JOP. J. Pancreas (Online) 2001; 2(4 Suppl):171-175.

Regulation of Na^+-Independent Cl^-/HCO^-_3 Exchangers by pH

Seth L Alper, Marina N Chernova, Andrew K Stewart

Departments of Medicine and Cell Biology, Harvard Medical School; Molecular Medicine and Renal Units, Beth Israel Deaconess Medical Center. Boston, MA, USA

Summary

Among human bicarbonate transporters, two major gene families encode Na-independent Cl^-/HCO_3^- exchangers: the SLC4 anion exchanger (AE) family [1], and the SLC26 "sulfate permease" anion transporter family [2]. The SLC4 AE family contains at least three genes, and comprises a subfamily within the larger and phylogenetically more ancient bicarbonate transporter superfamily that includes the Na bicarbonate cotransporters (NBC) and the Na-driven Cl/base exchangers. Mutations in the human AE1 gene cause autosomal dominant spherocytic anemia and distal renal tubular acidosis of both dominant and recessive forms. Anemia is also associated with AE1 mutations in mouse, cow, and zebrafish. Naturally occuring mutations in the human AE2 and AE3 genes have not been detected. The SLC26 family in humans consists of at least 10 members, and includes anion exchangers which exchange chloride for bicarbonate, hydroxyl, sulfate, formate, iodide, and/or oxalate. Mutations in three of these genes cause hereditary disease, including chondrodysplasia (SLC26A2, DTD), diarrhea (A3, down-regulated in adenoma/chloride-losing diarrhea protein: DRA/CLD), and goiter/deafness syndrome (A4, pendrin). Little is known about the acute regulation of these modulators of intracellular and compartmental pH and volume.

We have studied the structural elements required for short-term regulation of the SLC4 Cl^-/HCO_3^- exchangers AE1, AE2, and AE3, and have initiated similar studies of SLC26A3, the down-regulated in adenoma/chloride-losing diarrhea protein (DRA/CLD). We have detected regulation of some of these proteins by pH, tonicity, and NH_4^+. Small regions or single amino acid residues crucial to these regulations have been delineated and, in the case of AE2, mapped onto the published X-ray structure of the AE1 N-terminal cytoplasmic domain. Whereas AE1 shows little sensitivity to pH over the physiological range, intracellular and extracellular acidification both inhibit AE2. The particular structural requirements of AE2 inhibition by protons depend on whether only pH_i is varied, or pH_i change is caused by an imposed change in pH_o.

AE1 is expressed at highest levels in erythrocyte plasma membrane (eAE1) and in the basolateral plasma membrane of Type A intercalated cells of the renal cortical and medullary collecting ducts. kAE1 of intercalated cells lacks the N-terminal 65 (human) or 79 (mouse) residues of eAE1. AE1 is expressed at lower levels in heart, distal colon, and other tissues. AE2 is the most widely expressed form of AE, and in nearly all epithelial cells resides in the basolateral plasma membrane. A Golgi form of AE2 has been detected in situ and in transfected

cells, and exhibits distinct detergent extraction characteristics [3, 4]. Mammalian AE2 variant transcripts encode 5 distinct N-terminal amino acid sequences of as yet unknown functions [5, 6]. AE3 is expressed at highest level in excitable tissues, throughout the GI tract, and at lower levels in other tissues [7]. Mammalian AE3 transcripts encode two N-terminal amino acid sequences (bAE3 and cAE3) [8]. In epithelial cells of the kidney, bAE3 is basolateral, whereas in epithelial cells of the gut, bAE3 is detectable on both basolateral and apical surfaces. In contrast, cAE3 in kidney and gut preferentially localizes to apical membranes, where it could potentially contribute to apical bicarbonate secretion [9]. Mammalian AE3 transcripts also encode two C-terminal amino acid sequences. One of these (AE3-14nt) is the only AE to lack a transmembrane anion transport domain [10]. Although AE3-14nt is relatively abundantly expressed as mRNA and protein, its function remains unknown. cAE3-14nt does not exhibit a dominant negative phenotype when co-expressed in *Xenopus* oocytes with cAE3 [Papageorgiou, Shmukler, Alper: unpublished data]. The recently reported AE4 [11] is more closely related in sequence to the NBC Na-bicarbonate cotransporters than to AE anion exchangers, although reported to mediate Na+-independent Cl^-/HCO_3^- exchange.

