JOP. J. Pancreas (Online) 2001; 2(4 Suppl):165-170.

Carbonic Anhydrase: In the Driver's Seat for Bicarbonate Transport

Deborah Sterling[1], Reinhart AF Reithmeier[2], Joseph R Casey[1]

[1]Membrane Transport Group and CIHR Group in Molecular Biology of Membrane Proteins, Department of Physiology, University of Alberta. Edmonton, Canada. [2]CIHR Group in Membrane Biology, Department of Medicine, University of Toronto. Toronto, Canada

Summary

Carbonic anhydrases are a widely expressed family of enzymes that catalyze the reversible reaction: $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$. These enzymes therefore both produce HCO_3^- for transport across membranes and consume HCO_3^- that has been transported across membranes. Thus these enzymes could be expected to have a key role in driving the transport of HCO_3^- across cells and epithelial layers. Plasma membrane anion exchange proteins (AE) transport chloride and bicarbonate across most mammalian membranes in a one-for-one exchange reaction and act as a model for our understanding of HCO_3^- transport processes. Recently it was shown that AE1, found in erythrocytes and kidney, binds carbonic anhydrase II (CAII) via the cytosolic C-terminal tail of AE1. To examine the physiological consequences of the interaction between CAII and AE1, we characterized Cl^-/HCO_3^- exchange activity in transfected HEK293 cells. Treatment of AE1-transfected cells with acetazolamide, a CAII inhibitor, almost fully inhibited anion exchange activity, indicating that endogenous CAII activity is essential for transport. Further experiments to examine the role of the AE1/CAII interaction will include measurements of the transport activity of AE1 following mutation of the CAII binding site. In a second approach a functionally inactive CA mutant, V143Y, will be co-expressed with AE1 in HEK293 cells. Since over expression of V143Y CAII would displace endogenous wild-type CAII from AE1, a loss of transport activity would be observed if binding to the AE1 C-terminus is required for transport.

In any discussion of bicarbonate transport it is important to consider the role of the enzyme carbonic anhydrase. Carbonic anhydrase (CA) catalyzes the production of HCO_3^- from CO_2 and H_2O and the consumption of HCO_3^- by conversion [1]. Thus the enzyme supplies the HCO_3^- substrate for transport and removes HCO_3^- following transport. Modulation of carbonic anhydrase activity therefore provides a means to regulate the rate of HCO_3^- transport. The family of mammalian carbonic anhydrases consists of at least 10 members with both cytosolic forms and forms with catalytic site anchored to the extracellular surface of the cell [1, 2]. In studies of HCO_3^- transport physiology it is important to consider the location, expression level and regulation of CA as this can have profound effects on the rate of HCO_3^- transport.

One important family of HCO_3^- transporters are the sodium independent Cl^-/HCO_3^- exchangers, or anion exchangers (AE). The best known member of the family is erythrocyte AE1 (Band

3), but AE family members are very widely expressed across tissues, including isoforms AE2, AE3 [3, 4] and the very recently described AE4 [5]. AE proteins facilitate the one-for-one electroneutral exchange of Cl^- for HCO_3^- and thereby contribute to pH regulation, CO_2 metabolism, volume regulation and maintenance of Cl^- and HCO_3^- levels. Each AE protein has two large and one small domain (Figure 1). The N-terminal cytoplasmic domains of 43-77 kDa interact with glycolytic enzymes and cytoskeletal elements [6]. The approximately 55 kDa membrane domains alone carry out the anion transport function [7]. The C-terminal region of approximately 33 amino acids extends from the membrane on the cytosolic surface [8, 9] (Figure 1).

