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ScienceDirect
Respiratory Physiology & Neurobiology 154 (2006) 185–198
RESPIRATORY PHYSIOLOGY & NEUROBIOLOGY
www.elsevier.com/locate/resphysiol

The structure and function of carbonic anhydrase isozymes in the respiratory system of vertebrates☆

A.J. Esbaugh*, B.L. Tufts

Department of Biology, Queen's University, Kingston, Ont., Canada K7L 3N6

Accepted 20 March 2006

Abstract

Carbonic anhydrase is a ubiquitous metalloenzyme that catalyzes the reversible hydration/dehydration of carbon dioxide. To date, 16 different CA isozymes have been identified in mammals, and several novel isozymes have also been identified in non-mammalian vertebrates. These isozymes are involved in many physiological processes; however, one of the most important roles is facilitating the transport and subsequent excretion of carbon dioxide. As such, CA isozymes are found at virtually every step of the process, including the metabolic site of CO_2 production (muscle), the circulating red blood cells, and the primary respiratory surface (gills/lungs). This review will examine the structural characteristics that are integral to CAs participation in respiration, as well as highlight the specific roles and tissues that the different CA isozymes are involved in.

Keywords: Comparative physiology; Carbonic anhydrase; Isozymes; Vertebrates

1. Introduction

Carbonic anhydrase (CA) is a ubiquitous enzyme that catalyzes the reversible hydration/dehydration reactions of carbon dioxide. This enzyme has been found in virtually all living organisms, however only members of the α-CA gene family are found in mammals (Chegwidden and Carter, 2000; Hewett-Emmett, 2000). To date, 16 different CA isozymes have been identified in mammals by means of their sequence, biochemical properties and subcellular location. In addition, several novel isozymes have recently been identified in non-mammalian vertebrates.

In general, there are three distinct groups of CA isozymes within the α-CA gene family (Fig. 1). One of these groups contains the cytoplasmic CAs, which includes mammalian CA I, II, III, V, VII and XIII. These isozymes are found in the cytoplasm of various tissues, with the exception of the mitochondrial confined CA V. Another group of isozymes, termed the membrane-bound CAs, consists of mammalian CA IV, IX, XII, XIV and XV. These isozymes are associated with the plasma membranes of many different

☆ This paper is part of a special issue entitled "Frontiers in Comparative Physiology II: Respiratory Rhythm, Pattern and Responses to Environmental Change", guest-edited by William K. Milsom, Frank L. Powell, Gordon S. Mitchell.

* Corresponding author. Tel.: +1 613 533 6000x75398; fax: +1 613 533 6617.
E-mail address: esbaugha@biology.queensu.ca (A.J. Esbaugh).

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doi:10.1016/j.resp.2006.03.007