We have focused our studies on AE1 and AE2. These two anion exchangers exhibit similar maximal transport rates when expressed in *Xenopus* oocytes. Their anion selectivities and apparent affinities for extracellular anions are similar. AE2 is less sensitive than AE1 to inhibition by a wide range of drugs, with the notable exception (at least in *Xenopus* oocytes) of select polyaminosterol sulfates [12]. Notably, the AE2 anion exchanger expressed in *Xenopus* oocytes exhibits several modes of regulation not displayed by AE1. AE2 is strongly inhibited by protons, whereas AE1 is minimally regulated by protons within the near-physiological range [13]. AE2 is activated by hypertonicity [14] and can mediate regulatory volume increase in oocytes [15], but AE1 lacks these properties. AE2 is paradoxically activated by low concentrations of ammonium ion despite ammonium-mediated acidification in *Xenopus* oocytes, whereas AE1 is unaffected by ammonium [16]. We have set out to define the structural elements of AE2 which mediate these isoform-specific modes of regulation. Although we have measured AE function by fluorometric indices of Cl^-/HCO_3^- [17] and Cl^-/nitrate exchange in transfected mammalian cells and in *Xenopus* oocytes [unpublished data], we have performed most studies on regulation using unidirectional $^{36}Cl^-$ flux studies in *Xenopus* oocytes. These studies have been supplemented by fluorometric and microelectrode measurements of oocyte pH_i. Initial studies were performed by varying bath pH (pH_o) and allowing intracellular pH (pH_i) to achieve steady-state before measuring flux [13, 18]. More recent studies utilize weak acids to vary pH_i at constant pH_o. Current efforts are standardizing conditions to vary pH_o at constant pH_i.

Study of chimeric proteins assigned distinct pH-regulatory functions to the 705 aa N-terminal cytoplasmic domain and the 533 aa C-terminal transmembrane domain of AE2 [16]. When pH_o was varied between pH 9 and pH 5 in these studies, pH_i varied between pH 7.61 and 7.13, only 0.13 pH_i unit per pH_o unit. Across this range, AE2 was steeply regulated with a sigmoidal pattern characterized by a $pH_o(50)$ value of 6.93, whereas AE1 activity was not significantly altered. In these conditions, the $AE2_{cyto}/AE1_{memb}$ chimera exhibited an AE1 phenotype. In contrast, the $AE1_{cyto}/AE2_{memb}$ chimera exhibited an AE2 phenotype, modified only in its acid-shifted $pH_o(50)$ value of 6.3. Thus the sigmoidal pH-response of AE2 activity required the transmembrane domain, and the AE2 cytoplasmic domain served to increase sensitivity to protons of

the transmembrane domain. The transmembrane domain site was named a "sensor", and the N-terminal cytoplasmic domain site was named a "modifier", by analogy to similar studies with the NHE1 Na^+/H^+ exchanger [19].

Further studies have focused on the molecular definition of the "modifier site" in the N-terminal cytoplasmic domain of AE2. Removal of 99 N-terminal amino acids had no effect, whereas removal of 510 N-terminal residues produced a pH *vs*. activity curve identical in shape and $pH_o(50)$ to that of the $AE1_{cyto}/AE2_{memb}$ chimera. These studies, originally performed with $^{36}Cl^-$ influx experiments, have recently been reproduced with $^{36}Cl^-$ efflux experiments in which single oocytes were subjected to the full range of pH_o values. Stepwise analysis of additional N-terminal truncations with the efflux assay further narrowed the required region to residues between aa 328 and 347, and between aa 391 and 510. A hexa-Ala bloc scan restricted to the former stretch revealed that, even within this small region, the residues required to exhibit the wildtype pHo(50) value are non-contiguous in the primary sequence.

The above structure-function experiments could not reveal the role of pH_i in AE2 regulation. Another approach to measurement of AE2 regulation by pH has used nigericin high K^+ to clamp pH_i nominally equal to pH_o during measurement of Cl^-/nitrate exchange [20]. However, these experiments were similarly unable to determine the exclusive role of pH_i in AE regulation. Therefore, we standardized conditions of exposure to weak acids to vary pH_i at constant pH_o. In order to apply this method to study of AE function, we showed that weak acids were neither inhibitors of AE2-mediated $^{36}Cl^-$ transport, nor were they themselves substrates for AE2. With this procedure, we re-evaluated the regulation of Cl^-/Cl^- exchange activity by pH_i at constant pH_o. The AE2 regions required for regulation by pH_i overlapped with, but were not identical to those required for regulation by pH_o (with consequent variation of pH_i). In particular, removal of the N-terminal 310 aa reduced regulation by pH_i but had no effect on that by pH_o. Removal of the N-terminal 328 or 391 aa, which retained a nearly normal regulation by pH_o, abolished regulation by pH_i.