This C-terminal cytosolic region can be considered a separate domain given recent evidence of its important functional role. The C-terminal tail of AE1 was shown to bind to CAII, the most catalytically active CA isoform of the erythrocyte [17]. Evidence for the interaction was: 1) lectin-induced clustering of AE1 induced clustering of CAII in erythrocytes membranes; 2) CAII co-immunoprecipitated

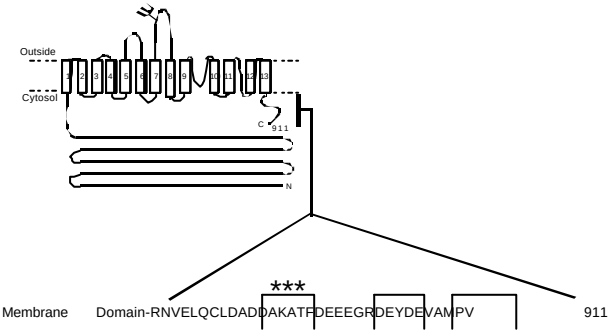

Figure 1. AE1 transmembrane topology model and amino acid sequence of C-terminal region.

Top left is a topology model for the arrangement of AE1 protein in the plasma membrane. Numbered boxes represent transmembrane segments [9, 10, 11,12 13, 14]. The C-terminal tail extends from the membrane on the cytosolic surface. The amino acid sequence shows the C-terminal tail region [15]. Boxes represent acidic sequences identified as potential CAII binding sites. Box marked with asterisks was identified as responsible for the AE1/CAII interaction [16].

with AE1; 3) an antibody directed against the C-terminal region of AE1 blocked the AE1/CAII interaction; 4) a glutathione-S-transferase (GST) AE1 C-terminus protein bound CAII [17]. Subsequent studies with GST fusion proteins of portions of the AE1 C-terminus indicated that the AE1/CAII interaction was ionic and mediated by the N-terminal most acidic sequence (DADD) of the AE1 C-terminus [16] (Figure 1). Mutation of the acidic residues of the DADD sequence resulted in loss of CAII binding [16]. Interaction between AE and CA may be common to all AE isoforms since similar acidic sequences are found in the C-termini of AE2, AE3 and AE4. However, a direct interaction with CAII has thus far been shown only for AE1 and AE2 [15]. A basic sequence at the CAII N-terminus was identified to interact with the DADD motif of AE1 [18]. Taken together this evidence lead to the proposal that the AE1/CAII complex forms a metabolon, a physical complex of enzymes in a linked metabolic pathway, for carbon dioxide metabolism [19]. What this proposal lacked is evidence for the physiological significance of the metabolon. Below is evidence that the interaction between CAII and AE1 is physiologically significant.

To examine the role of carbonic anhydrase in anion exchange activity, HEK293 cells were transfected with AE1 cDNA. Transport activity was measured in cells loaded with the pH-sensitive dye, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM). In each assay, cells were alternately perfused with Ringer's buffer containing Cl^-, or with Ringer's buffer with Cl^- replaced by gluconate. All solutions were bubbled continuously with 5% CO_2 in air. AE1-mediated efflux of HCO_3^- acidifies the cell, while influx alkalinizes the cell (Figure 2). In Figure 2 intracellular pH increases going up the Y-axis. Cells were incubated with the membrane-permeant CA inhibitor, acetazolamide (100 µM), and the transport

