



MIKA HILVO

Expression and Biochemical Properties of
Membrane-Bound Carbonic Anhydrase Isozymes
IX and XV



ACADEMIC DISSERTATION

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications that are referred to in the text by their Roman numerals (I–IV).

- I **Hilvo M**, Rafajová M, Pastoreková S, Pastorek J and Parkkila S (2004): Expression of carbonic anhydrase IX in mouse tissues. *J Histochem Cytochem* 52:1313–1321.
- II **Hilvo M***, Tolvanen M*, Clark A, Shen B, Shah GN, Waheed A, Halmi P, Hänninen M, Hämäläinen JM, Vihinen M, Sly WS and Parkkila S (2005): Characterization of CA XV, a new GPI-anchored form of carbonic anhydrase. *Biochem J* 392:83–92.
- III **Hilvo M**, Baranauskiene L, Salzano AM, Scaloni A, Matulis D, Innocenti A, Scozzafava A, Monti SM, Di Fiore A, De Simone G, Lindfors M, Jänis J, Valjakka J, Pastoreková S, Pastorek J, Kulomaa MS, Nordlund HR, Supuran CT and Parkkila S. Biochemical characterization of CA IX, one of the most active carbonic anhydrase isozymes. *J Biol Chem*, in press.
- IV **Hilvo M**, Innocenti A, Monti SM, De Simone G, Supuran CT and Parkkila S (2008): Recent advances in research on the most novel carbonic anhydrases, CA XIII and XV. *Curr Pharm Des* 14:672–678.

* = equally contributed.

ABBREVIATIONS

AE	anion exchanger
AP	activator protein
AZA	acetazolamide
BSA	bovine serum albumin
CA	carbonic anhydrase
<i>Car15</i>	mouse carbonic anhydrase 15 (gene or mRNA)
<i>Car9</i>	mouse carbonic anhydrase 9 (gene or mRNA)
CA-RP	carbonic anhydrase-related protein
ccRCC	clear cell renal cell carcinoma
CHO	Chinese hamster ovary
DMSO	dimethyl sulfoxide
<i>E. coli</i>	<i>Escherichia coli</i>
EGFR	epidermal growth factor receptor
ER	estrogen receptor
GABA	gamma-aminobutyric acid
Gal	galactose
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GPI	glycosylphosphatidylinositol
HIF	hypoxia inducible factor
HRE	hypoxia-responsive element
HRP	horseradish peroxidase
LB	Luria-Bertani
MALDI-TOF-MS	matrix-assisted laser desorption/ionization-time of flight-mass spectrometry
MAPK	mitogen-activated protein kinase
NeuAc	N-acetyl-neuraminic acid
NeuGc	N-glycolyl-neuraminic acid
PAP	peroxidase-antiperoxidase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	proteoglycan-like
PI3K	phosphatidylinositol 3'-kinase
PI-PLC	phosphoinositide-specific phospholipase C
PHD	prolyl-4-hydroxylase
PR	protected region
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene fluoride

RPTP	receptor-like protein tyrosine phosphatase
RCC	renal cell carcinoma
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SES-PCR	stepwise elongation of sequence-PCR
Sf	<i>Spodoptera frugiperda</i> (fall armyworm)
SP	specificity protein
VHL	von Hippel-Lindau

YHTEENVETO

Hiilihappoanhydraasit (CA:t) ovat metalloentsyymejä, joiden pääasiallinen tehtävä on katalysoida reversiibelisti hiilidioksidin hydraatiota bikarbonaatiksi ja protoniksi. Nämä entsyymit osallistuvat useisiin fysiologisiin prosesseihin, kuten pH:n säätelyyn, CO₂:n ja HCO₃⁻:n kuljetukseen, elimistössä olevien nesteiden tuotantoon, luun resorptioon ja erilaisiin metabolisiin reaktioihin. Nisäkkäissä on karakterisoitu kaksitoista aktiivista CA-isoentsyymiä. Nämä ovat sytosolissa sijaitsevat CA:t I, II, III, VII ja XIII, solukalvoon liittyneet CA:t IV, IX, XII ja XIV, mitokondriaaliset isoentsyymit VA ja VB sekä solusta erittyvä CA VI.

Tämän työn tarkoituksena oli tutkia isoentsyymien IX ja XV ilmentymistä ja biokemiallisia ominaisuuksia. CA IX poikkeaa muista CA-proteiiniperheen jäsenistä siten, että sitä tuotetaan eniten kasvaimissa ja sillä on klassisen CA-domeenin lisäksi niin sanottu proteoglykaanin kaltainen (PG) domeeni. pH:n säätelyn lisäksi tämän isoentsyymin on osoitettu liittyvän solujen väliseen adheesioon ja signaalointiin sekä solujen lisääntymiseen. CA XV taas on huonosti tunnettu isoentsyymi, josta tämän tutkimuksen alkaessa oli tiedossa ainoastaan genomitietokantaan tallennettu hiiren *Car15* cDNA-sekvenssi.

Näiden molempien isoentsyymien ilmentymistä tutkittiin hiiren kudoksissa. CA IX ilmentyi voimakkaimmin mahan limakalvolla, ja entsyymiä havaittiin myös paksusuolen enterosyyteissä sekä haiman rauhasoluissa. Munuaisissa ja luustolihaksessa havaittiin yllättävä ero CA IX:n lähetti-RNA (mRNA)- ja proteiinipitoisuuksien välillä: näissä kudoksissa oli paljon CA IX:ää koodaavaa mRNA:ta, mutta hyvin vähän tai ei lainkaan vastaavaa proteiinia. Tämä tulos viittaa siihen, että CA IX:ää voidaan säädellä transkription jälkeisillä mekanismeilla, joiden tehtävä on mahdollisesti vastata muuttuviin fysiologisiin olosuhteisiin. CA XV:n ilmentymistä tutkittiin mRNA-tasolla, ja tulosten mukaan tätä mRNA:ta tuotetaan hyvin harvoissa hiiren kudoksissa: sitä löydettiin paljon munuaisesta, erityisesti munuaiskuoren alueelta, ja jonkin verran ilmentymistä havaittiin myös aivoissa sekä kiveksissä. Ihmisen kudoksista ei löydetty CA XV:tä koodaavaa mRNA:ta. Tämä tulos oli sopusoinnussa sekvenssianalyysien kanssa, joiden mukaan CA XV näyttää olevan aktiivinen monissa selkärankaisissa, mutta ei kädellisissä lajeissa kuten ihmisissä, sillä niissä CA XV:tä koodaava geeni on muuttunut pseudogeeniksi.

Biokemiallisia tutkimuksia varten tuotettiin ihmisen CA IX- ja hiiren CA XV -rekombinanttiproteiineja. CA XV:n osoitettiin olevan CA IV:n lisäksi ainoa isoentsyymi, joka on liittynyt solukalvoon glykosyylifosfatidyli-inositoli (GPI) -ankkurilla. Sekä CA IX:n että XV:n näytettiin sisältävän niin sanottuja N-glykosylaatioita: isoentsyymillä XV näitä osoitettiin olevan kolme kappaletta ja isoentsyymillä IX yksi kappale. Tämä isoentsyymi IX:n CA-domeenista löytynyt

