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Mini Review

The Bicarbonate Transport Metabolon

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To allow cells to control their pH and bicarbonate levels, cells express bicarbonate transport proteins that rapidly and selectively move bicarbonate across the plasma membrane. Physical interactions have been identified between the carbonic anhydrase isoform, CAII, and the erythrocyte membrane Cl^-/HCO_3^- anion exchanger, AE1, mediated by an acidic motif in the AE1 C-terminus. We have found that the presence of CAII attached to AE1 accelerates AE1 HCO_3^- transport activity, as AE1 moves bicarbonate either into or out of the cell. In efflux mode the presence of CAII attached to AE1 will increase the local concentration of bicarbonate at the AE1 transport site. As bicarbonate is transported into the cell by AE1, the presence of CAII on the cytosolic surface accelerates transport by consumption of bicarbonate, thereby maximizing the transmembrane bicarbonate concentration gradient experienced by the AE1 molecule. Functional and physical interactions also occur between CAII and Na^+/HCO_3^- co-transporter isoforms NBC1 and NBC3. All examined bicarbonate transport proteins, except the DRA (SLC26A3) Cl^-/HCO_3^- exchange protein, have a consensus CAII binding site in their cytoplasmic C-terminus. Interestingly, CAII does not bind DRA. CAIV is anchored to the extracellular surface of cells via a glycosylphosphatidyl inositol linkage. We have identified extracellular regions of AE1 and NBC1 that directly interact with CAIV, to form a physical complex between the proteins. In summary, bicarbonate transporters directly interact with the CAII and CAIV carbonic anhydrases to increase the transmembrane bicarbonate flux. The complex of a bicarbonate transporter with carbonic anhydrase forms a "Bicarbonate Transport Metabolon."

INTRODUCTION

A metabolon is a weakly associated complex of sequential metabolic enzymes.[1,2] The weak associations of these enzymes can make it very difficult to identify the metabolon constituents. Identified metabolons are known to exist for the glycolytic chain, the citric acid cycle, and the urea cycle.[3] These metabolons exist to move metabolites from the active site of one enzyme to the next in an optimized manner; this is also referred to as substrate channelling.[3,4] Channelling limits the loss of intermediates by orienting the active sites of each enzyme in relatively close proximity to each other, effectively creating a chain of enzymes.[1,2,4,5] The maintenance of intermediates within the chain of enzymes ensures that the substrate flux through the metabolon occurs with maximum efficiency. Studies of the interaction between carbonic anhydrases and bicarbonate transporters now show that these proteins associate into functional bicarbonate transport metabolons.

BICARBONATE TRANSPORT PROTEINS

In mammals bicarbonate transport is facilitated by a superfamily of proteins that range in sequence relatedness from 10–90%. These cluster into three separate families, the classical Cl^-/HCO_3^- exchangers, Na^+-coupled transporters, and the novel SLC26A family (Figure 1). Bicarbonate is important in maintaining homeostasis by regulating cellular and whole body pH because it is a base.[6] Bicarbonate Transporters (BTs) are required to regulate transmembrane HCO_3^- flow, which would otherwise not occur because HCO_3^- is membrane impermeant.

The chloride/bicarbonate exchanger (AE) family (Figure 1) has three isoforms that share 65% amino

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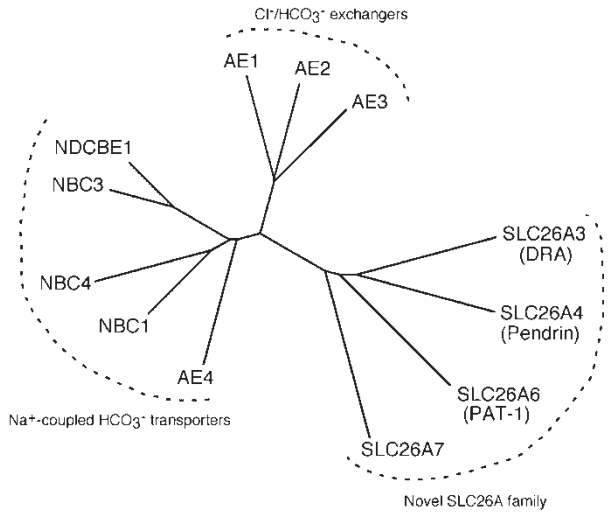

