellular acidification produced by extracellular acidification. Regulation of AE2 by pHo would allow the cell to down-regulate acid loading when first faced with extracellular acidosis, preferably before the cell has begun to experience adverse effects of an intracellular acid load. Nonetheless, an important role for NH2-terminal cytoplasmic domain residues is less easily envisioned for AE2’s response to changing pHo, but to changing pHi.

pHo regulates AE2-mediated Cl\(^{-}/HCO_3\) transport across a physiological range with a pHo(50) value of ~6.9, in contrast to the AE1 pHo(50) values of ~5.0 (Zhang et al., 1996) or 5.8 (as measured by a different method by Muller-Berger et al., 1995). As we show here for AE2, Muller-Berger et al. (1995) also found that AE1-mediated Cl\(^{-}\) transport activity is inhibited by lowering pHi at constant pHo, or by lowering pHo at near-constant pHi (although at much lower pH values than for AE2). Defined residues of the NH2-terminal cytoplasmic domain are absolutely required for inhibition of AE2-mediated Cl\(^{-}\) transport by acidic pHi as assessed by the butyrate removal assay. These residues must either interact with or be required to maintain integrity of the pH sensor of the AE2 transmembrane domain (Zhang et al., 1996). The postulated interaction with or conformational change in the transmembrane sensor (resulting from either mutation or deletion of these critical residues of the NH2-terminal cytoplasmic domain) must sufficiently reduce the effective pK of sensor residues to render it insensitive to the 0.5 pH unit intracellular acidification produced by addition of 40 mM butyrate to a pHo 7.4 bath (Stewart et al., 2001). Among the candidate residues contributing to this transmembrane pH sensor might be E1007 (Sekler et al., 1995), the residue corresponding in AE1 to human E681 and mouse E699. In AE1, mutation of this glutamate to

![Figure 9](image_url)

Figure 9. Mutation of corresponding glutamate residues in the NH2-terminal cytoplasmic domain of the chimeric anion exchanger AE1cyto/AE2memb similarly alters regulation by pHi. (A) Schematic showing site of alanine substitutions for conserved glutamate residues in the NH2-terminal cytoplasmic domain of the chimera AE1cyto/AE2memb. (B) Representative 36Cl\(^{-}\) efflux time course from oocytes expressing "wild-type" AE1cyto/AE2memb (filled circles) or the corresponding E99A/E100A double mutant (open circles) during intracellular alkalinization (butyrate removal) and subsequent inhibition by DIDS (200 μM). (C) Mean fold stimulation (± SEM) of 36Cl\(^{-}\) efflux by intracellular alkalinization (butyrate removal) in n oocytes expressing either wild-type AE2, wild-type AE1, or the indicated chimeras or mutant polypeptides.

3Muller-Berger et al. (1995) found that reduction of bath pH to 6.1 either from 7.2 or from 8.3 did not change oocyte pHi when measured after 90 min preincubation (in 110 mM KCl Barth’s solution). Thus, 90 min preincubation at the desired pH achieved conditions of nominal pHi-clamp which were suitable for their experiments in which each oocyte was exposed first to control pH and then to a single altered bath pH. Our experimental protocol exposed each oocyte to the complete range of bath pH values in the course of a single flux experiment (Stewart et al., 2001 and the current work). In this setting, pHi measured either by pH-sensitive microelectrode (Fig. 1) or by BCECF ratio fluorimetry (Zhang et al., 1996; unpublished data) did change acutely in parallel with changing pHo. Our experimental conditions required coincident changes in pHo and butyrate concentration to achieve oocyte pHi clamp. These and other experimental differences might contribute to the difference of ~1 pH unit between the AE1 pHo(50) values reported by Muller-Berger et al. (1995) and Zhang et al. (1996). Muller-Berger et al. (1995) lowered pH at constant pHi, through use of 40 or 60 mM NH4Cl. In our experiments, 20 mM NH4Cl did not inhibit AE1. The use of NH4Cl for intracellular acidification at constant pHi, could not be applied to experiments with AE2, since NH4Cl activates AE2 despite its accompanying acidification of the oocyte (Humphreys et al., 1997).
glutamine (Chernova et al., 1997b) or its chemical modification to hydroxyxynorvaline with Woodward’s reagent K (Jennings, 1995) not only alters anion selectivity, but also converts H⁺-sulfate cotransport into proton-independent sulfate transport. This observation has led to the hypothesis that this glutamate at or near the cytoplasmic face of putative transmembrane span 8 is or contributes to the proton binding site. Such direct, pH-dependent interactions (with their presumed conformational changes) have been demonstrated in K⁺ channels, where it is suggested that at acidic pH, specific residues are protonated in the COOH-terminal cytoplasmic tail, leading to interaction with the NH₂-terminal tail and in turn causing conformational changes in the protein (Qu et al., 2000; for review see Jiang et al., 2002).