N-glykosylaatio karakterisoitiin massaspektrometrian avulla. Lisäksi osoitettiin, että CA IX:n PG-domeeni sisältää niin sanotun O-glykosylaation. Näin kyettiin kokeellisesti osoittamaan ensimmäistä kertaa, että kyseinen domeeni todella näyttäisi olevan PG-domeeni. CA IX:llä todistettiin olevan yksi molekyyllinsisäinen disulfididisidos, ja tämän lisäksi se on ainoa CA-isoentsyymi, joka muodostaa molekyylien välisten disulfididisidosten avulla oligomeerejä. Toisaalta CA XV:n kolmiulotteisesta rakenteesta tehty malli taas viittasi siihen, että tällä isoentsyymillä on kolme molekyyllinsisäistä disulfididisidosta stabiloimassa proteiinin rakennetta. CA XV:n CO₂-hydraatioaktiivisuuden havaittiin olevan keskitasoa ja verrattavissa isoentsyymeihin XII ja XIV. Asetatsoliamidin, klassisen CA-inhibiittorin, sitoutumisvakio tälle entsyymille oli hyvin lähellä isoentsyymien IV arvoa. Aiemmissä tutkimuksissa CA IX:n aktiivisuus on mitattu käyttäen ainoastaan CA-domeenia, ja tutkimukset vahvistivat aikaisemmat tulokset, joiden mukaan CA IX:n katalyyttisen domeenin aktiivisuus on keskitasoa. Tehdyissä tutkimuksissa pystyttiin kuitenkin ensimmäistä kertaa mittaamaan katalyyttinen aktiivisuus CA IX:n koko solun ulkoiselle osalle. Tulokset näyttivät, että sen k_{cat}/K_M -arvo oli sama kuin CA II:lla, jonka on aiemmin julkaistu olevan kaikkein aktiivisin CA-isoentsyymi ja myös yksi nopeimmista luonnosta löydetyistä entsyymeistä. Asetatsoliamidi inhiboi hieman voimakkaammin CA IX:ää, jossa oli PG-domeeni mukana. Tämän lisäksi havaittiin, että tiettyjen metalli-ionien lisääminen puskuriliuokseen lisäsi huomattavasti PG-domeenin sisältävän CA IX:n aktiivisuutta ja nosti sen arvoon, jollaista ei ole mitattu millekään isoentsyymille aikaisemmin.

ABSTRACT

Carbonic anhydrases (CAs) are metalloenzymes whose main function is to catalyze the reversible hydration of carbon dioxide to bicarbonate and a proton. These enzymes participate in a variety of physiological processes, such as pH regulation, CO₂ and HCO₃⁻ transport, production of biological fluids, bone resorption and metabolic processes. In mammals, twelve active CA isozymes have been characterized. These include the cytosolic CAs I, II, III, VII and XIII; the membrane-bound CAs IV, IX, XII and XIV; the mitochondrial isozymes VA and VB; and the secreted CA VI.

The aim of this study was to investigate the expression and biochemical properties of isozymes IX and XV. CA IX is an exceptional member of the CA family because it is a tumor-related isozyme that contains a proteoglycan-like (PG) domain in addition to its CA domain. This isozyme has been suggested to participate in cell adhesion, proliferation and signaling processes, in addition to its classical role in pH regulation. CA XV, however, is a poorly understood enzyme. Intriguingly, the only information available has been a cDNA sequence coding for the mouse isozyme that has been deposited in a genome database.

The expression of these two isozymes was investigated in mouse tissues. The strongest expression of CA IX was observed in the gastric mucosa, while moderate expression was observed in the colon enterocytes and pancreatic acini. A surprising discrepancy was observed in regard to the mRNA and protein levels of CA IX in the kidney and skeletal muscle. Specifically, these tissues showed significant levels of the mRNA but very low levels or a complete lack of the corresponding protein. This implies that CA IX may be regulated post-transcriptionally, possibly according to the physiological demands. The expression of CA XV was investigated at the level of transcription, and indicated that the mRNA for this isozyme showed very limited distribution in mouse tissues, where it was found predominantly in the kidney, especially in the cortex region, with a lower level of expression in the brain and testis. Human tissues, however, did not express the mRNA for this isozyme; this was in agreement with the results of sequence analyses. These analyses indicated that CA XV appears to be an active enzyme in several vertebrate species, although its gene has become a pseudogene in primates, such as humans.

The recombinant enzymes of the human CA IX and mouse CA XV were expressed and isolated for biochemical analysis. In addition to isozyme IV, CA XV was shown to be the other member of the CA isozyme family that is attached via a glycosylphosphatidylinositol (GPI)-anchor to the cell membrane. Both CA IX and CA XV were shown to contain N-linked glycosylations. While CA XV possesses three N-linked glycosylations, isozyme IX has a single N-linked

glycosylation located within the CA domain, as characterized by mass spectrometric analysis. The PG domain of CA IX was shown to contain an O-linked glycosylation site, thus providing the first experimental evidence that this domain is a *bona fide* proteoglycan domain. CA IX was shown to possess one intramolecular disulfide bond, and it is the only CA isozyme that forms oligomers through the creation of intermolecular disulfide bonds. Conversely, the structural prediction of CA XV suggested that this isozyme contains three intramolecular disulfide bonds that stabilize its molecular structure. The CO₂ hydration activity of CA XV was found to be moderate and, thus, comparable to those of isozymes XII and XIV. The inhibition constant of acetazolamide, a well-known CA inhibitor, was very similar for both CA XV and CA IV. The catalytic activity of CA IX has been previously measured only for its CA domain, and the results presented here confirmed that the activity is moderate. However, the CO₂ hydration activity for the full-length extracellular domain of CA IX was measured for the first time and found to have a k_{cat}/K_M identical to that for isozyme II, which has been reported to be the most active CA isozyme and one of the fastest enzymes found in nature. Acetazolamide appeared to inhibit the activity of the full-length CA IX slightly more than that of the catalytic domain alone. Furthermore, addition of certain metal ions to the buffer solution increased the catalytic activity of the full-length extracellular domain considerably, achieving a level of activity that has never been reported for any other CA isozyme.

1. INTRODUCTION

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate according to the following reaction: $\text{CO}_2 + \text{H}_2\text{O} \Leftrightarrow \text{HCO}_3^- + \text{H}^+$ (Supuran 2008). CA enzymes are ubiquitous in nature and can be found in organisms all over the phylogenetic tree (Hewett-Emmett 2000). In mammals, all of the characterized CA enzymes belong to the α -CA family, and twelve active members of the family have been characterized, including CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII and XIV (Lehtonen et al. 2004). Each isozyme has a unique distribution within tissues, meaning that some of these isozymes are expressed in certain cell types of nearly all tissues, whereas others are expressed only in a very limited number of tissues. Moreover, the enzymes differ in their subcellular localization, with five cytosolic members and four membrane-bound members; two are located in the mitochondria, and one is a secretory isozyme. All of these isozymes possess distinct catalytic activities and inhibition profiles. For instance, CA II has been reported to be the most active isozyme, possessing an activity close to the diffusion-controlled limit, whereas CA III is an enzyme possessing negligible activity in comparison to that of CA II (Chegwidden and Carter 2000).

The membrane-bound isozyme CA IX has gained much interest since its identification as a tumor-associated protein. While this isozyme has relatively limited distribution throughout human tissues, it is overexpressed in several cancers, especially under hypoxic conditions (Pastoreková and Zavada 2004). CA IX is an exceptional member of the CA family because, in addition to its CA domain, it also contains a proteoglycan-like (PG) domain, which has been suggested to contribute to the cell adhesion and proliferation processes. Due to these unique properties, CA IX has been considered an attractive target in cancer therapeutics, and, at the moment, two drug candidates possessing anti-CA IX activity are in clinical trials (Pastoreková and Zavada 2004, Supuran 2008). Despite all the information that has been reported for CA IX, no studies have characterized the biochemical properties of this enzyme expressed from a eukaryotic expression system. The goals of this study include performing these aforementioned analyses of CA IX expressed from a eukaryotic system, in addition to investigating the expression of CA IX in mouse tissues, since CA IX expression in the rodents has only been previously reported for the tissues of the rat alimentary tract (Pastoreková et al. 1997).