FIGURE 1 Phylogenetic relationships of human bicarbonate transport proteins. Amino acid sequences for human bicarbonate transporters were analysed with the program Phylip on the ClustalW website.[50] Plotted are evolutionary relationships for the transporters, where the length of the line is proportional to the degree of sequence similarity between the proteins. Separate lines represent divergence from common ancestors. Dashed lines surround each of the three clearly separate branches of the human bicarbonate transporter superfamily (Cl^-/HCO_3^- exchangers: AE1;[38] AE2;[51] AE3,[52] Na^+-coupled bicarbonate transporters: NBC1;[53,54] NBC3;[55] NBC4;[56] AE4;[18] and NDCBE1,[57] and Novel transporters: SLC26A3;[58] SLC26A4;[59] SLC26A6;[60] and SLC26A7[61]).

acid sequence identity.[7] AEs exchange bicarbonate for chloride with a 1:1 stoichiometry, resulting in an electroneutral transfer of anions across the cell membrane. AE1 is found in erythrocytes and a truncated version of AE1 is found on the basolateral surface of renal α-intercalated cells.[8] AE2 is the most widely expressed AE isoform, present in nearly all human tissues.[6] AE3 is found in the brain,[9] heart,[10] retina[11] and other excitable tissues.[6] Although located in different tissues, all of the AE proteins share a similar structure consisting of three major domains.[12] The amino-terminal cytosolic domain is important for protein–protein interactions with cytosolic proteins, metabolic enzymes, and cytoskeletal elements.[13] The carboxyl-terminal membrane domain consists of 12 transmembrane segments[14] and is responsible for anion transport.[15] The C-terminal cytosolic tail can be considered a third domain as it folds independently and has a separable function. The C-terminal tail contains the binding site for CAII,[16,17] forming the basis for the intracellular bicarbonate transport metabolon.

The second family of the BT superfamily are the sodium-coupled bicarbonate transporters (Figure 1). Na^+/HCO_3^- co-transporters (NBC) co-transport sodium and bicarbonate across the plasma membrane with either an electroneutral (NBC3) or electrogenic (NBC1, NBC4) mechanism ($2/3HCO_3^-$:1 Na^+). All NBC isoforms are expressed in the kidney implying their importance. NBCs are also located in heart, skeletal muscle, and parts of the intestinal tract. Phylogenetic analysis groups AE4, which is less genetically similar to the AE family than to the NBC family, with the NBCs.[18]

The third branch of the BT phylogenetic tree shows selected members of the SLC26 family (Figure 1). The SLC26 anion transport gene family,[19] also mediate anion exchange at the plasma membrane of mammalian cells. The family is comprised of eleven genes, SLC26A1-A11, which transport Cl^-, SO_4^{2-}, OH^-, HCO_3^-, I^-, oxalate and formate anions with different preferences.[20–25] Only SLC26A3, SLC26A4, SLC26A6 and SLC26A7 family members have been reported as Cl^-/base (HCO_3^- and OH^-) exchangers, which suggests common features between these transporters and members of the bicarbonate transport superfamily.

CARBONIC ANHYDRASES

Carbonic anhydrases (CA) are zinc metalloenzymes that catalyze the reversible reaction of: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$.[26] There are currently 14 CA isoforms identified.[27–29] CAII, one of the most efficient biological catalysts (turnover rate of 10^6 s^{-1} at 37°C [30]), uses a zinc-activated hydroxide ion. The metal ion stabilizes the highly reactive hydroxide ion, thereby ensuring that the nucleophile (the zinc-hydroxide complex) is available for rapid catalysis.