Regulation of AE2 by pH involves protonation of extracellular residues, likely distinct from external Cl⁻ binding site(s) (Milanick and Gunn, 1984). These protonation(s) could alter Cl⁻ binding affinity or Cl⁻ translocation rate, a function of the conformational change between inward-facing and outward-facing forms of the transporter. Substitution of the entire AE2 NH₂-terminal cytoplasmic domain with that of AE1 (Zhang et al., 1996; Stewart et al., 2001), or deletion or mutation of those NH₂-terminal cytoplasmic domain residues essential for wild-type AE2 response to changing pH₄ (including many residues not subject to titration by protons near the physiological range) shift the wild-type AE2 pH₄ₜ₅₀ to more acidic values by 0.3–0.8 pH units. This shift, representing altered proton affinity of the external pH sensor (Gunn et al., 1973), might be accomplished through loss of its direct interaction with the cytoplasmic face of the AE2 transmembrane domain, resulting in altered conformation (and pKa) of protonatable residues at AE2’s extracellular face or within the aqueous vestibule thought to have E1007 at its base. Alternately, a distinct polypeptide may mediate this interaction. The effects of AE2 mutations in the mutations in the NH₂-terminal cytoplasmic domain upon AE2 pH₄ₜ₅₀ sensitivity resemble the alterations in ligand binding affinity of transmembrane hormone receptors secondary to mutations in receptor cytoplasmic loops or tail, or to receptor interaction with cytoplasmic regulatory polypeptide(s).

**Putative Structure of the pH-regulatory Region of the AE2 NH₂-terminal Cytoplasmic Domain**

AE2 polypeptide has been purified from porcine stomach (Zolotarev et al., 1996, 1999), and is present in gastric membranes as a dimer or higher order oligomer (Zolotarev et al., 1999), but its structure remains unknown. We have mapped the AE2 sequences implicated in regulation of AE2 by pH upon the X-ray structure of crystallized erythroid AE1 NH₂-terminal cytoplasmic domain reported by Zhang et al. (2000). This alignment predicts that AE2 aa 336–347 occupies a region corresponding to most of the AE1 structure’s β strands β2 and β3 along with their short connecting loop. This region is adjacent to AE1 residue 356, the COOH-terminal extent of structured sequence. Although structure remains uncertain for the disordered aa 357–379 in the AE1 NH₂-terminal cytoplasmic domain and the ~20 subsequent amino acids that link the cytoplasmic domain to the first transmembrane span of AE1, the loop connecting β strands β2 and β3 is plausibly oriented close to the cytoplasmic face of the AE1 transmembrane domain (Zhang et al., 2000). We postulate that AE2 aa 336–347, although hundreds of residues away (in linear sequence) from the first transmembrane domain of AE2, may adopt a similar conformation with respect to elements of the internal pH sensor proposed to reside in the AE2 transmembrane domain. The pattern of altered pH₄-regulatory phenotype produced by the alanine scan of AE2 aa 336–347 is consistent with a structure of two short β sheets connected by a loop, showing one short patch of alternating phenotype within each predicted β-sheet. This pattern is distinct from that of altered pH₄ sensitivity across the same region.