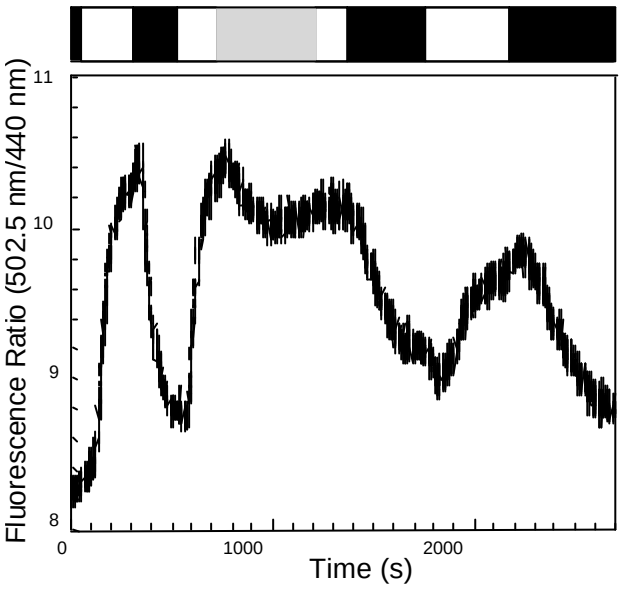

Figure 2. Effect of the carbonic anhydrase inhibitor, acetazolamide, on AE1-mediated chloride/bicarbonate exchange activity.
HEK293 cells transfected with AE1 cDNA were grown on glass coverslips loaded with BCECF-AM, suspended in a fluorescence cuvette and intracellular pH monitored following alternate perfusion with chloride-containing (solid bar) and chloride-free Ringer's buffer (open bar). The gray bar represents perfusion with solutions containing 100 µM acetazolamide. The Y-axis shows the ratio of emission recorded at 520 nm with excitation wavelengths 502.5 nm and 440 nm. The ratio is directly proportional to intracellular pH.

activity was measured. Acetazolamide does not inhibit anion exchange activity, as opposed to other CA inhibitors which inhibit anion exchange [20]. Comparison of the transport rates before and after treatment with acetazolamide indicated that the rate of HCO_3^- efflux was inhibited by 85±1%, while HCO_3^- influx was inhibited by 48±6%. The endogenous expression level of CA is not rate-limiting to anion exchange since overexpression of CAII 10-20 fold did not increase transport activity (not shown). These data clearly indicate that CA activity has a large role in maximizing the rate of AE1 activity. The asymmetry of the effect, that CA has a larger effect during HCO_3^- efflux than during influx, is also interesting. This data shows that CA is important to HCO_3^- transport because it produces the substrate for HCO_3^- efflux. CA has a lower effect on the rate of HCO_3^- influx. However, the two fold decrease in the rate of HCO_3^- influx after treatment with acetazolamide indicates that CA enhances HCO_3^- influx by conversion of HCO_3^- to CO_2, decreasing the intracellular HCO_3^- concentration, increasing the size of the inward-directed HCO_3^- gradient, and thereby driving HCO_3^- transport forward.
Although it is now clear that CAII interacts with the C-terminus of AE1, the next question is whether this interaction has physiological significance. CAII can alternately interact with AE1 or be found free in the cytosol (Figure 3). One possible role of the AE1/CAII interaction is to maximize the rate of bicarbonate transport, by limiting the distance that bicarbonate needs to diffuse to reach the transport site and to minimize local [HCO_3^-] during influx (Figure 3). To test the significance of the AE1/CAII interaction we have preliminary results from two types of experiments. First we have measured the anion exchange rate of AE1 mutants of the acidic CAII binding site (Figure

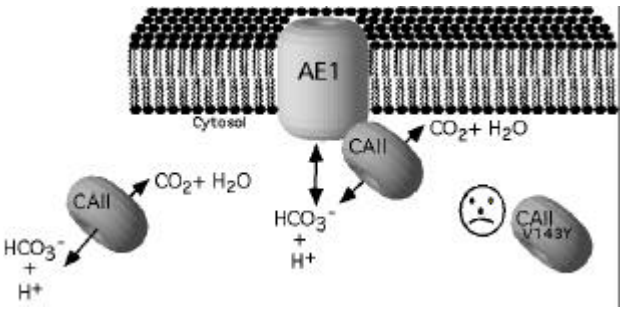

Figure 3. Model for the interaction of CAII with the cytosolic C-terminal tail of AE1.
Carbonic anhydrase II (CAII) catalyses the reversible conversion of CO_2 and water to HCO_3^- and H^+, while AE1 moves HCO_3^- in exchange for Cl^- reversibly across the plasma membrane. During HCO_3^- efflux, CAII produces the substrate for AE1 transport while during influx, CAII consumes HCO_3^-. The model depicts CAII as either associated with the plasma membrane via AE1, or free in the cytosol. CAII V143Y is a catalytically inactive mutant of CAII [21]. Interaction of CAII with the C-terminal tail of AE1 minimizes the distance HCO_3^- must diffuse prior to efflux and minimizes the local concentration of HCO_3^- during influx mode.