CA isoforms differ in their cellular localization. For example, CAI and II are cytosolic while CAV localizes to mitochondria.[6,31,32] Uniquely, CAIV is anchored to the extracellular surface via a glycosyl phophatidyl inositol (GPI) anchor. Similarly, CAIX, XII, and CAXIV are anchored to the extracellular surface, but via a transmembrane protein anchor. There is a broad expression of CA isoforms throughout the body similar to the broad BT expression.[33] There are many tissues where there are multiple isoforms of CA and other tissues where there is only one.[33] For example, in erythrocytes CAI and CAII are both present in the cytosol and CAIV is bound to the extracellular membrane,[16,34] but in the adult heart CAIV is the only isoform expressed.[35,36]

Although CAI is the dominant isoform in the erythrocyte, the more catalytically active isoform, CAII, accounts for the majority of activity in erythrocytes. CAII and CAIV also work in concert in the kidney to regulate acid–base balance.[27] CAII catalyzes the hydration of carbon dioxide (CO_2) in the cytoplasm and CAIV catalyzes the reverse reaction on the extracellular surface of plasma membrane. Cytosolic CAII comprises about 95% of carbonic anhydrase activity in kidney cells. The remaining 5% is largely due to the activity of the membrane bound CAIV.[37] CAII and CAIV are the focus for this discussion since they have been identified to be a part of one or more metabolons. The remaining CA

isoforms are still under investigation for their role in bicarbonate transport metabolons.

PHYSIOLOGY OF ERYTHROCYTE BICARBONATE TRANSPORT

CO_2, produced by cells as the end product of respiratory oxidation, is membrane permeable and thus diffuses from the cell into erythrocytes in the bloodstream. In the erythrocyte, CAII readily hydrates CO_2 to HCO_3^-. HCO_3^- would also quickly saturate the cell if not for AE1,[38] which rapidly transports HCO_3^- out of the erythrocyte into the plasma where it travels to the lungs. The process is reversed in erythrocytes, once they reach the lungs where CO_2 is produced for exhalation. Because HCO_3^- is more soluble than CO_2 this pathway allows for a greater concentration of CO_2 to be absorbed and transported in blood.[6] This scenario led to the investigation of a direct link between CAII and AE1.

THE BICARBONATE TRANSPORT METABOLON

The initial observations of CA/BT interactions were made in the erythrocyte, with AE1 and CAII.[16] Several lines of evidence have shown that the two proteins associate. Firstly, tomato lectin will bind the carbohydrate on AE1 and cause clustering of the protein in the plane of the erythrocyte membrane. Interestingly, in tomato lectin treated erythrocyte membranes, immunofluorescence showed that CAII clustered on the cytosolic surface in a pattern very similar to AE1, indicating co-localization.[16] More direct evidence for an AE1/CAII association was the coimmunoprecipitation of CAII with AE1, when solubilized from erythrocyte membranes.[16] Finally, a GST fusion protein of the terminal 33 amino acids of the C-terminus of AE1, but not GST alone, interacted with CAII with high affinity in both GST pull down assays and in a solid phase binding assay.[16] Taken together this data showed that CAII and AE1 interact with high affinity in the erythrocyte membrane.

Further studies revealed the nature of the CAII/AE1 interaction. The pH dependence of CAII/AE1 interaction suggested a requirement for electrostatic interactions. Acidic regions of the AE1 C-terminal region were the likely candidates for the CAII binding site. This hypothesis was borne out by peptide competition assays, a sensitive micro-titre binding assay, and functional assays that tested the activity of AE1 mutated by truncation or point mutations at the Carboxyl-termimal binding sequence.[17] When the acidic region D887ADD was removed from the C-terminal region, binding of CAII was lost.[17] When mutating the motif to DANE, the analogous motif from AE2, binding of CAII was retained indicating that AE2 was also capable of CAII binding.[17] Further analysis demonstrated that AE3 also bound CAII.[33] The motif for CAII binding was thus identified as a hydrophobic residue followed by four residues, at least two of which are acidic. This motif has been found in the C-terminus of all examined bicarbonate transporter sequences, except SLC26A3 (DRA).[5]