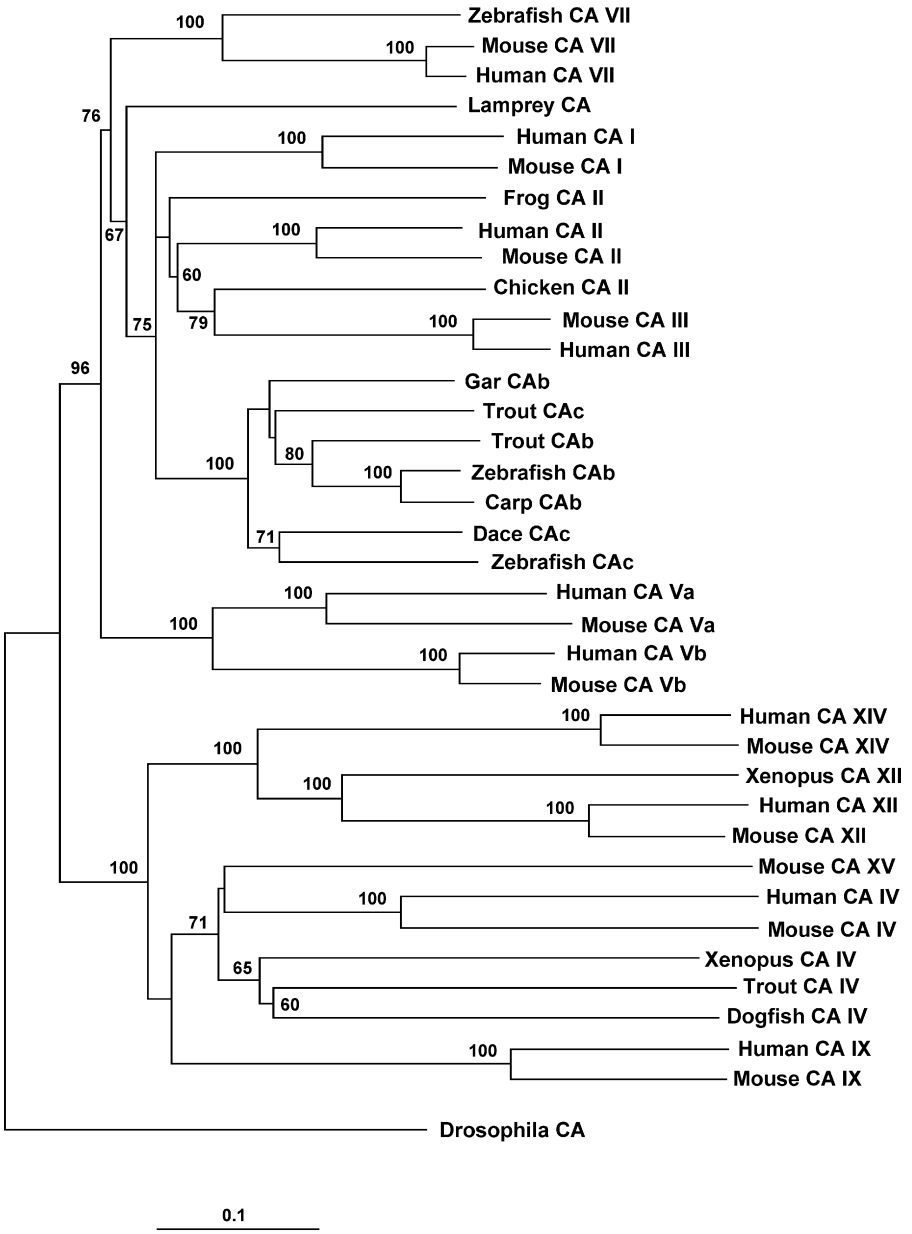

Fig. 1. A phylogenetic analysis of the cytoplasmic and membrane-bound carbonic anhydrase isozymes of the α-carbonic anhydrase gene family. The phylogenetic tree was constructed using neighbour joining (reproduced using parsimony analysis) with support for nodes assessed using bootstrap analysis (1000 pseudoreplicates), and ordered using drosophila CA as an outgroup. Bootstrap values below 50 were not included, denoting poor branch support. Branches are drawn to scale with 0.1 approximating replacement of 10% of the amino acids in the protein alignment.

tissue types. The final group (not illustrated in Fig. 1) contains several very intriguing isozymes, CA VIII, X and XI, which are termed the CA-related proteins (CA-RP; Tashian et al., 2000). These isozymes have lost classical CA activity – the hydration/dehydration of CO_2 – and have no known physiological function; however, their highly conserved nature does suggest a very important role in vertebrates (Tashian et al., 2000).

The various CA isozymes are found in many different tissues and are involved in a number of different physiological processes, including bone resorption, calcification, ion transport, acid–base transport, and a number of different metabolic processes. One of the most important and well studied roles of CA, however, involves the movement of respiratory gases within the body. The work in this field has primarily examined the mechanisms by which different CA isozymes function to facilitate respiratory gas transport in a variety of tissues. This review will examine the structural characteristics that are integral to CAs participation in these physiological functions, as well as highlight the specific roles that the different isozymes play with respect to respiratory gas exchange in vertebrates.

2. Structure

2.1. Catalytic mechanism

The general catalytic mechanism of all members of the α-CA gene family revolves around the presence of a zinc bound hydroxide ion. This mechanism has been reviewed in detail by Lindskog and Silverman (2000), and thus will be only briefly described here. In short, a substrate molecule (CO_2) is brought into the active site pocket of CA where it undergoes a nucleophilic attack by the zinc-bound hydroxide (Silverman and Lindskog, 1988; Christianson and Fierke, 1996). This directly forms a bicarbonate ion, thereby bypassing the slower uncatalyzed reaction that sees the formation, and subsequent disassociation, of carbonic acid. The bicarbonate ion is then replaced in the active site by a water molecule. It is currently unclear whether bicarbonate is actively removed from the active site, or if water simply displaces the ion from zinc after its formation. In either case, the zinc ion binds a water molecule. The zinc-bound hydroxide is then regenerated by the removal of a proton via the proton shuttling mechanism (Tu and Silverman, 1989). The proton is then absorbed by buffers outside of the active site. It should be noted that the regeneration of zinc-bound hydroxide in the active site is the rate limiting step in the reaction, and is highly dependent on the presence of buffer agents in the cytoplasm to absorb the proton (Lindskog and Silverman, 2000).

There are four main components of the molecular structure of CA that are important to its catalytic function. These are: the zinc binding site, the substrate association pocket, the threonine-199 loop, and the proton shuttling mechanism (Table 1). The zinc binding site consists of three histidine amino acid residues (His-94, His-96 and His-119; Christianson and Alexander, 1989), which directly bind to the zinc ion. In addition, each histidine residue has an associated ligand (Gln-92, Asn-244 and Glu-117) that provides a hydrogen bond to help stabilize the zinc ion (Kiefer and Fierke, 1994; Kiefer et al., 1995). The entirety of this motif is conserved within the α-CA gene family, with the exception of inactive CA-RP isozymes (Tashian et al., 2000) and trout CA IV, which has unknown catalytic ability (Table 1). The substrate association pocket is a series of six non-polar amino acids that create a hydrophobic pocket (Krebs et al., 1993). This pocket is adjacent to the zinc binding site and is responsible for isolating CO_2 and channeling it toward the zinc-bound hydroxide. The major function of the threonine-199 loop is to orient the zinc-bound hydroxide for catalysis by donating a hydrogen bond (Merz, 1990), while the proton shuttle residue simply accepts a proton from the zinc-bound water molecule and proceeds to donate it to buffer agents in the cytoplasm (Tu and Silverman, 1989). It should be noted that the proton shuttle residue does not accept the proton directly from the zinc-bound water, but instead the proton moves through a shuttle chain consisting of three water molecules. The proton shuttling residue in high activity CA isozymes is His-64, and changes in this residue have been shown to dramatically decrease CA activity (reviewed by Lindskog and Silverman (2000)). In addition, amino acid substitutions of the neighbouring amino acid (residue 65) with amino acids with bulky side chains have also been shown to decrease the catalytic efficiency.