The decreased pH₄ sensitivity exhibited by the (β3 strand) double mutant E99A/E100A of the chimeric AE1cyto/AE2memb transporter (Fig. 8) further supports this hypothesis. The absence of pH₄ dependence for AE1-mediated Cl⁻ efflux in the butyrate removal assay highlights the functional interaction between pH-regulatory residues in NH₂-terminal cytoplasmic domain and the yet undefined pH sensor structure present in the AE2 transmembrane domain, but lacking in that of AE1.

The different effects on AE2 regulation by pH₄ of E346 substitution by alanine or by aspartate suggest that in this position side chain packing may be more important than charge to the structure of a functional regulatory domain. A similar result has been presented for the electrogenic SGLT1 Na⁺-glucose cotransporter (Quick et al., 2001). The importance of packing in the folding of this regulatory domain is further suggested by the modified pH regulation exhibited by alanine substitution of conserved tryptophan residues. In contrast, substitution of the adjacent residue E347 with Ala or with Asp each led to altered regulation by pH₄ (Figs. 3 F and 5 C). These two adjacent glutamates likely comprise part of a β-sheet structure, projecting their side chains in opposite directions (Zhang et al., 2000). Thus, the different consequences of mutagenesis of these adjacent residues likely reflects their different nearest neighbors.

Important roles for glutamate residues in conferring sensitivity to changing pH₄ and pH₃ have been demon-
strated previously in potassium channels (Xu et al., 2000), chloride channels (Stroffekova et al., 1998), and the capsaicin receptor (Jordt et al., 2000). Although histidine residues play major roles in regulation of several K+ channels by pH (Coulter et al., 1995; Doi et al., 1996; Chanhevalap et al., 2000; Qu et al., 2000; Xu et al., 2000), alanine substitution of AE2 His residues 314 and 317, either individually or in tandem, was without effect on AE2 regulation by pH (not depicted). The lack of effect of T339 mutation to Val or to Glu similarly failed to support a role for T339 phosphorylation in AE2 regulation by pH.

Conserved Amino Acids of the NH2-terminal Cytoplasmic Domain Contribute to the pH regulation of other Anion Exchangers

The region of aa 336–347 is among the most highly conserved of the NH2-terminal cytoplasmic domains of SLC4 gene family members. Its functional importance was confirmed in the AE2 homologue cAE3 by mutating the two glutamate residues in cAE3 corresponding to E346 and E347 of AE2. Both mutations greatly attenuated the pH dependence of anion exchange in 293 cells transiently transfected with AE3 (Lee et al., 1991), but considerably reduced AE3-mediated Cl− efflux from Xenopus oocytes. However, whereas the AE2 mutant E346A exhibited a wild-type response to butyrate removal, the corresponding mutation in cAE3 (E150) showed considerably reduced pH dependence (only two-fold increase upon butyrate removal) compared with an increase exhibited by wild-type cAE3. This difference between the functional consequences of mutation within this highly conserved region in AE2 and AE3 may reflect sequence differences in immediately adjacent flanking regions, but very likely reflects different effects on interacting structures. These binding sites may be at the cytoplasmic face of the AE2 and AE3 transmembrane domains, or may represent other polypeptides.

These results are consistent with the initial report of pH-sensitive Cl−/HCO3− exchange in 293 cells transiently transfected with AE3 (Lee et al., 1991), but contrast with the pH-insensitive AE3-mediated Cl−/nitrate exchange in 293 cells reported by Sterling and Casey (1999). The latter measurements were made in high K+/nigericin pH-clamp conditions in which pHi = pHo. The activation of AE3 by intracellular alkalization in Xenopus oocytes is consistent with its hypothesized role in cardiac myocytes in recovery from alkaline load (Leem et al., 1999). As anion exchange in guinea pig cardiomyocytes is oppositely regulated by pHi and by pHo (Sun et al., 1996; Vaughan-Jones, 1986; Leem and Vaughan-Jones, 1998), the individual effects of pHi and pHo on heterologous AE3 in transfected 293 cells (Sterling and Casey, 1999) may have been masked by the chosen experimental conditions. Interestingly, whereas the pH dependence of anion exchange in guinea pig myocytes is similar to that exhibited by mouse AE2 expressed in Xenopus oocytes their responses to varying pH differ. In guinea pig myocytes, low pH stimulates and high pH inhibits Cl−/HCO3− exchange, with a pHo(50) value of 7.03 (Wilson and Vaughan-Jones, 2000), a value similar to that of AE2-mediated Cl−/Cl− exchange oppositely regulated in oocytes by pHo. The functionally dominant anion exchanger protein of guinea pig myocytes has not yet been identified, although AE3 (Kopito et al., 1989; Kudrycki et al., 1990; Yannoukakos et al., 1994) and AE1 (Richards et al., 1999) have both been proposed.