Since AE1 and AE2 have an acidic CAII binding site it was reasoned that CAII would contain a corresponding basic region that binds AE. Truncation mutations of the amino-terminal region of CAII localized the binding region to a basic patch in the first 17 amino acids.[39] Point mutation analysis confirmed that CAI, which lacks 5 histidine residues in the first 17 amino acids of the N-terminus, did not bind AE1.[39] These 5 His residues are the major differences between CAI and CAII. X-ray crystallography revealed that the N-terminus of CAII is found on the surface of the protein.[40] Thus, CAII mutations outside the N-terminal can impair catalytic activity, without effect on AE1 binding capacity. For example, V143Y CAII mutant can still bind to AE1. The suggestion of a metabolon came with the structural basis for CAII/AE1 binding[3] but the question of functional significance of the CAII/AE1 interaction still needed to be addressed.

The functional significance of the CAII/AE1 interaction later became apparent. When AE1 transfected HEK293 cells were treated with acetazolamide, a carbonic anhydrase inhibitor, bicarbonate transport was nearly completely inhibited.[41] This indicated that endogenous CAII was essential for efficient AE1 activity,[41] identifying an intracellular component of the bicarbonate transport metabolon (Figure 2A and B). To assess the functional importance of the CAII/AE1 interaction, a dominant negative approach was used. HEK293 cells, which endogenously express CAII, were transfected to express AE1 with CAII or a functionally inactive human CAII mutant, V143Y. V143Y CAII retained its ability to bind AE1.[5,41] V143Y CAII was expressed at levels 20-fold higher than the endogenous wild-type CAII in the cells and could thus compete with the endogenous CAII for the CAII binding site on the AE1 C-terminus. Since the V143Y CAII-expressing cells still expressed wild-type CAII, the expression of V143Y CAII would not affect total CA activity in the cells; V143Y CAII would affect only the location of functional CAII. AE1 activity was inhibited by 39% when HEK293 cells were co-transfected with functionally inactive V143Y CAII.[5] This indicates that displacement of endogenous CAII from the AE1 C-terminus into the cytosol greatly reduced AE1 HCO_3^- transport activity. In another set of experiments, the CAII