To delineate further the regions required for AE2 regulation by pH_i, we performed hexa-alanine bloc substitutions between residues 310 and 347. In contrast to the N-terminal truncation experiments, only Ala substitution for residues between 336-341 and 342-347 abolished regulation by pH_i. Once more, the 6 amino acid blocks required for wildtype $pH_o(50)$ value were not identical to those required for regulation by pH_i. This region is among the most highly conserved in the N-terminal cytoplasmic domain of the bicarbonate transporter superfamily. Within this region, point mutations have been identified which modify only AE2 regulation by pH_i, or which modify regulation by both pH_i and pH_o. The region involved aligns with a segment of the AE1 N-terminal cytoplasmic domain X-ray structure predicted to abut or to reside nearby the endofacial surface of the AE transmembrane domain at the cytoplasmic surface of the plasma membrane [21]. In this position, the candidate modifier region would be well situated to interact directly with the AE2 pH sensor(s) in the membrane-spanning domain. Future studies will test the consequences to Cl^-/HCO_3^- exchange of mutants shown to modify regulation of Cl^-/Cl^- exchange by pH_i and pH_o.

We have initiated comparison of the anion exchange processes and mechanisms of the two distinct structural classes of anion exchanger, the band 3-related AE (SLC4) and the "sulfate permease" SLC26 family [22]. We have found that Cl^-/Cl^- exchange mediated by the $Cl^-/formate/OH^-/HCO_3$ exchanger, pendrin [23, 24] is insensitive to bath pH change, and that mediated by the Cl^-/HCO_3^- exchanger DRA [25] is minimally

sensitive to bath pH change. Whereas pendrin is also insensitive to intracellular acidification at constant pH_o, DRA is inhibited by intracellular acidification and activated by intracellular alkalinization. DRA is also stimulated by ammonium and, less robustly, by hypertonicity. Although insensitive to the stilbene inhibitor DIDS, DRA is rather potently inhibited by the anti-inflammatory drugs, tenidap and niflumate. The carboxy-terminal 9 aa of DRA (including a candidate PDZ domain recognition sequence) are dispensable for wildtype Cl^-/Cl^- exchange function in the *Xenopus* oocyte. However, truncation of four additional residues greatly reduces DRA-mediated Cl^- transport.

Comparison of mechanism, regulation, and structure-function relationships between SLC4 and SLC26 anion exchangers should enhance understanding of how two distinct sets of structures mediate very similar ion exchange reactions across lipid bilayers. The availability of microbial homologs of the SLC26 gene family may prove particularly helpful for structural study of this class of proteins.

Key words Acidosis, Renal Tubular; Band 3 Protein; Spherocytosis, Hereditary

Abbreviations AE: anion exchanger; CLD: chloride-losing diarrhea; DTD: diastrophic dysplasia; DIDS: 4,4'-di-isothiocyanostilbene-2,2'-disulfonic acid; DRA: down-regulated in adenoma; NBC: Na bicarbonate cotransporter; NHE: Na^+/H^+ exchanger; PDZ: postsynaptic density-95, discs large, zonula occuldens-1; SLC: HUGO designation for "solute carrier" gene family

Correspondence
Seth L Alper
Department of Medicine (Cell Biology)
Harvard Medical School
Molecular Medicine and Renal Units
RW763 Beth Israel Deaconess Med. Ctr. East Campus
330 Brookline Ave
Boston, MA 02215
USA
Phone: +1-617.667.2930; +1-617.667.2918
Fax: +1-617.667.8040; +1-617.667.2913
E-mail address: salper@caregroup.harvard.edu

References

1. Alper SL. The band 3-related AE anion exchanger gene family. Cell Physiol Biochem 1994; 4:265-81.

2. Everett LA, Green ED. A family of mammalian anion transporters and their involvement in human genetic diseases. Hum Mol Genet 1999; 8:1883-91. [99400527]

3. Alper SL, Stuart-Tilley AK, Biemesderfer D, Shmukler BE, Brown D. Immunolocalization of AE2 anion exchanger in rat kidney. Am J Physiol 1997; 273:F601-14. [98026661]

4. Holappa K, Suokas M, Soininen P, Kellokumpu S. Identification of the full-length AE2 (AE2a) isoform as the Golgi-associated anion exchanger in fibroblasts. J Histochem Cytochem 2001; 49:259-69. [21068591]

5. Stuart-Tilley AK, Shmukler BE, Brown D, Alper SL. Immunolocalization and transcript analysis of AE2 anion exchanger in mouse kidney. J Am Soc Nephrol 1998; 9:946-56. [98284297]

6. Lecanda J, Urtasun R, Medina JF. Molecular cloning and genomic organization of the mouse AE2 anion exchanger gene. Biochem Biophys Res Commun 2000; 276:117-24. [20462926]

7. Kudrycki KE, Newman PR, Shull GE. cDNA cloning and tissue distribution of mRNAs for two proteins that are related to the band 3 Cl^-/HCO_3^- exchanger. J Biol Chem 1990, 265:462-71. [90094439]

8. Kopito RR, Lee BS, Simmons DM, Lindsey AE, Morgans CW, Schneider K. Regulation of intracellular pH by a neuronal homolog of the erythroid anion exchanger. Cell 1989; 59:927-37. [90075236]

9. Alper SL, Stuart-Tilley AK, Yannoukakos D, Brown D. AE3 anion exchanger immunolocalization in rodent kidney: evidence for apical and basolateral isoforms. J Am Soc Nephrol 1995; 6:372A.