Analyses of genome databases suggested that, in addition to the twelve characterized isozymes, mammals may possess another CA isozyme, CA XV. The cDNA sequence of this enzyme was submitted to the National Center for Biotechnology Information (NCBI) by Hewett-Emmett and Shimmin in 2000

(GenBank[®] accession no. AF231122). During the present study, it became clear that CA XV is a novel member of the CA isozyme family. Since it was discovered that CA XV is active in several species, but not in humans, we used mouse as a model organism to investigate the expression of this isozyme. Furthermore, we also illuminated the subcellular localization and several biochemical properties for the mouse CA XV.

2. REVIEW OF THE LITERATURE

2.1 Regulation of the physiological acid-base balance

Due to the fact that most biologically important molecules contain chemical groups that can act either as weak acids or bases, even small changes in pH may have significant physiological consequences (Boron 2005). A pH shift may, therefore, change the net electrical charge of these groups and subsequently have an effect on the conformation and biological activity of the molecule. For most proteins, pH sensitivity is modest, but, for some proteins, even a slight shift in the pH by 0.1 units can have a profound effect on protein function. Changes in the intracellular pH values affect several cellular processes, including cell proliferation, cell cycle progression, differentiation, apoptosis and malignant transformation (Srivastava et al. 2007). The cytosolic acid-base homeostasis is tightly regulated; nevertheless, it is affected by changes in the extracellular pH (Guyton and Hall 2006).

The intra- and extracellular acid-base balance is regulated over long periods by the kidneys, while the lungs can adjust the pH of the extracellular fluid within minutes. Several buffer systems, which act instantaneously, form the first line of defense against changes in pH. The systems important to human physiology include bicarbonate, phosphate and ammonia buffer systems. In addition, proteins, like hemoglobin in the blood, can also buffer against pH changes. (Guyton and Hall 2006)

While all of these buffer systems are related to each other, the most important of them is the bicarbonate buffer system. This is an open system, meaning that, due to the volatility of carbon dioxide, the lungs can maintain a stable CO₂ concentration throughout the blood plasma, despite physiological reactions that produce or consume CO₂ (Boron 2005). The chemical reactions underlying the bicarbonate buffer system are as follows: $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3^- + \text{H}^+$. The dissociation of carbonic acid to form bicarbonate and a proton is a fast reaction even in the absence of enzyme catalysis. The hydration of carbon dioxide, however, occurs too slowly to meet the physiological needs, and, therefore, evolutionary processes have generated the carbonic anhydrase enzyme family to catalyze this reversible reaction, in which carbon dioxide is directly hydrated to form a proton and bicarbonate: $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$ (Boron 2005, Supuran 2008).

2.2 Carbonic anhydrase isozyme family

2.2.1 General and historical aspects

The first indication of CA activity was observed in the late 1920s, when experiments performed with hemolyzed blood and serum demonstrated that the rate of carbon dioxide release from the blood was so high that the blood was likely to contain a catalyst for this reaction (Henriques 1928, Van Slyke and Hawkins 1930). A few years later it became evident that this catalyst was an enzyme, given the name carbonic anhydrase; it was then partially isolated and purified for the first time (Meldrum and Roughton 1932, Meldrum and Roughton 1933). This enzyme was shown to contain one zinc ion per molecule and appeared to have a molecular mass of approximately 30 kDa (Keilin and Mann 1939).

However, it took about twenty years before a CA enzyme was purified for the first time, from bovine erythrocytes (Lindskog 1960). Purification from human erythrocytes subsequently revealed three CA isoforms that were distinct enzymes on the basis of electrophoretic analysis and were thus designated as the A, B and C isoforms (Nyman 1961, Laurent et al. 1962, Rickli and Edsall 1962, Rickli et al. 1964, Laurent et al. 1965, Kannan et al. 1975). The amino acid composition of the A and B isoforms were indistinguishable from each other, while the C isoform contained a unique amino acid composition as well as a higher catalytic activity for carbon dioxide hydration when compared to the A and B enzymes (Nyman and Lindskog 1964). The B and C enzymes were later designated as CA I and CA II, respectively. During the 1970s, the amino acid sequences and x-ray crystal structures were reported for both CA I and II (Andersson et al. 1972, Liljas et al. 1972, Henderson et al. 1973, Lin and Deutsch 1973, Lin and Deutsch 1974). During the same decade, a sulfonamide-resistant CA activity was discovered in male rat liver homogenates (Garg 1974a, Garg 1974b) and in chicken muscle tissue (Holmes 1976). It later became clear that the sulfonamide-resistant CA enzyme was the basic muscle protein that had been purified from rabbit skeletal muscle (Blackburn et al. 1972), and this enzyme was subsequently named CA III (Koester et al. 1977).

These three enzymes were shown to be just the beginning of the CA isozyme family. In 1979, a secreted CA enzyme (CA VI) was isolated from the saliva of sheep (Fernley et al. 1979), and the human enzyme was characterized in 1987 (Murakami and Sly 1987). During the 1980s, a membrane-associated enzyme, CA IV, was purified for the first time from bovine (Whitney and Briggie 1982) and human tissues (Wistrand and Knuutila 1989, Zhu and Sly 1990). In the 1990s cytosolic CA VII (Montgomery et al. 1991), the transmembrane isozymes CA IX (Pastoreková et al. 1992, Opavský et al. 1996), XII (Türeci et al. 1998) and XIV (Fujikawa-Adachi et al. 1999b, Mori et al. 1999), in addition to mitochondrial CAs VA (Nagao et al. 1993, Heck et al. 1994) and VB (Fujikawa-Adachi et al. 1999c), were discovered. The most recent member of the family to be characterized is cytosolic CA XIII (Lehtonen et al. 2004). A cDNA sequence

that may encode yet another uncharacterized isozyme, CA XV, was submitted to the National Center for Biotechnology Information (NCBI) by Drs. Hewett-Emmett and Shimmin in 2000 (GenBank[®] accession no. AF231122).

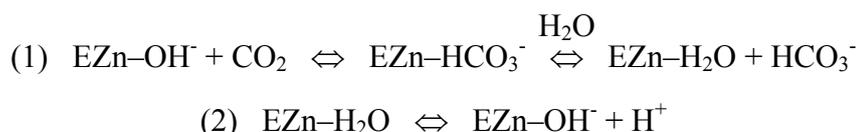
While all of these mammalian CA isozymes belong to the α -CA family, CA activity is not limited to mammals. In fact, the reversible hydration of carbon dioxide seems to be such a fundamental biological reaction that CAs are present in virtually all living organisms (Chegwidden and Carter 2000). To date, functional α -CA enzymes, or sequences showing homology to this family, have been found in animals, plants, algae, some eubacteria and even in viruses (Hewett-Emmett 2000). But in addition to α -CAs, there are other CA enzyme families, known as the β -, γ - and δ -CAs. Although the members of these enzyme families catalyze the same reaction, the families have evolved independently (Chegwidden and Carter 2000). The β -CAs are found predominantly in eubacteria, algae and chloroplasts of both mono- and dicotyledonous plants (Supuran 2004). In addition, sequences coding for putative β -CAs have been found in the animal kingdom (e.g., *Caenorhabditis elegans*), some fungi and archaeobacteria (Hewett-Emmett 2000). The γ -CA family is found primarily in archaeobacteria and in some eubacteria (Hewett-Emmett 2000, Supuran 2004). Among the α -, β - and γ -CA proteins, there are differences in the folding of the protein subunits, active site structure and oligomerization state, suggesting that these enzyme classes have evolved independently to catalyze the same reaction (Supuran 2004). Studies of the marine diatom *Thalassiosira weissflogii* revealed that this organism contains a CA enzyme that does not show homology to the other CA families (Roberts et al. 1997), although the structure of its active site is strikingly similar to that of α -CAs (Cox et al. 2000). It has been proposed that this enzyme is an example of convergent evolution at the molecular level, and, therefore, it has been suggested to be a prototype for a new CA family, called the δ -family (Cox et al. 2000, Tripp et al. 2001).