1). The sequence DADD of the AE1 C-terminus was identified as responsible for the CAII/AE1 interaction. Two GST fusion proteins of the C-terminal region, with DADD mutated to NANN and AAAA, were previously constructed and shown not to bind CAII [16]. Using the anion transport assay described in Figure 2 we measured the anion exchange rate of wild-type AE1 and AE1 mutants containing the NANN and AAAA sequences in place of DADD. Each of these mutants had greatly reduced anion exchange activity (flux 2.9±0.6 and 4.0±1.1 mM H^+ equivalents/min, respectively) relative to wild-type AE1 (40±0.6 mM H^+ equivalents/min), yet both mutants were processed to the cell surface as well as wild-type AE1. The simplest explanation for the observation is that loss of the CAII binding site on the mutants results in a reduced transport rate. No role of the C-terminal region in anion transport mechanism has ever been shown. However, it is also possible that these mutants compromise the AE1 mechanism or protein folding, resulting in reduced transport activity.

To examine further the functional role of the CAII/AE1 interaction we performed a dominant negative CAII experiment (Figure 3). In this experiment HEK293 cells were transfected with AE1 + vector, AE1 + wild-type CAII and AE1 + CAIIV143Y. The V143Y mutant of CAII has catalytic activity that is approximately three thousand fold lower than wild-type CAII [21]. The rate of AE1 transport activity when expressed alone was 40±0.6 mM H^+ equivalents/min and co-expressed with wild-type CAII was 38±1.4 mM H^+ equivalents/min, which indicates that overexpresion of wild-type CAII had no significant effect on AE1 transport activity. In contrast, overexpresion of V143Y CAII reduced AE1 transport activity to 16±2.8 mM H^+ equivalents/min, which represents a 60% reduction in transport activity. We interpret these results to mean that overexpression of CAII V143Y results in displacement of endogenous wild-type CAII from its binding site on the AE1 C-terminus. The resulting decrease in bicarbonate transport rate indicates that AE1 transport activity is most efficient when CAII is localized to the cytoplasmic surface of AE1.

Three lines of evidence indicate a significant role of carbonic anhydrase in the transport of bicarbonate by AE1. Treatment of cells with the CA inhibitor, acetazolamide, resulted in a major decrease in bicarbonate transport rate by AE1. Mutants of AE1 that lack the ability to bind CAII have decreased transport activity relative to wild-type AE1. Overexpression of a catalytically inactive form of CAII exerts a dominant negative effect upon the wild-type CAII and results in loss of anion exchange activity by wild-type AE1. We conclude that interaction of CAII with AE1 potentiates the rate of bicarbonate transport.

The significance of these findings to the study of bicarbonate transport and cystic fibrosis is three-fold. First, clearly it is important to consider the impact of CA in the cell physiology of bicarbonate transport processes. Which CA isoforms are expressed in the relevant tissues, where is it expressed and when? In studies of bicarbonate transporters expressed in heterologous expression systems, it is important to determine whether the physiologically relevant CA isoforms are present in the expression system cells. These findings are also important to the study of cystic fibrosis because they suggest that other bicarbonate transport proteins may interact directly with CA. Do other bicarbonate transporters also bind CA? What effect does this interaction have on transport rate? Finally, these findings suggest that one way to regulate bicarbonate transport is to regulate CA activity, by changes of CA expression, or modulation of the interaction between CA and the bicarbonate transporter.

Key words Carbonate Dehydratase, Band 3 Protein, Ion Transport

Abbreviations AE: anion exchanger; BCECF-AM: 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester; CA: carbonic anhydrase; GST: glutathione-S-transferase

Acknowledgements Research in the laboratories of J.R.C. and R.A.F.R is funded by the Canadian Institutes of Health Research (CIHR) and Heart and Stroke Foundation (J.R.C.). J.R.C. is a Scholar of the Alberta Heritage Foundation for Medical Research (AHFMR) and a New Investigator of CIHR. D.S. is supported by Graduate Studentships from AHFMR and the Heart and Stroke Foundation of Canada.