10. Morgans CW, Kopito RR. Generation of truncated brain AE3 isoforms by alternate mRNA processing. J Cell Sci 1993; 106:1275-82. [94171936]

11. Tsuganezawa H, Kobayashi K, Iyori M, Araki T, Koizumi A, Watanabe SI, et al. A new member of the HCO_3^- transporter superfamily is an apical anion exchanger of beta-intercalated cells in the kidney. J Biol Chem 2001; 276:8180-9 [PMID 11102437]

12. Alper SL, Chernova MN, Williams J, Zasloff M, Law FY, Knauf PA. Differential inhibition of AE1 and AE2 anion exchangers by oxonol dyes and by novel polyaminosterol analogs of the shark antibiotic, squalamine. Biochem Cell Biol 1998; 76:799-806. [99280181]

13. Humphreys BD, Jiang L, Chernova MN, Alper SL. Functional characterization and regulation by pH of murine AE2 anion exchanger expressed in *Xenopus* oocytes. Am J Physiol 1994; 267:C1295-307. [95068311]

14. Humphreys BD, Jiang L, Chernova M, Alper SL. Activation of murine AE2 anion exchanger in *Xenopus* oocytes by increased pH_i secondary to hypertonic activation of Na^+/H^+ exchange. Am J Physiol 1995; 268:C201-9.

15. Jiang L, Chernova MN, Alper SL. Secondary regulatory volume increase conferred on *Xenopus* oocytes by expression of AE2 anion exchanger. Am J Physiol 1997; 272:C191-202. [97190866]

16. Humphreys BD, Chernova MN, Jiang L, Zhang Y, Alper SL. NH_4Cl activates AE2 anion exchanger in *Xenopus* oocytes at acidic pH_i. Am J Physiol 1997; 272:C1232-40. [97287720]

17. Jiang L, Stuart-Tilley AK, Parkash J, Alper SL. AE2-mediated Cl^-/HCO_3^- exchange in CHOP cells of defined, transient transfection status is regulated by pH_i and serum. Am J Physiol 1994; 266:C845-56.

18. Zhang Y, Chernova MN, Stuart-Tilley AK, Jiang L, Alper SL. The cytoplasmic and transmembrane domains of AE2 both contribute to regulation of anion exchange by pH. J Biol Chem 1996; 271:5741-9. [96215037]

19. Ikeda T, Schmitt B, Pouyssegur J, Wakabayashi S, Shigekawa M. Identification of cytoplasmic subdomains that control pH-sensing of the Na^+/H^+ exchanger (NHE1): pH-maintenance, ATP-sensitive, and flexible loop domains. J Biochem (Tokyo) 1997; 121:295-303. [97244627]

20. Sterling D, Casey JR. Transport activity of AE3 chloride/bicarbonate anion-exchange proteins and their regulation by intracellular pH. Biochem J 1999; 344:221-9. [20018036]

21. Zhang D, Kiyatkin A, Bolin JT, Low PS. Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3. Blood 2000; 96:2925-33. [20504031]

22. Lohi H, Kujala M, Kerkela E, Saarialho-Kere U, Kestila M, Kere J. Mapping of five new putative anion transporter genes in human and characterization of SLC26A6, a candidate gene for pancreatic anion exchanger. Genomics 2000; 70:102-12. [20541715]

23. Scott DA, Karniski LP. Human pendrin expressed in *Xenopus* oocytes mediates chloride/formate exchange. Am J Physiol 2000; 278:C207-11. [20113293]

24. Soleimani M, Greeley T, Petrovic S, Wang Z, Amlal H, Kopp P, Burnham CE. Pendrin: an apical $Cl^-/OH^-/HCO_3^-$ exchanger in the kidney cortex. Am J Physiol 2001; 280:F356-64. [21128101]

25. Melvin JE, Park K, Richardson L, Schultheis PJ, Shull GE. Mouse down-regulated in adenoma (DRA) is an intestinal Cl^-/HCO_3^- exchanger and is up-regulated in colon of mice lacking NHE3 Na^+/H^+ exchanger. J Biol Chem 1999; 274:22855-61. [99357827]