2.2.2 Catalytic mechanism, inhibition and activation

The α -CAs are globular proteins whose structure is characterized by a central ten-stranded antiparallel β -sheet (Di Fiore et al., in press). The enzyme active site is located in a cavity toward centre of the protein molecule. A Zn^{2+} ion is located at the base of this cavity and plays an essential role in the catalytic mechanism of these proteins. This Zn^{2+} ion is coordinated by three histidine residues (His 94, His 96 and His 119 in CA II) and a molecule of water (or hydroxide ion) (Stams and Christianson 2000). These histidine residues are illustrated in Figure 1.

The catalytic mechanism for all the CA isozymes is referred to as the zinc-hydroxide mechanism (Lindskog and Silverman 2000). The central catalytic step involves a reaction between CO_2 and the zinc-bound OH^- ion that yields a coordinated HCO_3^- ion, which is subsequently displaced from the metal ion by a water molecule. This reaction is shown in Equation 1, where E denotes the enzyme (Supuran 2004). The regeneration of OH^- involves the transfer of a H^+

from the zinc-bound water molecule to the solvent as shown in Equation 2 (Lindskog and Silverman 2000, Supuran 2004).



The zinc-bound water molecule is engaged in hydrogen bond interactions with the hydroxyl moiety of Thr 199, which, in turn, interacts with the carboxylate moiety of Glu 106 (the residues are shown in Figure 1). These interactions enhance the nucleophilicity of the zinc-bound water molecule and orient the molecule of CO₂, which is located in a hydrophobic pocket, to a location favourable for nucleophilic attack. In the active form, the enzyme has a hydroxide ion bound to the Zn²⁺ ion. This strong nucleophile attacks the CO₂

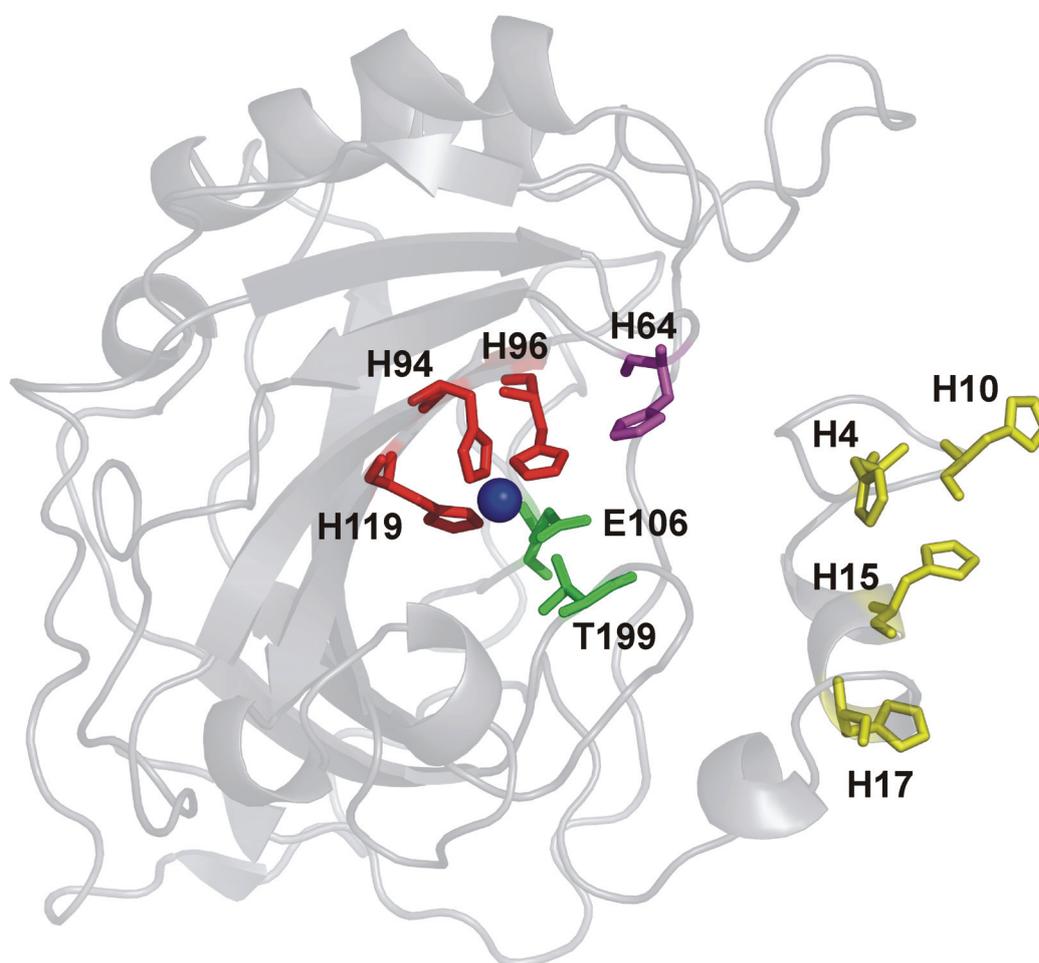


Figure 1. The overall three-dimensional structure of the CA II enzyme, including catalytically important amino acid residues. The figure has been generated from the x-ray coordinates reported by Eriksson et al. (1988), PDB entry 1CA2, using PyMOL 0.97 (DeLano, W.L. The PyMOL Molecular Graphics System [2002] DeLano Scientific, Palo Alto, CA, USA, <http://www.pymol.org>). The colors indicate the following ions or amino acid residues: blue, Zn²⁺ ion; red, the histidine residues coordinating the metal ion; green, the Glu and Thr residues involved in the hydrogen bond interactions with Zn-bound water molecule; purple, the proton shuttle residue; yellow, the histidine cluster (His 3 is not shown in these x-ray data).

molecule, which leads in the formation of HCO_3^- coordinated to the Zn^{2+} ion. The bicarbonate ion is then displaced by a water molecule and liberated into solution, creating the acid form of the enzyme, with water coordinated to the Zn^{2+} ion (Equation 1). This form of enzyme is inactive, and in order to regenerate the basic form (EZn-OH^-), a proton must be transferred from the active site to the surrounding solvent. (Supuran 2004)

The rate-limiting step of the reaction involves the proton transfer reaction (Equation 2), and in the isozymes possessing high activity this step is assisted by a histidine residue (His 64), located at the opening of the active site (Figure 1) (Supuran 2004). This histidine, referred to as a proton-shuttle residue, is located approximately 8 Å from the zinc-bound solvent; thus, the actual proton immediately transferred away from zinc-bound water does not diffuse toward His 64 (Stams and Christianson 2000). Instead, this proton is transferred to a hydrogen-bonded water molecule, which subsequently transfers a different proton to a second hydrogen-bonded water molecule. This transfer then promotes the movement of yet another proton to the His 64 residue. Finally, His 64 shuttles this latter proton from the enzyme to the surrounding solvent. His 64 can adopt two different conformations with respect to the zinc ion (one pointing toward the active site in the direction of the Zn^{2+} ion, and the other oriented away from the active site), an ability crucial for its role as a proton shuttle (Lindskog and Silverman 2000, Stams and Christianson 2000). In addition to His 64, a unique histidine cluster (His 3, His 4, His 10, His 15, His 17; four of these residues are illustrated in Figure 1) is likely to be important for the high catalytic activity of the CA II isozyme. It has been suggested that the histidine cluster together with His 64 forms some sort of channel that efficiently transfers the protons from the active site to the reaction solvent (Di Fiore et al., in press), thus increasing the rate of the rate-limiting step in the reaction.