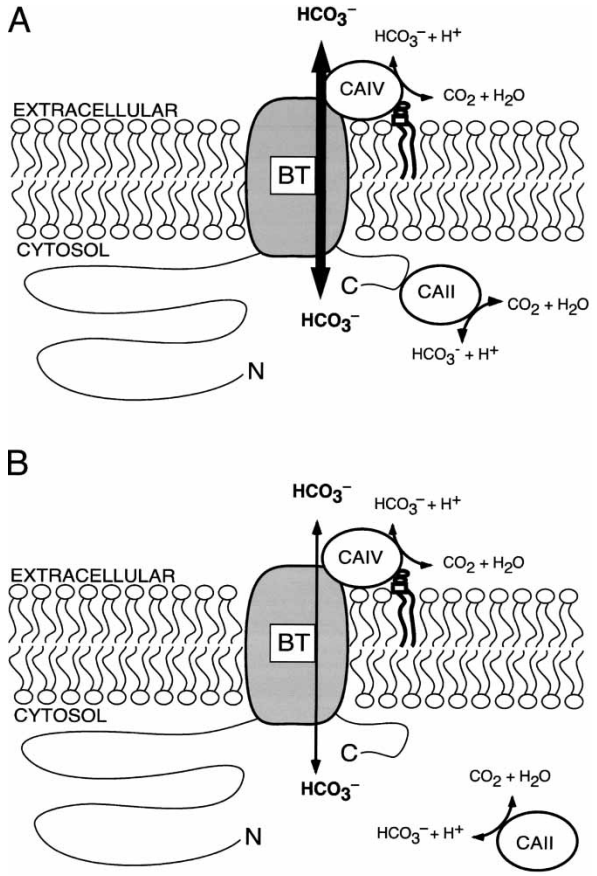

FIGURE 2 Model of the bicarbonate transport metabolon. Bicarbonate transporters (BT) transport bicarbonate across cell membranes. Carbonic anhydrases (CA) catalyze the reversible hydration of CO_2 to HCO_3^- and H^+. The CAIV isoform associates with the plasma membrane via a glycosyl phosphatidyl inositol (GPI) linkage. Although CAIV binds both AE proteins and NBC1, the reversibility of the process in the membrane has not been studied. BTs bind CAII at their cytosolic carboxyl terminus; CAII can also exist free in the cytosol. When CAII and CAIV are bound to BT the transport rate is maximized (A). When CAII is not bound to BT and CAIV is not expressed the transport rate is reduced, but not abolished (B). The physical complex between the BT and the CAs facilitates efficient transport by minimizing the distances the substrates must diffuse, thereby maximizing the local concentration of substrate both for the transporter and the enzyme.

binding site on AE1 was mutated so that it could not bind CAII. HCO_3^- transport activity was reduced by 90% relative to wild-type AE1 activity.[5] This indicated that binding of CAII was necessary for full HCO_3^- transport activity.

CAII/AE1 interaction was also demonstrated when the proteins were expressed in *Xenopus* oocytes. The bicarbonate transport activities of inactive AE1 mutants were monitored with the expression of CAII.[42] As expected, inhibition of CAII inhibited AE1 transport activity as did overexpression of V143Y CAII in *Xenopus* oocytes.[42] CAII affected Cl^-/HCO_3^- exchange activity but had no effect on Cl^-/Cl^- exchange activity of AE1, consistent with the bicarbonate transport metabolon model.[42] Although there are differences between transport assays in *Xenopus* oocyte and HEK293 cells, these findings support the role of CAII as a bicarbonate channelling activator.

Interestingly, this activating relationship is reciprocal. Evidence suggests that CAII is not fully active without binding to AE1. This was demonstrated when di-/tri- and tetrapeptides containing the LDADD acidic motif of AE1 were shown to improve CAII activity.[43] However, binding of the peptides did not affect CAI or CAIV activity. This would be expected since CAI does not bind to AE1 and CAIV is extracellular and would not have access to the C-terminus of AE1. These results indicated that AE1 was both a bicarbonate transporter and a CAII activator[43] and perhaps could activate other CAs.

The bicarbonate transport metabolon extends beyond CAII. CAIV, which is the extracellular isoform that is bound through a GPI linkage to the extracellular surface, interacts with extracellular loop 4 of AE1.[33] This interaction was suspected when CAIV was recruited to the position of AE1 in Triton X-100 HEK293 extracts resolved by sucrose gradient ultracentrifugation.[33] Gel overlay assays demonstrated a specific physical interaction between CAIV and AE1, AE2, and AE3.[33] A functional interaction was identified when CAIV restored AE1, AE2, and AE3 to full HCO_3^- transport activity after activity was inhibited by the overexpression of inactive V143Y CAII.[33] Glutathione *S*-transferase pull down-assays localized the physical interaction of CAIV to extracellular loop 4 of AE1.[33] These experiments identified the extracellular component of the bicarbonate transport metabolon (Figure 2A and B).

The bicarbonate metabolon has broad significance since CAII and CAIV have now been shown to bind to other members of the bicarbonate superfamily. A physical relationship exists between CAII and NBC1,[44,45] NBC3,[46] and human putative anion transport 1 (SLC26A6/PAT-1).[47] It has also been demonstrated that NBC1 and CAIV have a physical and functional relationship.[44] CAIV binds the fourth extracellular loop of NBC1, in a region conserved between BT.[44]

Important support for the bicarbonate metabolon comes from the failure of DRA and CAII to interact. DRA lacks a consensus CAII binding motif in its C-terminus, in contrast to all of the other HCO_3^- transporters.[48] DRA (SLC26A3) did not bind CAII and the dominant negative V143Y CAII had no effect on DRA transport activity. Thus, the absence of a CAII binding motif was predictive of the absence of CAII binding.[48]