Table 1
Comparison of the amino acid residues of the putative active site pocket from the various cytoplasmic and membrane isozymes of vertebrates

Consensus II	Y	S	N	N	H	S	F	N	E	I	Q	H	H	E	H	E	H	V	F	L	V	G	W	Y	L	T	T	P	P	L	C	V	W	V	N	R	
	7	29	61	62	64	65	66	67	69	91	92	94	96	106	107	117	119	121	131	141	143	145	192	194	198	199	200	201	202	204	206	207	209	211	244	246	
					+							z	z				z	~		~	~				~								~	~				Diff
Cytoplasmic																																					
Trout	.	.	.	.	.	.	.	Q	G	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.		4
Lamprey	.	.	.	.	.	.	.	S	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	S	.	.	.	.	.		4
Gar	.	.	.	.	.	.	.	Q	D	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.		4
Xenopus	.	.	.	.	.	A	.	.	.	K	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		3
Chicken	_	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.		2
Human I	.	.	.	V	.	.	.	H	N	F	.	.	.	.	.	.	.	.	L	.	I	.	.	.	.	.	H	.	H	S	.	.	I	.	.		10
Human III	.	.	.	.	K	T	C	R	V	R	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	F	.	.	.	E	.	I	.	L	.	.		11
Human Va	W	.	.	T	Y	L	F	Q	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	S	.	.	I	.	.		10
Human Vb	.	.	.	.	Y	S	F	L	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	S	.	.	I	.	.		8
Human VII	.	.	.	.	.	.	V	Q	D	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	S	.	.	.	.	.		6
Membrane																																					
Trout IV	.	.	.	.	R	T	V	Q	D	.	.	.	.	.	.	.	.	.	L	V	.	.	F	.	.	.	.	.	E	A	.	.	.	T	.		11
Dogfish IV	.	.	.	.	.	.	V	Q	T	V	.	.	.	.	.	.	.	.	I	.	.	.	Y	.	.	.	.	.	G	F	A	.	.	.	.	.		9
Xenopus IV	.	.	.	.	.	.	A	Q	D	.	.	.	.	.	.	.	.	.	D	.	.	.	Y	.	.	.	.	.	.	N	T	.	.	L	.	.		8
Human IV	.	.	.	.	.	.	V	M	L	K	.	.	.	.	.	.	.	.	V	I	.	A	F	.	.	.	.	.	T	D	K	.	.	.	.	.		11
Human IX	H	.	.	.	.	.	V	Q	T	L	.	.	.	.	.	.	.	.	V	.	.	A	F	.	.	.	.	.	.	A	G	.	.	.	.	.		10
Human XII	.	.	.	.	.	.	V	K	N	T	.	.	.	.	.	.	.	.	A	.	.	A	Y	.	.	.	.	.	.	N	T	.	.	.	.	.		9
Human XIV	.	.	.	.	.	T	V	Q	S	A	.	.	.	.	.	.	.	.	L	.	.	.	F	.	.	.	.	.	T	Y	Q	.	.	.	.	.		10
Mouse XV	.	.	.	D	.	T	V	L	R	L	.	.	.	.	.	.	.	.	M	F	I	A	Y	.	.	.	.	.	G	E	A	.	.	.	.	.		14

z: zinc binding ligand; +: proton shuttling ligand; ∼: substrate associated pocket. The CA II consensus sequence from Tashian et al. (2000). Genbank accession numbers for the other sequences are as follows: trout CAb (AY307082) and IV (AY514871), lamprey CA (DQ157849), gar CA (AY125007), Xenopus CA II (BC041213) and IV (BC054242), chicken CA II (X12639), dogfish CA IV (DQ092628), mouse CA XV (AF231113), and human CA I (X05014), III (NM_005181), IV (NM_000717), Va (NM_001739), Vb (NM_007220), VII (AY075019), IX (NM_001216), XII (BC001012) and XIV (012113).

2.2. Cytoplasmic CAs

In mammals, there are six known catalytically active cytoplasmic CA isozymes (CA I, II, III, V, VII and XIII). Of these six isozymes, two are high turnover (CA II and VII), three are low turnover (CA I, V and XIII) and CA III has an extremely low turnover rate (Chegwidden and Carter, 2000). Structural modifications to the proton shuttling mechanism are the major causes for less efficient catalysis in CA I, III and V (Lindskog and Silverman, 2000; Stams and Christianson, 2000). In the cases of both CA III and V, an amino acid substitution at residue 64 to a less efficient proton shuttle resulted in decreased efficiency (Table 1). In addition, both isozymes also contain a mutation at residue 65 – from serine to an amino acid with a more bulky side chain – that further inhibits the proton shuttle mechanism. For CA III, these changes, along with the Phe-198 substitution, have resulted in complete elimination of the shuttling mechanism, which causes the proton to be donated directly to cytoplasmic buffers (Tu et al., 1990; Lindskog and Silverman, 2000; Duda et al., 2005). In the case of CA I, however, both residues 64 and 65 are identical to those found in the high activity CA II isozyme (Table 1). The major modifications in this case appear to be the addition of three histidine amino acids at residues 67, 200 and 204 of the active site cavity, which causes the size of the cavity to be reduced (Engstrand et al., 1995). In this case, the proton shuttle is believed to be His-200.

Phylogenetic analyses of the α-CA gene family reveal that CA V and VII are the most ancestral cytoplasmic isozymes (Fig. 1). Interestingly, these two isozymes also appear to have limited roles within vertebrates. CA V is confined to liver and muscle mitochondria where it provides bicarbonate ions for various biochemical pathways, while the role of CA VII has yet to be determined (Chegwidden and Carter, 2000). Interestingly, however, the highly conserved structure of CA VII and its presence throughout the vertebrate lineage suggest that it should have a very important function. To date, much of the research on the evolution of the

structure and function of CA has been focused on the widely distributed CA I and II isozymes.