In addition to the reversible hydration of carbon dioxide, CAs can also act on other carbonyl compounds, such as esters and aldehydes (Lindskog and Silverman 2000). There is evidence that both ester hydrolysis and aldehyde hydration are facilitated via a zinc-hydroxide mechanism, but the details of these reactions remain unsolved. In addition, it is still unclear whether other CA-catalyzed reactions besides CO_2 hydration are physiologically relevant (Supuran 2004).

Since CAs are involved in several physiological and pathophysiological processes, it was realized decades ago that their inhibition has biomedical relevance (Chegwidden and Carter 2000). Although it is feasible to develop inhibitors specific to the CAs, the high sequence similarity of the enzymes in this family has made it rather challenging to develop isozyme-specific inhibitors (Mansoor et al. 2000). At present, there are two main classes of CA inhibitors: the unsubstituted sulfonamides, including their bioisosteres (e.g., the sulfamates and sulfamides) and the metal-complexing anions (Supuran 2008). The sulfonamide inhibitors bind to the Zn^{2+} ion through a substitution mechanism where the sulfonamide exchanges with the water molecule (Equation 3), while the anionic inhibitors simply become a part of the metal coordination sphere

(Equation 4; in the equations below, E denotes the enzyme and I denotes the inhibitor).



Sulfonamides are the most important CA inhibitors, and they bind in a tetrahedral geometry to the Zn^{2+} ion in a deprotonated state, with the nitrogen atom of the sulfonamide moiety coordinated to the metal ion (Supuran 2004, Supuran, 2008). Moreover, they form an extended network of hydrogen bonds involving the residues Thr 199 and Glu 106, which also participate in anchoring the inhibitor molecule to the Zn^{2+} ion. The aromatic portion of the sulfonamide inhibitor interacts with both the hydrophilic and hydrophobic residues of the active site cavity, while the anionic compounds typically form trigonal-bipyramidal adducts. CA inhibitors have been used primarily as diuretics and to treat glaucoma, epilepsy and acute mountain sickness in addition to intracranial hypertension. Recently, it has emerged that these inhibitors may have a potential role in treating obesity, cancer and various infections (Swenson 2000, Malomo et al. 2006, Supuran 2008).

Although the inhibition of CAs has been studied for decades, activation of these enzymes has garnered interest only quite recently. CA activators are molecules that bind within the active site cavity, at a location distinct to that of the inhibitor or substrate-binding sites, and function to facilitate the proton shuttle step. Many physiologically relevant compounds, such as biogenic amines (histamine, serotonin and catecholamines), amino acids, oligopeptides or small proteins, act as efficient activators of the CA isozymes. It has been suggested that CA activators might have use in treating Alzheimer's disease. (Supuran 2008)

2.2.3 Carbonic anhydrase-related proteins

Proteins that have homologous sequences with the α -CA family but lack one or more of the zinc-binding histidine residues critical for CA activity are known as carbonic anhydrase-related proteins (CA-RPs). The CA-RPs found in mammals include CA-RP VIII, X and XI in addition to the N-terminal domains of the receptor-like protein tyrosine phosphatases β and γ (RPTP β and RPTP γ). (Tashian et al. 2000)

The subcellular localization of CA-RPs VIII, X and XI has remained somewhat unclear. In comparison to the cytosolic CA isozymes, CA-RPs possess N-terminal sequence extensions consisting of at least twenty amino acid residues long, yet do not contain a known translocation signal motif (Nishimori 2004). Several experimental results have suggested that CA-RPs may be cytosolic proteins. CA-RPs X and XI have a similar expression pattern in humans and in mice; they are primarily expressed in the brain (Fujikawa-Adachi et al. 1999d,

Okamoto et al. 2001, Taniuchi et al. 2002a, Taniuchi et al. 2002b). CA-RP VIII has shown a high level of expression in the brain, but, in contrast to CA-RPs X and XI, CA-RP VIII has a considerably broader distribution in human and mouse tissues (Tashian et al. 2000, Nishimori 2004). The role of CA-RP VIII in brain physiology was elucidated upon analysis of the waddles (*wdl*) mouse model (Jiao et al. 2005). This mouse model, which exhibits ataxia and appendicular dystonia, was discovered to have a deficiency in CA-RP VIII activity. Indeed, it was recently demonstrated that the *wdl* mice have aberrant synaptic morphology and excitatory synaptic function in the cerebellum, which is the primary motor coordination center in the brain (Hirasawa et al. 2007). CA-RP VIII has also been associated with cancer. It has been shown to be overexpressed in the non-small cell lung and colorectal cancers (Akisawa et al. 2003, Miyaji et al. 2003) and been demonstrated to contribute to the growth and invasiveness of tumors (Lu et al. 2004, Ishihara et al. 2006, Nishikata et al. 2007).

The functional role of CA-RPs has been puzzling researchers for many years. While CA-RPs have high sequence identity with the CA isozymes, they are unable to catalyze the reversible hydration of carbon dioxide (Nishimori 2004). Moreover, CA-RPs have been highly conserved during evolution, implying that they possess important physiological functions. It has been proposed that CA-RPs may function in protein-protein interactions. In fact, it was demonstrated that CA-RP VIII interacts with inositol 1,4,5-trisphosphate (IP₃) receptor type 1 (IP₃R₁), which is a IP₃-gated Ca²⁺-channel located on the intracellular Ca²⁺-stores and whose function is to convert IP₃-signaling into Ca²⁺-signaling (Hirota et al. 2003).

Of the protein tyrosine phosphatase (PTP) protein family, the extracellular portion of two RPTPs, namely RPTPβ and RPTPγ, contains a CA-like domain (Barnea et al. 1993, Tashian et al. 2000, Nishimori 2004). In addition to this CA domain, the extracellular portion consists of a fibronectin III (FN III) repeat domain and a Cys-free domain. The long extracellular region is joined by a transmembrane region to the intracellular D1 and D2 phosphatase domains, and, through this, ligand binding to the extracellular region controls the activity of the intracellular phosphatase domains. The expression of RPTPβ is restricted to the central nervous system (Levy et al. 1993). The CA-like domain has been reported to bind contactin, a GPI (glycosylphosphatidylinositol)-anchored protein expressed on the surface of neuronal cells, and this, in addition to several other findings, indicate that the CA-RP domain of RPTPβ plays an important role in cell-to-cell communication between glial cells and neurons during development (Peles et al. 1995, Nishimori 2004). Altogether it is conceivable that the molecular function of CA-RPs is primarily linked to cell signaling mediated through protein-protein interactions.

2.3 Expression and function of carbonic anhydrase isozymes

2.3.1 Cytosolic isozymes (I, II, III, VII and XIII)

Mammals have five cytosolic CA isozymes: CA I, II, III, VII and XIII.