The pH-regulatory subdomains within the AE2 NH2-terminal cytoplasmic domain whose function requires the integrity of aa 318–323 and aa 336–347 likely interact with or control the conformation of effector moieties. These may include binding site(s) at the cytoplasmic face of the AE2 transmembrane domain not present in AE1, binding sites elsewhere within the AE2 cytoplasmic NH2-terminal domain, and/or binding surfaces on separate, regulatory polypeptides. These possibilities are currently under investigation. Although the structure of the transmembrane domain of AE1 is known only to a resolution of 18 Å, several mutations of human AE1 associated with distal renal tubular acidosis (dRTA) (Alper et al., 2002) suggest sites at the cytoplasmic face of the transmembrane domain that are sensitive to the structure of the NH2-terminal cytoplasmic domain, and so either influence or themselves might represent sites of intramolecular interaction. Thus, the AE1 mutants R589H (Bruce et al., 1997; Jarolim et al., 1998) and R901X (Karet et al., 1998) are both functional in the context of the erythroid AE1 NH2-terminal cytoplasmic domain. In contrast, within the context of the renal type A intercalated cell, in which the NH2-terminal cytoplasmic domain of kidney AE1 lacks the first 65 residues present in erythroid AE1, these mutants can exhibit impaired surface accumulation (Toye et al., 2002) and/or a dominant-negative trafficking phenotype (Quilty et al., 2002).

As judged by the butyrate removal assay, pHi regulates both Cl−/Cl− and Cl−/HCO3− exchange mediated by AE2. The recent demonstrations that Cl−/HCO3− exchange mediated by AE2 and by AE3 can be regulated by bound carbonic anhydrase II (Sterling et al., 2002a) and by carbonic anhydrase IV (Sterling et al., 2002b) adds to the potential complexity of AE regulation by pH. These AE binding proteins might in some contexts exert allosteric effects on AE activity, in addition to their presumed functions in channeling transport substrate to and from the anion translocation pathway through the polypeptide.

Conclusion

The present study has defined individual, conserved amino acid residues in the NH2-terminal cytoplasmic
domain of the AE2 anion exchanger whose mutation alters the independent regulation of AE2 activity by pH\textsubscript{i} and by pH\textsubscript{o}. The corresponding conserved glutamate residues were shown also to be critical for regulation by pH\textsubscript{i} of anion exchange mediated by cAE3 and of the AE1\textsubscript{cyt/AE2\textsubscript{memb}} chimera. The importance of one of these glutamates was further demonstrated for the regulation of AE2-mediated Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange by pH\textsubscript{i}. The conservation of these residues in yet other bicarbonate transporters involved in intracellular and extracellular compartmental pH regulation suggests similar mechanisms of functional control. These results confirm that integrity of residues in both the NH\textsubscript{2}-terminal cytoplasmic domain and in the COOH-terminal transmembrane domain of AE2 is necessary for wild-type regulation of AE2 by pH\textsubscript{i} and by pH\textsubscript{o}. Integrity of individual residues of the NH\textsubscript{2}-terminal cytoplasmic domain which contribute to the “pH-modifier site” previously proposed by Zhang et al. (1996) is necessary but not sufficient for the wild-type pattern of independent sensing of pH\textsubscript{i} and pH\textsubscript{o}. Also required is the presence of the appropriate “pH\textsubscript{i}/pH\textsubscript{o} sensor(s)” in the transmembrane domain which mediates anion translocation. Future experiments will seek to identify the transmembrane domain pH sensor(s) of AE2 and their modes of communication directly with modulatory residues of the AE2 NH\textsubscript{2}-cytoplasmic domain, or indirectly via distinct regulatory polypeptides.

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