The basis for a metabolon is to create a more efficient passage of substrate from one enzyme to the next in an enzyme chain. This paper has discussed in detail the relationship of CAII/AE1 and CAIV/AE1 and has illustrated their intimate interactions

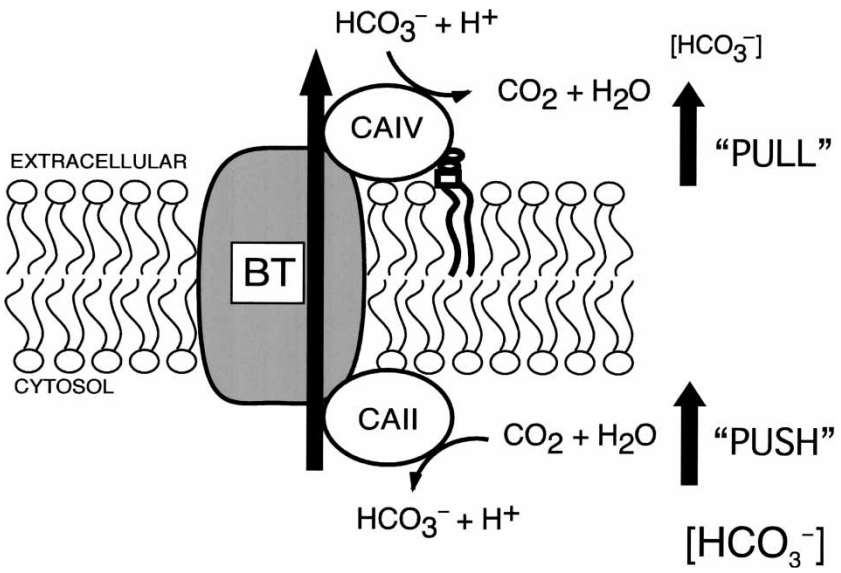

FIGURE 3 The metabolon maximizes the size of the transmembrane bicarbonate gradient. The carbonic anhydrases that interact with the bicarbonate transporter are responsible for the generation of a concentration gradient of HCO_3^- via a "push-pull" mechanism. In HCO_3^- efflux mode, CAII rapidly converts CO_2 to HCO_3^- establishing a local high concentration of HCO_3^- to "push" transport of bicarbonate out of the cell. At the extracellular face, CAIV rapidly converts HCO_3^- to CO_2, which depletes the local extracellular HCO_3^- concentration, thereby "pulling" bicarbonate into the extracellular space. Combined, these effects drive bicarbonate transport out of the cell. The reverse of this process also occurs to drive bicarbonate transport into the cell.

(Figure 2). CAII is physically located very near to the AE1 active site (Figure 2A). This allows bicarbonate to be released from either protein in a region that allows for quick binding to the other protein, a channelling effect. Having the proteins so closely associated creates a microenvironment around AE1 that allows for a tightly controlled bicarbonate concentration gradient. CAII or CAIV can generate the substrate for AE1 creating a forward "push" of the transport, or they can consume the substrate passing through AE1 shifting the flux of bicarbonate in a reverse "push", or "pull" (Figure 3). When CAII is present it is responsible for the transmembrane concentration gradient that determines the direction and magnitude of the "push" or "pull" driving force for bicarbonate through AE1 or any BT that it binds to.[33] The driving force of the metabolon is the high concentration of HCO_3^- at the active site of the transporter, and the low concentration of HCO_3^- on the opposite side of the membrane.

Metabolon interactions of CA have now extended beyond bicarbonate transporters. CAII interacts in a regulatory fashion with the sodium/hydrogen exchanger (NHE1).[49] This may hold true for the other CAs as well. These interactions may have profound implications in disease research. Since full activity of the pH-regulatory transporters is dependent on a precise arrangement of protein interactions there may be very important links between the malfunction of the metabolon and many significant diseases.

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