CA I possesses moderate catalytic activity that is approximately 15% of the activity determined for CA II (Khalifah 1971, Nishimori et al. 2007a). CA I is a highly abundant protein in human erythrocytes (Tashian 1992), but its relatively low activity only contributes to approximately 50% of the total CA activity in these cells (Dodgson et al. 1988). Nevertheless, the presence of CA I may explain why individuals with CA II deficiency syndrome show no phenotype in their erythrocytes (Sly and Hu 1995). Besides erythrocytes, CA I is also expressed in several other tissues. In the human alimentary tract, CA I is expressed in the A cells of the Langerhans islets, the subepithelial capillary endothelial cells and in the epithelium of the esophagus, small intestine and colon (Lönnerholm et al. 1985, Parkkila et al. 1994, Christie et al. 1997). Other tissues and cells expressing CA I include the placenta and foetal membranes, the corneal endothelium, the lens of the eye, the sweat glands, adipose tissues, neutrophils and the zona glomerulosa of the adrenal glands (Venta et al. 1987, Parkkila et al. 1993a, Campbell et al. 1994, Mühlhauser et al. 1994, Sly and Hu 1995). Individuals lacking CA I activity were reported in 1977 (Kendall and Tashian 1977). Later it was shown that the loss of this activity was caused by a missense mutation at residue 246 (from Arg to His) (Wagner et al. 1991). This arginine residue is conserved in all isozymes, and, therefore, it appears to be critical for the CA activity. There is no apparent phenotype in individuals lacking CA I activity, indicating that the other CA isozymes or alternative processes can substitute for the function of CA I (Tashian 1992). Overall, the functional importance of this isozyme is still unclear (Sly and Hu 1995). The human and mouse genes encoding CA I contain two tissue-specific promoters. One of these promoters is functional in erythrocytes, while the other is functional in non-erythroid tissues (Fraser et al. 1989, Brady et al. 1991). The coding region of the mRNAs transcribed from these promoters is identical. It was recently shown that the erythroid-specific promoter of CA I contains a target sequence for the transcription factor c-Myb that is encoded by the proto-oncogene *Myb* (Chen et al. 2006). c-Myb is an essential transcription factor in normal hematopoiesis, and it regulates the expression of CA I in mouse erythroleukemia cells. Thus, it was suggested that CA I may be involved in regulating the differentiation and proliferation of these cells.

CA II is an enzyme possessing high activity and has the widest distribution of all CA isozymes, being expressed in specific cell types of virtually every tissue or organ (Tashian 1992). Due to its high level of activity and pervasive expression, CA II has been proposed to contribute to several distinct physiological processes. It was first discovered in erythrocytes, where it catalyzes the reversible hydration of carbon dioxide, a reaction that is

responsible for both converting the CO₂ produced by the metabolic processes to HCO₃⁻ in the peripheral tissues and also for catalyzing the reverse reaction in the lungs (Swenson 2000). CA II is widely expressed in the renal tissues, where it has an essential role in the acidification of the urine, together with the assistance of membrane-bound isozymes (Parkkila 2000a). In the human alimentary tract, CA II is proposed to perform several additional roles. It is expressed in the serous acinar cells of the parotid and submandibular glands and is thought to generate bicarbonate for saliva (Parkkila et al. 1990, Ogawa et al. 1993). CA II is produced by the squamous epithelium cells of the esophagus, where it may participate in endogenous bicarbonate production, thereby protecting the mucosa against acidity (Meyers and Orlando 1992, Parkkila et al. 1994, Christie et al. 1997). In the stomach, the gastric mucosa secretes high concentrations of bicarbonate and protons. These bicarbonate ions primarily originate from the surface epithelial cells, and while the protons originate from the parietal cells of the gastric glands, both express CA II (Davenport 1939, Kumpulainen 1981, Parkkila et al. 1994). In addition, the bicarbonate ions and mucus produced from the surface epithelial cells and the duodenal Brunner's glands form a layer that protects the epithelial cells from acid digestion (Flemström 1986, Swenson 1991, Parkkila et al. 1994, Leppilampi et al. 2005). CA II has also been found in the surface epithelial cells of the jejunum and colon (Lönnerholm et al. 1985, Parkkila and Parkkila 1996). The non-goblet cells are known to express CA II in the colon, where it is suggested that it participates in the electroneutral reabsorption of NaCl (Swenson 1991, Parkkila et al. 1994, Parkkila and Parkkila 1996).

CA II also contributes to the production of numerous biological fluids. In the liver, CA II has been observed in the hepatocytes and in the epithelium of the bile ducts (Carter et al. 1989, Parkkila et al. 1994) where it is thought to produce bicarbonate for the alkalization of bile (Swenson 1991). In the gallbladder, CA II is involved in concentrating and acidifying the bile (Juvonen et al. 1994, Parkkila et al. 1994, Parkkila and Parkkila 1996). These processes are considered important in preventing gallstone formation. CA II is expressed in the epithelial duct cells of the pancreas, and it is known to contribute to bicarbonate secretion into the pancreatic juice (Kumpulainen and Jalovaara 1981, Spicer et al. 1982, Swenson 1991). In the brain, CA II expression has been demonstrated in several cell types, and one of its functions is to participate in the formation of the cerebrospinal fluid (Maren 1967, Kumpulainen and Korhonen 1982, Cammer and Brion 2000). Moreover, together with CA IV, CA II has been proposed to contribute to the formation of the aqueous humour (Kumpulainen 1983, Matsui et al. 1996, Wu et al. 1997, Chegwiddden and Carter 2000).

CA II is also expressed in the osteoclasts where it produces protons needed for bone resorption, and it has been also suggested to play a role in the differentiation of the osteoclasts (Väänänen and Parvinen 1983, Väänänen 1984, Lehenkari et al. 1998). In metabolic processes, CA II has been suggested to contribute to fatty acid and amino acid synthesis (Cammer 1991, Sly and Hu 1995). CA II can also be found in the reproductive tissues (Härkönen and Väänänen 1988, Kaunisto et al. 1990, Parkkila et al. 1991), the placenta and the

foetal membranes (Mühlhauser et al. 1994) and the adrenal glands (Parkkila et al. 1993a).

The importance of CA II in several aspects of human physiology is emphasized by the fact that its deficiency produces a syndrome that is characterized by osteopetrosis, renal tubular acidosis, cerebral calcification and varying levels of mental retardation and impaired growth (Sly et al. 1983, Sly et al. 1985b, Tashian 1992). To date, CA II deficiency is the only known inherited CA deficiency that has been reported to be of clinical significance (Sly and Hu 1995). CA II deficiency is a rare autosomal recessive disorder, and the loss of CA II catalytic activity may result from several different mutations in the *CA2* gene (Shah et al. 2004). Although CA II is a highly active and abundant isozyme in the alimentary tract, no gastrointestinal symptoms have been reported in CA II-deficient patients. However, recent results with CA II-deficient mice demonstrated that these animals have an abnormal histological phenotype and display impaired prostaglandin E₂-mediated HCO₃⁻ secretion in the duodenum, which suggests that further studies could reveal some gastrointestinal phenotype in CA II-deficient patients (Leppilampi et al. 2005).

CA III is a unique member of this family of isozymes because its catalytic activity is very low. This low activity results partly from the absence of the proton shuttle residue (His 64) and partly from the presence of Phe 198 in place of Leu 198 (Phe 197 in Swiss-Prot entry P07451) (Tu et al. 1989, Chen et al. 1993, LoGrasso et al. 1993). The presence of Phe 198 has also been thought to cause the resistance of CA III to sulfonamide inhibitors (Sly and Hu 1995). CA III is expressed in the skeletal muscle, where high amounts can be found in the type I (slow-twitch) fibers and low amounts in the other fiber types (Väänänen et al. 1985, Laurila et al. 1991). It is abundant in adipose cells (Stanton et al. 1991) and has also been found in the rodent liver (Garg 1974b, Garg 1974a). Several other human or rodent tissues producing various amounts of CA III have been reported, such as the myoepithelial cells of the mammary and prostate glands, the smooth muscle cells of the uterus, the salivary glands, colon, testis, lung, cardiac muscle and erythrocytes (Jeffery et al. 1980, Väänänen and Autio-Harmainen 1987, Spicer et al. 1990, Sly and Hu 1995). CA III can be used to diagnose whether serum myoglobin is originating from skeletal muscle injury or from myocardial infarction, since myocardial infarction patients show a significantly increased ratio of myoglobin to CA III (Väänänen et al. 1990, Beuerle et al. 2000). The same ratio has also been used as a marker of reperfusion after myocardial infarction (Vuotikka et al. 2003).