Early phylogenetic analyses have shown that mammalian CA I, II and III evolved via a series of gene duplication events (Hewett-Emmett and Tashian, 1991). In these studies, however, the ancestral state of the cytoplasmic isozymes was uncertain. The intron/exon patterns of the genes themselves suggested that a high activity isozyme was likely the original gene, as CA II, VII and the more derived low activity CA III had the same pattern (Tashian, 1992). It was therefore concluded that the different structural pattern in CA I was the result of an intron insertion sometime after the duplication events. This conclusion, however, was based on a small number of species (human and mouse). In contrast, biochemical evidence of red blood cell (rbc) CA isozymes from a modern representative of an ancient vertebrate lineage (agnathans) suggested that the slow activity CA I isozyme was the ancestral state (Maren et al., 1980; Henry et al., 1993). This hypothesis arose from the finding that the ancestral agnathans, such as hagfish and lamprey, had only a single rbc CA isozyme that had biochemical characteristics similar to those of mammalian CA I (Maren et al., 1980; Henry et al., 1993). Recent studies into the structure and function of lamprey cytoplasmic CA, however, have used modern protein expression methods to conclude that lamprey rbc CA is, in fact, a high activity CA isozyme (Esbaugh and Tufts, 2006). It is therefore likely that the ancestral cytoplasmic CA isozyme was a high activity enzyme, and the catalytic structure has been conserved throughout the vertebrate lineage.

Several other interesting trends arise when the available sequence data for cytoplasmic CA isozymes from non-mammalian vertebrates is examined. One such trend is that phylogenetic analyses of the cytoplasmic CA isozymes do not group non-mammalian and mammalian isozymes together (Fig. 1; Lund et al., 2002; Tufts et al., 2003; Esbaugh et al., 2004; Esbaugh et al., 2005). That is to say, the high activity CA isozymes found in fish do not appear to be the same isozymes as those in mammals. Thus, it is not appropriate to use mammalian nomenclature to describe non-mammalian cytoplasmic CA isozymes (Tufts et al., 2003; Esbaugh et al., 2005). Examination of the amino acid residues of the active site pocket, however, shows that the key elements of the catalytic mechanism are almost entirely conserved throughout vertebrates, with only 2–4 differences between CA II and representative cytoplasmic isozymes from agnathans, teleosts, amphibians and birds (Table 1). This strongly suggests that the catalytic mechanism of CA is highly conserved throughout the vertebrate lineage.

In light of the structural conservation of the cytoplasmic CA active site, it would seem prudent to re-examine the properties of elasmobranch cytoplasmic CA. Biochemical analysis of the rbcs of elasmobranchs suggest that these species contain only a single low activity CA isozyme (Maren et al., 1980). There has been no effort, however, to use modern molecular methods to re-enforce these findings, as in the study recently performed for lamprey rbc CA (Esbaugh and Tufts, 2006). In view of the highly conserved nature of the cytoplasmic CA active site, it seems likely that rbc CA in elasmobranchs is also a high activity isozyme. This is supported by the fact that elasmobranch rbc membranes contain a high activity anion exchange protein (Obaid et al., 1979) that would cause low activity rbc CA to be rate limiting during gas transport. Future studies of elasmobranch rbc CA will therefore provide valuable information concerning the early evolution of rbc CA isozymes.

2.3. Membrane-bound CAs

In mammals, there are five CA isozymes (IV, IX, XII, XIV and XV) that are associated with plasma membranes (Sly, 2000; Hilvo et al., 2005). As shown in Fig. 1, the membrane-associated CAs diverged from the cytoplasmic isozymes at a very early stage in the evolution of the α-CA gene family. Of these isozymes, three (IX, XII and XIV) have transmembrane domains while two (IV and XV) are bound to membranes via a glycosylphosphatydlinositol (GPI) anchor. Interestingly, the catalytic rate of the membrane-associated isozymes is generally high – the only exception being CA XV, which has a low catalytic rate (Maren et al., 1993; Chegwidden and Carter, 2000; Whittington et al., 2004; Hilvo et al., 2005). This is in spite of the large number of amino acid differences between the active site pockets of the membrane-associated isozymes and that of the high activity CA II isozyme (Table 1). Upon closer examination, however, it is clear that there are no major changes to amino acid residues in the membrane-bound isozymes that are directly implicated in the catalytic mechanism, although there is the possibility of steric

effects within the pocket. An explanation for the low activity of CA XV is not immediately clear from the active site pocket analysis; however, it is possible that the Asn-62 → Asp mutation may alter the orientation of His-64, making it unable to function as a proton shuttle, however this has yet to be demonstrated. It should be noted that CA III and V also show a mutation at this residue, and both isozymes have low CA activity.

In most cytoplasmic CA isozymes, a mutation of the Pro-202 residue would lead to the destabilization of the Thr-199 loop, and thus a dramatic decrease in catalytic efficiency. Interestingly, such mutations are found in mammalian CA IV, XIV and XV, but the proper conformation of the Thr-199 loop is maintained in these isozymes despite the energetically unfavorable mutation at residue 202 (Tweedy et al., 1993). This is largely due to the presence of a disulfide bridge between Cys-23 and 203. This bridge is conserved in all of the membrane-bound CA isozymes, and is believed to confer extra stability to these isozymes in the face of the harsh extracellular environment (Stams and Christianson, 2000). It is also thought to be responsible for the resistance of the membrane-bound CA isozymes to denaturation by SDS (Whitney and Briggle, 1982). A second disulfide bridge (Cys-6 and 11) in human CA IV is also thought to participate in stabilizing the enzyme in the extracellular environment, but this bridge is absent in all other membrane-bound CA isozymes.