Correspondence
Joseph R Casey
University of Alberta
Department of Physiology
Edmonton, AB
Canada T6G2H7
Phone: +1-780-492.7203
Fax: +1-780-492.8915
E-mail address: joe.casey@ualberta.ca

References

1. Geers C, Gros G. Carbon dioxide transport and carbonic anhydrase in blood and muscle. Physiol Rev 2000; 80:681-715. [20211948]

2. Kivela A, Parkkila S, Saarnio J, Karttunen TJ, Kivela J, Parkkila AK, et al. Expression of a novel transmembrane carbonic anhydrase isozyme XII in normal human gut and colorectal tumors. Am J Pathol 2000; 156:577-84. [20132828]

3. Alper SL. The Band 3-related anion exchanger family. Annu Rev Physiol 1991; 53:549-64.

4. Kopito RR. Molecular biology of the anion exchanger gene family. Int Rev Cytol 1990; 123:177-99. [91147304]

5. 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. [P11102437]

6. 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]

7. Grinstein S, Ship S, Rothstein A. Anion transport in relation to proteolytic dissection of Band 3 protein. Biochim Biophys Acta 1979; 507:294-304. [78103968]

8. Lieberman DM, Reithmeier RAF. Localization of the carboxyl terminus of Band 3 to the cytoplasmic side of the erythrocyte membrane using antibodies raised against a synthetic peptide. J Biol Chem 1988; 263:10022-8. [88257067]

9. Fujinaga J, Tang X-B, Casey JR. Topology of the membrane domain of human anion exchange protein, AE1. J Biol Chem 1999; 274:6626-6633. [99156948]

10. Mori A, Okubo K, Kang D, Hamasaki N. A structural study of the carboxyl terminal region of the human erythrocyte band 3 protein. J Biochem (Tokyo) 1995; 118:1192-8. [96362157]

11. Casey JR, Reithmeier RA. Anion exchangers in the red cell and beyond. Biochem Cell Biol 1998; 76:709-13. [99280169]

12. Tang XB, Fujinaga J, Kopito R, Casey JR. Topology of the region surrounding Glu681 of human AE1 protein, the erythrocyte anion exchanger. J Biol Chem 1998; 273:22545-53. [98380478]

13. Popov M, Tam LY, Li J, Reithmeier RAF. Mapping the ends of transmembrane segments in a polytopic membrane protein. Scanning N-glycosylation mutagenesis of extracytosolic loops in the anion exchanger, band 3. J Biol Chem 1997; 272:18325-32. [97364763]

14. Popov M, Li J, Reithmeier RA. Transmembrane folding of the human erythrocyte anion exchanger (AE1, Band 3) determined by scanning and insertional N-glycosylation mutagenesis. Biochem J 1999; 339:269-79. [99208513]

15. Tanner MJ, Martin PG, High S. The complete amino acid sequence of the human erythrocyte membrane anion-transport protein deduced from the cDNA sequence. Biochem J 1988; 256:703-12. [89134172]

16. Vince JW, Reithmeier RA. Identification of the carbonic anhydrase II binding site in the Cl^-/HCO_3^- anion exchanger AE1. Biochemistry 2000; 39:5527-33. [20281302]

17. Vince JW, Reithmeier RAF. Carbonic anhydrase II binds to the carboxyl-terminus of human band 3, the erythrocyte Cl^-/HCO_3^- exchanger. J Biol Chem 1998; 273:28430-7. [98447702]

18. Vince JW, Carlsson U, Reithmeier RA. Localization of the Cl^-/HCO_3^- anion exchanger binding site to the amino-terminal region of carbonic anhydrase II. Biochemistry 2000; 39:13344-9. [20519029]

19. Reithmeier RAF. A membrane metabolon linking carbonic anhydrase with chloride/bicarbonate anion exchangers. Blood Cells Mol Dis 2001; 27:85-9.

20. Cousin JL, Motais R. The role of carbonic anhydrase inhibitors on anion permeability into ox red blood cells. J Physiol 1976; 256:61-80. [76217400]

21. Fierke CA, Calderone TL, Krebs JF. Functional consequences of engineering the hydrophobic pocket of carbonic anhydrase II. Biochemistry 1991; 30:11054-63. [92031568]