The high amount of CA III in the adipocytes (approximately 30% of the total soluble protein) (Kim et al. 2004) and skeletal muscle, coupled with its very low catalytic activity, has raised speculations that pH regulation may not be the primary function of this enzyme. CA III may have a specialized role in protecting liver hepatocytes from oxidative damage because chronic alcohol abuse has been shown to induce the expression of *CA3* mRNA in perivenous hepatocytes (Chen et al. 1992, Parkkila and Parkkila 1996) in the same region where acetaldehyde-modified proteins resulting from excessive ethanol consumption also occur (Niemelä et al. 1991, Halsted et al. 1993, Niemelä et al.

1994). Furthermore, CA III has two sulfhydryl groups (Cys 183 and 188) that can conjugate to the tripeptide glutathione (GSH) through a disulfide link in a process called S-glutathiolation (Mallis et al. 2000). S-glutathiolation has emerged as an important mechanism that can adjust the intracellular redox state and protect cellular components from reactive oxygen and nitrogen species (Klatt and Lamas 2000). Several studies have accordingly promoted the idea that CA III may be an antioxidative agent. CA III has been found to be a major participant in the liver's response to oxidative stress (Chai et al. 1991, Lii et al. 1994), and studies with NIH/3T3 cells have indicated that it can protect the cells from hydrogen peroxide-induced apoptosis (Räisänen et al. 1999). It has also been shown that the S-glutathiolation of CA III is increased in aged rats, along with a corresponding decrease in the protective GSH (Mallis et al. 2002). However, an interesting observation is that CA III knock-out mice appeared to be healthy and presented no obvious phenotype (Kim et al. 2004), although microarray analyses revealed that CA III deficiency in skeletal muscle altered gene expression related to the glutathione redox cycle, thus further suggesting that CA III is an antioxidant (Zimmerman et al. 2004). However, a very recent paper has indicated that the lack of this enzyme may impair mitochondrial ATP synthesis (Liu et al. 2007).

With respect to its amino acid sequence, **CA VII** appears to be the most highly conserved isozyme in mammals (Earnhardt et al. 1998). In fact, it has been shown that fish CAs probably diverged after the evolutionary events that gave rise to CA VII. This evidence supports the hypothesis that CA VII could be the most ancient isozyme of the α -CA family (Lund et al. 2002, Tufts et al. 2003). Nevertheless, very little is known about the mammalian CA VII. There has been no thorough survey of CA VII expression in human or mouse tissues, but its expression has been reported in the human salivary gland and in the mouse brain, in both the cerebrum and cerebellum (Montgomery et al. 1991, Lakkis et al. 1997). In addition, CA VII appears to be a key molecule in the GABA (gamma-aminobutyric acid)-mediated signaling pathway, since studies have shown that it enables the synchronous firing of CA1 pyramidal neurons (Ruusuvoori et al. 2004, Rivera et al. 2005).

CA XIII was characterized in 2004, and, therefore it is the member of the CA family most recently identified (Lehtonen et al. 2004). Phylogenetic analyses indicate that it is most closely related to the isozymes I, II and III. The expression of CA XIII has been investigated in human and mouse tissues using immunohistochemistry. Although the expression pattern is slightly different between the two species, CA XIII is widely expressed in the gastrointestinal tract of both species (Lehtonen et al. 2004, Pan et al. 2007). CA XIII is also expressed in both the renal cortex and medulla of the kidney, while the strongest expression has been observed in the collecting ducts (Lehtonen et al. 2004). In mice, CA XIII is expressed in the brain (cerebrum and cerebellum), in the oligodendrocytes and nerve fiber bundles. It has also been observed in the lungs of mice. Interestingly, CA XIII is expressed in the reproductive tissues. In humans, all stages of the developing sperm cell produce CA XIII, and, in the tissues of female reproductive tract, CA XIII is expressed in the uterine cervix

and endometrium. In contrast to humans, CA XIII is absent in the mouse testis but present in the endometrium.

The expression of CA XIII in the reproductive tissues suggests that CA XIII may play a role in the fertilization process. The bicarbonate present in the ejaculate has been proposed to maintain the sperm motility until these cells enter the lumen of the uterus through the cervical canal (Okamura et al. 1985, Kaunisto et al. 1990). In the female genital tract, the endometrial and oviductal epithelium can produce an alkaline environment in order to maintain the sperm motility (Lehtonen et al. 2004). CA XIII may contribute to the normal fertilization process by maintaining the appropriate concentration of HCO_3^- in the cervical and endometrial mucus. Support for this theory was gleaned from inhibition studies of mouse CA XIII, where it was observed that the inhibition constant of bicarbonate for this enzyme is exceptionally high (Innocenti et al. 2004). It therefore seems plausible that this enzyme catalyzes the reaction in physiological environments characterized by high bicarbonate concentration, such as in the reproductive tissues.

2.3.2 Mitochondrial and secretory isozymes (VA, VB and VI)

CAs VA and VB are mitochondrial isozymes, while CA VI is the only secreted isozyme of the CA family.

Mitochondrial CA activity was observed several decades ago (Karler and Woodbury 1960, Dodgson et al. 1980), yet this activity, attributed to a mitochondrial enzyme, was only later given the designation CA V (Dodgson and Forster 1986). However, it was not known until 1999 that mammals possessed two nuclear-encoded mitochondrial CAs (Fujikawa-Adachi et al. 1999c). After this discovery, CA V was designated as **CA VA**, and the novel enzyme was given the name **CA VB**. The distribution of these two enzymes can be generalized such that CA VA is expressed primarily in the liver and to a lesser extent in the skeletal muscle and kidney, while CA VB is ubiquitously expressed in several tissues but not in the liver (in humans it appears that CA VB is not expressed in the liver, but it is expressed in the mouse liver) (Fujikawa-Adachi et al. 1999c, Shah et al. 2000). Considering the high sequence identity between these two isozymes and the fact that polyclonal antibodies have been used to detect their expression, it is difficult to explicitly identify the isozyme whose expression has been reported in those publications, where mitochondrial CA has been referred to only as CA V. Mitochondrial CA (VA or VB) has been discovered in the beta cells of the pancreas (Parkkila et al. 1998), in the parietal cells and gastrin-producing G-cells of the stomach, in the intestinal enterocytes (Karhukorpi et al. 1992, Saarnio et al. 1999) and in the neuronal and glial cells of the brain (Ghandour et al. 2000).

Several cellular metabolic pathways require an early carboxylation step. The mitochondrial CA isozymes have been suggested to contribute to these biosynthetic processes by providing the substrate, bicarbonate, used for this requisite carboxylation reaction (Chegwiddden et al. 2000). In gluconeogenesis,

for example, pyruvate carboxylase converts pyruvate and bicarbonate into oxaloacetate, and the rate of this reaction has been observed to slow down upon CA inhibition (Dodgson and Forster 1986, Dodgson and Cherian 1989, Chegwiddden et al. 2000). In lipogenesis, acetyl-CoA carboxylase converts acetyl-CoA and bicarbonate into malonyl-CoA in the cytosol (Chegwiddden et al. 2000). However, the mitochondrial pyruvate carboxylase is required to help enable citrate (which is converted to acetyl-CoA in the cytosol) to be transferred from the mitochondria to the cytosol (Chegwiddden et al. 2000). The participation of mitochondrial CA in lipogenesis has been confirmed experimentally, although it is possible that cytosolic CA II may also be involved in this process (Lynch et al. 1995, Hazen et al. 1996). In ureagenesis, carbamoyl phosphate synthetase I converts NH_4^+ and bicarbonate to carbamoyl phosphate in the mitochondria. CA activity has been suggested to facilitate bicarbonate production, as a result of studies that indicate a decrease in ureagenesis when CA is inhibited (Rognstad 1983, Häussinger and Gerok 1985, Metcalfe et al. 1985, Bode et al. 1994). Carbamoyl phosphate II is a cytosolic enzyme that uses glutamine and bicarbonate as substrate to produce carbamoyl phosphate and glutamate, and carbamoyl phosphate II functions in the *de novo* pyrimidine nucleotide synthesis. It has been suggested that the carbamoyl phosphate generated in the mitochondria could also become available for pyrimidine synthesis (Cammer and Downing 1991, Chegwiddden et al. 2000). Thus, it is possible that either cytosolic CA, mitochondrial CA, or some combination of these two may contribute to the pyrimidine synthesis. The inhibition of insulin secretion by a CA inhibitor has suggested that mitochondrial CA found in the pancreatic beta cells may play some role in this process (Parkkila et al. 1998).