As previously mentioned, both CA IV and XV are bound to plasma membranes via a GPI anchor; these isozymes therefore undergo significant post-translational modification in the endoplasmic reticulum prior to being delivered to the membrane. First, endoproteolytic enzymes cleave off the N-terminal signal peptide. This peptide consists of the first 18–20 amino acids of CA IV and XV isozymes, and is responsible for directing the post-translational transport of the immature protein (Waheed et al., 1996). The C-terminal hydrophobic motif is also cleaved to provide a binding site for the GPI anchor. This binding site is Ser-266 (numbered for the mature protein; Okuyama et al., 1995), which is almost entirely conserved throughout vertebrate CA isozymes. Although this binding site has only been experimentally documented for human CA IV, the conserved nature of the Ser-266 residue leads to the conclusion that this binding site is probably similar for all CA IV and XV isozymes. Both the signal peptide and hydrophobic region are necessary for proper in vivo function of the protein, but it is also noteworthy that a functional cytoplasmic protein of similar catalytic efficiency can be created by removing both of these sections (Waheed et al., 1996). This knowledge may be useful for future biochemical characterization of non-mammalian vertebrate CA IV and XV isozymes because cytoplasmic forms that do not require post-translational modifications could be expressed in bacterial cultures as opposed to the mammalian cell lines that are currently used.

Similar to CA IV and XV, the transmembrane isozymes also contain an N-terminal signal peptide (Sly, 2000). In addition, these isozymes have a hydrophobic region in the C-terminal region that forms a single transmembrane domain. The length of this transmembrane domain varies from 20 amino acids in CA IX, to 26 amino acids in CA XII. There is also a small intracellular domain consisting of 25–30 amino acids (Sly, 2000). Similar to the GPI anchored CA isozymes, the transmembrane CAs have an extracellular domain that resembles a typical CA structure. Interestingly, CA IX also contains a proteoglycan binding site at the N-terminus of the mature protein. This site interacts with beta-catenin (Svastova et al., 2003), but the physiological relevance of this interaction remains unclear.

There has recently been significant effort put into the study of membrane-bound CA isozymes in non-mammalian vertebrates. The presence of a GPI anchored membrane CA has been documented in a variety of tissues from several different fish species (Gervais and Tufts, 1998; Gilmour et al., 2001; Gilmour et al., 2002; Tufts et al., 2002). Evidence for a non-GPI anchored membrane CA has also been obtained for the heart of the ancient vertebrate, the lamprey (Esbaugh and Tufts, 2004). Only recently, however, have full length cDNA sequences become available for non-mammalian membrane-bound CA isozymes. Thus far, most of the information available is for CA IV-like isozymes, with the exception of a single amphibian CA XII sequence. Interestingly, unlike the cytoplasmic CA isozymes, the non-mammalian CA IV sequences group closely with their mammalian counterparts, suggesting that these are, in fact, CA IV isozymes. Although no catalytic data are yet available

for these sequences, it is apparent from active site analysis that both dogfish and Xenopus CA IV are likely high activity isozymes. The trout isozyme, however, appears to have suffered a mutation to the proton shuttle residue, and thus is likely a low activity isozyme with a turnover number more similar to that of human CA III.

2.4. Metabolon complexing

Over the last number of years, a great deal of effort has been dedicated to the study of protein–protein interactions involving various CA isozymes. The majority of this work has been performed in humans; where intracellular CA II has been shown to transiently bind to the intracellular domain of various transport proteins, including AE1 (Vince and Reithmeier, 1998), AE2 (Reithmeier, 2001), NHE1 (Li et al., 2002), kNBC1 (Gross et al., 2002), NBC3 (Loiselle et al., 2004), and the DRA bicarbonate transporter (Sterling et al., 2002b). All of these interactions have been collectively termed metabolon complexing. The exact mechanism of protein binding is unique to each particular interaction; however, in general a basic motif found in the N-terminal region of CA binds to an acidic motif in the intracellular C-terminal region of the transport proteins. In human CA II, the first 17 amino acids contain five histidine residues that form a basic patch on the protein surface. Truncation studies have shown that removal of these residues results in complete loss of binding to AE1 (Vince et al., 2000). Although no such studies have examined the other interactions, it is likely that the basic motif on the N-terminal region of CA II is integral, as the binding site on all the transport proteins is an acidic motif (Vince and Reithmeier, 2000; Gross et al., 2002; Li et al., 2002; Loiselle et al., 2004).

There is also evidence that metabolon complexes have an extracellular component, as membrane-associated CA IV has been shown to transiently bind to AE1. Although very little is known about the mechanism of this particular interaction, it is clear that the binding site for CA IV is on the fourth extracellular loop of AE1 (Sterling et al., 2002a); however, the section of CA IV that participates in this interaction has yet to be elucidated. It should also be noted that CA IV has not yet been shown to bind to any other transport proteins.

3. Function

3.1. The role of red blood cell CA

One of the most important roles of CA is in the rbc, where the enzyme was first found (Brinkman et al., 1932; Meldrum and Roughton, 1933). In the rbc, CA is strictly cytoplasmic and is primarily involved in the process of CO_2 transport and excretion (Fig. 2; reviewed by Tufts and Perry (1998), Henry and Swenson (2000), and Swenson (2000)). It should be noted that human rbc ghost membranes have been shown to contain small amounts of membrane-bound CA IV (Wistrand et al., 1999); however this has not been shown to be true cell specific expression. The process of CO_2 transport begins with molecular CO_2 diffusing out of the tissues and into the blood stream and subsequently into the rbcs along its partial pressure gradient. In the rbcs CO_2 is hydrated into bicarbonate and a proton; a process catalyzed by CA. The bicarbonate ion is then shuttled out of the cell by band 3, while the proton is buffered by either haemoglobin or non-carbonic acid buffers, such as phosphate buffers. These processes remove both end products of CO_2 hydration from the rbc, allowing a maximal amount of CO_2 to be loaded into the blood. At the respiratory surface (gill, lung and/or skin), these reactions are reversed and CO_2 is eliminated from the body along its partial pressure gradient.

Although the general strategy for CO_2 transport is similar across most vertebrate groups, there are some notable exceptions. The major exception to this strategy occurs in the ancient vertebrate group, the agnathans (reviewed by Tufts and Perry (1998)). In agnathans, bicarbonate is transported within the rbc, and not shuttled into the plasma, due to the absence of band 3 in the rbc membrane (Nikinmaa and Railo, 1987; Tufts and Boutilier, 1989, 1990; Cameron and Tufts, 1994; Cameron et al., 1996). Another notable exception is found in the Antarctic icefishes (*C. aceratus* and *C. hamatus*) that lack red blood cells and circulating CA (Maffia et al., 2001; Tufts et al., 2002); however little is known about the exact mechanisms of CO_2 transport in this species.

In addition to the important role of rbc CA in CO_2 transport, CA also has another, often overlooked role in the rbc. In cooperation with haemoglobin, CA is integral in providing the link between O_2 transport and

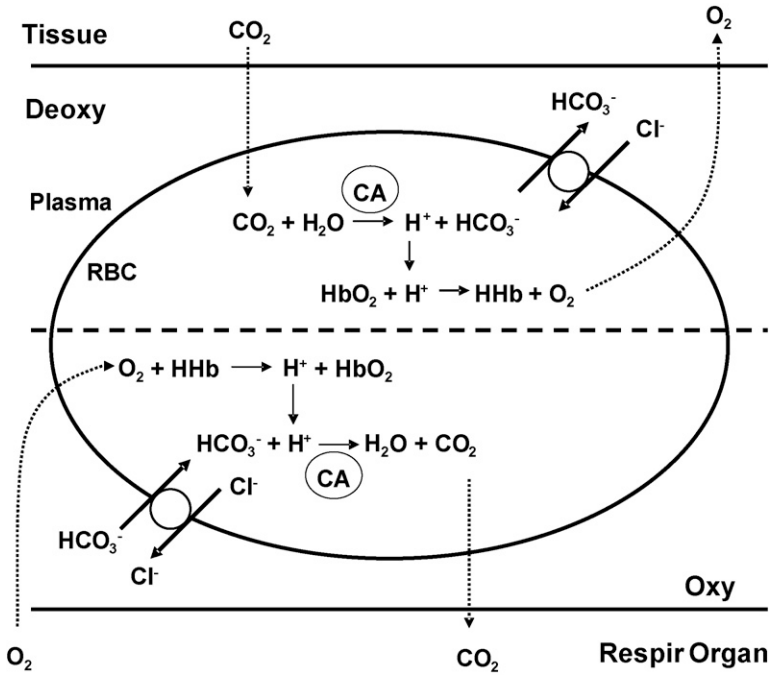

Fig. 2. The role of red blood cell carbonic anhydrase (CA) in vertebrate gas transport. At the tissue site of production (deoxy), CO_2 diffuses into the rbc along its concentration gradient, where CA catalyzes its conversion to bicarbonate. The resulting proton is buffered by haemoglobin (Hb), and bicarbonate is transported into the plasma by band 3. At the respiratory surface (oxy) these reactions happen in reverse resulting in the elimination of molecular CO_2 from the body.

CO_2 transport within the rbc (reviewed by Brauner and Randall (1998)). When molecular CO_2 diffuses into the rbc, it is dehydrated by CA into bicarbonate and a proton. The excess protons cause the intracellular pH to drop, causing haemoglobin to buffer free protons. The affinity of haemoglobin for oxygen, however, is decreased when protons bind (the Bohr effect), and thus oxygen is released from the rbc as CO_2 is taken up at the tissues. At the respiratory surface, the large amount of O_2 taken into the cell reduces haemoglobin's ability to bind protons. This is due to the Haldane effect, which refers to the fact that deoxygenated blood carries more CO_2 than oxygenated blood (Christianson et al., 1914). The combination of the release of free protons and the removal of molecular CO_2 across the respiratory surface cause CA to catalyze the dehydration reaction. This produces more molecular CO_2, which is removed from the body along its concentration gradient. Because the transit time of blood through both the tissue and pulmonary capillary beds (0.5–1 s; Swenson, 2000), or gills (0.5–2 s; Cameron and Polhemus, 1974), is very short, the ability to quickly hydrate and dehydrate CO_2 is integral to the proper release of both O_2 at the tissues and CO_2 at the respiratory surface.

Interestingly, there are two cytoplasmic CA isozymes present in the rbcs of most mammals – a high activity CA II and a low activity CA I. The CA I isozyme is far more abundant, but the majority of CO_2 hydration is performed by CA II (Chegwidden and Carter, 2000). The distinct roles of each isozyme within the rbc are currently unclear. Even more intriguing is that no disruption of gas transport occurs in the absence of rbc CA I, as found during human CA I deficiency syndrome (Sly and Hu, 1995). In addition, various animals devoid of rbc CA II activity, such as the naked mole rat and beluga whale, retain normal gas transport ability (Yang et al., 1998; Chegwidden and Carter, 2000). Recent studies on the potential protein interactions of CA II within the rbc may have provided the best insight into the presence of multiple isozymes in the cytoplasm of mammalian rbcs. As previously mentioned, the high activity CA II isozyme has been found

to transiently bind to various transport proteins in rbc membranes (Vince and Reithmeier, 1998; Vince et al., 2000; Reithmeier, 2001; Sterling et al., 2001, 2002a; Li et al., 2002). There is also evidence that suggests cytoplasmic CA may bind to haemoglobin (Silverman et al., 1979; Backman, 1981). It is therefore possible that multiple isozymes are present within the rbc to ensure the presence of CA at various intracellular locations. This theory may also help to explain why there appears to be an over-abundance of CA within the rbc. Some estimates suggest that more than 80% of the rbc CA activity can be inhibited without physiological consequence (Maren and Swenson, 1980; Swenson, 2000). These estimates, however, do not consider that CA may be limiting at a certain intracellular location during inhibition.

Similar to mammals, some non-mammalian vertebrates, such as rainbow trout, also have multiple rbc CA isozymes (Esbaugh et al., 2005). Unlike mammals, however, trout appear to have one isozyme in much greater abundance within the rbc. It is also interesting to note that both rbc CA isozymes are high activity in trout. In an effort to discern the function of each of these rbc isozymes, studies are currently underway to examine whether metabolon-like associations involving CA are present in rainbow trout rbcs. Preliminary examinations of the molecular structure of these isozymes, however, suggest that these interactions are unlikely. This is primarily due to a lack of histidine residues in the N-terminus of either isozyme. It is possible, however, that an analogous system with a novel binding mechanism arose in these animals.

3.2. The role of pulmonary/branchial CA

In mammals, intracellular CA II makes up the vast majority of pulmonary CA activity (Henry et al., 1986). Most functional studies in this area, however, have focused on the contributions of extracellular CA to CO_2 excretion. This activity is the result of membrane-bound CA IV that is associated with the pulmonary capillary bed (Whitney and Briggle, 1982). It has been suggested that membrane-bound CA could facilitate the excretion of CO_2 across the capillary walls, and lung perfusion experiments on a variety of vertebrates appear to support this view (Heming et al., 1993, 1994; Stabenau and Heming, 1999, 2003). In contrast, in vivo experiments using selective CA inhibitors have failed to support these conclusions (Swenson et al., 1993; Swenson and Hughes, 1993). Access of plasma HCO_3^- to the large pool of rbc CA also makes it unlikely that pulmonary CA would be necessary for CO_2 excretion. Another potential role proposed for pulmonary membrane-bound CA is to ensure complete plasma pH/PCO_2 equilibrium during capillary transit (Henry and Swenson, 2000). More simply, membrane-bound CA may be present in the pulmonary capillaries to ensure that all chemical species are in complete equilibrium in plasma when blood leaves the lung. Chemoreceptors in the peripheral and central nervous system that are associated with respiration can detect changes in these chemical species (Lahiri and Forster, 2003), thus, the main role of CA IV in the lung may be involved with the regulatory control of ventilation; however, this has yet to be thoroughly investigated.

In fish, there appears to be several different patterns of branchial CA expression depending on the species. In teleosts, a modern fish group, the gill CA activity is exclusively cytoplasmic (Henry et al., 1988, 1993) with some CA expression found in virtually every cell type; however, expression is highest in the chloride and pavement cells (Dimberg et al., 1981; Conley and Mallatt, 1988). The main role of cytoplasmic CA is to provide counter ions for transport processes involved with ion regulation and pH balance (Perry, 1986; Henry and Swenson, 2000; Claiborne et al., 2002; Marshall, 2002; Evans et al., 2005). Thus, the primary role of cytoplasmic CA activity in teleost gills is not CO_2 excretion. A similar pattern of CA expression is found in the agnathans, an ancient vertebrate lineage, with the exception that branchial cytoplasmic CA appears to be confined to the chloride cells (Conley and Mallatt, 1988; Henry et al., 1993). In contrast, several species of elasmobranchs have been shown to contain a CA IV-like isozyme on the basolateral membrane of the gill (Henry et al., 1997a; Gilmour et al., 2001; Gilmour et al., 2002). The role of this isozyme with regard to gas exchange has been much debated since its discovery, but in vivo experiments using selective CA inhibitors have now provided clear evidence for its participation in CO_2 exchange (Gilmour et al., 2001; Gilmour and Perry, 2004). It is unclear, however, to what extent this isozyme is necessary for proper respiratory gas exchange, and therefore it is unclear if CO_2 exchange is, in fact, the predominant role of branchial CA IV.

The presence of a CA IV isozyme in the basolateral membrane of elasmobranchs whose primary role is CO_2 excretion is an intriguing prospect. One major question, however, is why such a system would only be present in elasmobranchs, and not in other early vertebrate groups such as agnathan or teleosts. It is known that elasmobranchs do possess an anion exchange protein in the rbc membranes that has similar activity to that found in humans and teleosts (Obaid et al., 1979). Thus, elasmobranchs possess all the cellular components necessary for efficient gas transfer in the absence of branchial CA IV. One possible explanation for this finding may be related to the osmoregulatory strategy of elasmobranchs. The osmolarity of elasmobranch plasma is kept equal to that of seawater through the maintenance of high amounts of urea and TMAO in the plasma. It has also been shown that high intracellular protein concentrations can have an inhibitory effect on the diffusion rates of bicarbonate within a cell (Geers and Gros, 2000). It is therefore conceivable that CA IV is necessary to compensate for the diffusion limitations of bicarbonate ions in elasmobranch plasma, caused by the high plasma urea content. In view of this possibility, it would be interesting to compare the CA IV expression in the gills of elasmobranchs of sea water and fresh water origins.

3.3. The role of muscle CA

In general, vertebrate muscle has three pools of CA that are involved in respiratory gas exchange (reviewed by Geers and Gros (2000)). The first pool, GPI anchored CA IV, is located on the capillary walls with an extracellular orientation (Ridderstrale, 1979). The function of CA on the capillary walls is two fold. The first function of this pool of CA is to facilitate the transport of CO_2 into the capillaries by hydrating CO_2 at the capillary wall and thus maintaining the concentration gradient of CO_2 between the muscle and the blood. In mammals, this role appears to be small at rest, but becomes more important as the CO_2 load within muscle increases, such as during exercise (Geers and Gros, 2000). The second function of CA at the capillary walls is to help equilibrate the post-capillary pH. In the absence of plasma accessible CA, a pH disequilibrium develops between the rbcs and plasma after capillary transit. This is due to the fast production of protons within the rbcs, while protons are produced slowly in the plasma via the uncatalyzed hydration of CO_2 (Geers and Gros, 2000). The capillary bound CA IV produces protons within the plasma that participate in pH equilibration of the blood during capillary transit. This capillary pH equilibration – similar to that found in pulmonary capillary beds – may be involved in the integrative control of breathing; however this has yet to be investigated.

A second pool of membrane-bound CA is found on the extracellular surface of the sarcolemma and sarcoplasmic reticulum (Effros et al., 1978). This isozyme appears to be CA IV in red and white muscle fibres (Chegwidden and Carter, 2000), however, CA XIV has also been detected in the fibres of cardiac muscle (Fujikawa-Adachi et al., 1999). In mammals, this pool of CA is not involved in gas transport. Instead, the enzyme is involved in providing the interstitial space with bicarbonate buffers (Geers and Gros, 2000). The presence of bicarbonate buffers in the interstitial space is thought to be extremely important for maintaining lactate efflux. These bicarbonate buffers absorb protons that diffuse out of the cell with lactate through the H^+-lactate co-transporter. Interestingly, experiments performed on white muscle trunk preparations of rainbow trout did provide evidence for a role of membrane-bound CA in CO_2 excretion across the sarcolemma (Henry et al., 1997b; Wang et al., 1998). In contrast to CAs role in the capillary walls, however, these studies suggest that membrane-bound CA in teleost fish only contributes to CO_2 excretion under steady state conditions, and not during exercise. As is the case in mammals, studies on fish have also provided evidence for a role for membrane-bound CA in facilitating lactate efflux from muscle during exercise, as well as ammonia efflux under steady state conditions.

Muscle fibres also have significant amounts of cytoplasmic CA. In mammals, the isozymes present in the muscle cytoplasm are CA II (white muscle) and CA III (red muscle; Chegwidden and Carter, 2000). Neither of these isozymes, however, has been found to play a major role in gas transport. The primary role of CA III in red muscle appears to be to confer resistance against oxidative stress from radical oxygen species (Raisanen et al., 1999). In white muscle, CA II is involved in the facilitated diffusion of intracellular CO_2. This is evident from the inhibitory effect of acetazolamide on the intracellular CO_2 diffusion rates (Geers and Gros, 2000); however the importance of facilitated diffusion

to overall gas transport is unclear. Interestingly, intracellular muscle CA inhibition in rainbow trout did not result in any changes in CO_2 efflux versus control groups, both at rest and under exercise conditions (Henry et al., 1997b; Wang et al., 1998). It is therefore possible that intracellular muscle CA is not integral to gas transport in vertebrates.

Interestingly, the skeletal muscle fibers of an ancient vertebrate, the lamprey, may be unique in that they have not yet been shown to contain any CA activity (Esbaugh and Tufts, 2006). Northern blot analysis of cytoplasmic CA from lamprey showed no expression in skeletal muscle, and this was confirmed by biochemical analysis of tissue lysates. In addition, unpublished data examining the biochemical properties of lamprey skeletal muscle membranes revealed no membrane-bound activity (Esbaugh and Tufts, unpublished data). This is contrary to lamprey cardiac muscle, which appears to contain a non-GPI anchored membrane-bound CA (Esbaugh and Tufts, 2004). It should be noted, however, that more sensitive molecular approaches to address this issue will be required before any firm conclusions can be made in this area. Nonetheless, these results may suggest that the role of muscle CA first originated in cardiac muscle, and that a role in skeletal muscle evolved later in response to the increase in metabolic capacity of more derived vertebrates.

4. Conclusion

CA is a crucial enzyme in many physiological functions, including the transport of CO_2 throughout the body. Although the structural components of the CA catalytic mechanism are remarkably conserved throughout vertebrates, much remains to be learned about the way that structural differences in CA influence the specific physiological function of the different isozymes. This knowledge will be needed in order to understand the reason why so many different isozymes of CA have evolved in vertebrates. With regard to respiration, we believe that future studies should focus on determining whether the various pools of CA are actually necessary for proper gas transport in early (non-mammalian) vertebrate groups. Additional studies should also examine whether CA plays an important role in metabolon complexes in non-mammalian vertebrates. Finally, it would be interesting to know how increased respiratory demand has influenced the presence, relative distribution and concentrations of the different CA isozymes during vertebrate evolution. Clearly, such studies will be necessary to help ascertain the selective pressures that have led to the plethora of CA isozymes in vertebrates.

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