CA VI, the only secretory isozyme of the CA family, was first characterized in the ovine parotid gland and saliva (Fernley et al. 1979). Interestingly, another zinc-containing salivary protein, gustin, which had been studied in parallel with CA VI, was later determined to be CA VI (Thatcher et al. 1998). CA VI is produced in the serous acinar and ductal cells of the parotid and submandibular glands, and is secreted into the saliva (Parkkila et al. 1994). A competitive time-resolved immunofluorometric assay was developed to measure the concentration of CA VI (Parkkila et al. 1993b). The assay revealed that secretion of CA VI into saliva followed a circadian period, where CA VI levels were low at night and increased rapidly to daytime levels upon awakening (Parkkila et al. 1995). Experimental results suggest that CA VI does not regulate the pH of the secreted saliva (Kivelä et al. 1997). Instead, it has been demonstrated to localize in a region composed of a thin layer of proteins between the enamel of the tooth and the bacterial plaque known as the enamel pellicle. Therefore, CA VI is located at an optimal site on dental surfaces and is hypothesized to catalyze the conversion of salivary bicarbonate and microbe-delivered hydrogen ions to carbon dioxide and water (Leinonen et al. 1999, Parkkila 2000b). This hypothesis was supported by the discovery that low salivary concentrations of CA VI were associated with an increased prevalence of dental caries, particularly in subjects who neglected oral hygiene (Kivelä et al. 1999). Saliva containing CA VI also appears to offer mucosal protection in the upper alimentary tract because patients suffering from

an acid peptic disease have a lower concentration of CA VI in their saliva when compared to healthy control subjects (Parkkila et al. 1997). In addition, CA VI appears to retain its activity in the harsh environment of the gastric lumen. CA VI may also be an important enzyme for the growth and development of the infant alimentary tract, due to the fact that it can be found in both human and rat milk, with particularly high concentrations in the colostrum (Karhumaa et al. 2001b). CA VI has also been linked with taste and smell functions, because a lower concentration of CA VI has been associated with gustatory and olfactory dysfunctions as well as with abnormalities of the taste buds (Shatzman and Henkin 1981, Henkin et al. 1999a). Accordingly CA VI has been proposed to be linked to the growth and development of the taste buds (Henkin et al. 1999b). In fact, the expression of CA VI has been implicated in several parts of the rat tongue, such as the von Ebner's glands, which produce saliva for the taste buds of the circumvallate, in addition to the foliate papillae, which are known to be rich in taste receptor cells (Leinonen et al. 2001). CA VI may also have a mucosa-protective role in the respiratory tract, since its expression has been observed in the rat lower airways and lungs (Leinonen et al. 2004) as well as in the mouse nasal gland, where it may also possess an olfactory function (Kimoto et al. 2004). CA VI has also been observed in the lacrimal gland (Ogawa et al. 1995, Ogawa et al. 2002). In 1999, a stress-inducible form of CA VI was reported (Sok et al. 1999). This form is expressed from an internal promoter in the gene and encodes an intracellular form of the protein. However, the physiological importance of the stress-inducible CA VI has not yet been elucidated.

2.3.3 Membrane-bound isozymes (XII and XIV)

Four membrane-bound mammalian CA isozymes, CAs IV, IX, XII and XIV, have been characterized thus far. In all of these membrane-bound isozymes, the catalytic domain is located on the cell exterior (Chegwidden and Carter 2000). CAs IX, XII and XIV have a single membrane-spanning helix, while CA IV is bound to the membrane via a GPI-anchor. In addition to these characterized isozymes, the cDNA sequence for CA XV, a possible membrane-bound isozyme, can be found in the NCBI genome database, but there are no published reports regarding enzyme characterization. **CA IV** and **CA IX** are reviewed in detail in sections 2.3.4 and 2.3.5, respectively.

The gene for the transmembrane enzyme **CA XII** was cloned and the corresponding protein characterized in 1998 (Türeci et al. 1998). In normal tissues, CA XII is expressed on the basolateral membrane of the epithelial cells in the endometrium, where it is likely to play a role in reproductive physiology (Karhumaa et al. 2000a, Hynninen et al. 2004). Furthermore, CA XII is present on the basolateral plasma membrane of the epithelial cells in human efferent ducts, and its expression has also been observed in the rat epididymis, especially in the corpus and proximal cauda regions (Karhumaa et al. 2001a, Hermo et al. 2005). The expression of CA XII differs between human and rodent renal tissues

(Parkkila et al. 2000a, Kyllönen et al. 2003). While it is expressed in the epithelial cells of the proximal tubule in both humans and rodents, only in humans it is also expressed in the thick ascending limb of Henle and in the distal convoluted tubules. Moreover, in the rodent collecting ducts, CA XII is expressed in the H⁺-secreting intercalated cells, whereas in the human kidney it is expressed in the principal cells. Therefore, CA XII may perform different physiological roles in the human and rodent kidneys. CA XII is highly expressed in the surface epithelial cells of the colon, where it may participate in the reabsorption of NaCl (Kivelä et al. 2000a, Halmi et al. 2004). The expression of CA XII has also been reported in several other cells and tissues, such as the mesothelial cells of the body cavity and the epithelium of the breast (Ivanov et al. 2001). In the eye, the expression of CA XII is low, but it has been observed to increase in the non-pigmented epithelial cells of patients with glaucoma (Liao et al. 2003). In addition, during mouse embryogenesis, the expression of CA XII has been reported in the brain, stomach, pancreas, liver and kidney (Kallio et al. 2006).

As with CA IX, CA XII is a tumor-related protein that is assumed to be upregulated under hypoxic conditions, although a functional hypoxia-responsive element (HRE) has never been published for *CA12* gene (Ivanov et al. 1998, Ivanov et al. 2001, Hynninen et al. 2006). Nevertheless, the overexpression of this enzyme has been reported in several cancers (Ivanov et al. 2001). The expression of CA XII has been reported in 75% of invasive breast carcinoma cases, and it is associated with several favorable prognostic parameters, such as low tumor grade, ER (estrogen receptor)-positive status, EGFR (epidermal growth factor receptor)-negative status and absence of necrosis (Watson et al. 2003). CA XII is expressed in the majority of renal carcinomas and the expression of this protein has a slight, although not statistically proven, correlation with histological grade (Parkkila et al. 2000a). CA XII is slightly overexpressed in the colorectal tumors (Kivelä et al. 2000a). In adenomas, the amount of CA XII expressed increases along with the grade of dysplasia, and nearly all of the investigated malignant lesions have shown diffuse immunostaining. CA XII has also been shown to be overexpressed in ovarian tumors (Hynninen et al. 2006). In brain tumors, the overexpression of CA XII has been implicated in gliomas, meningiomas, hemangioblastomas and brain metastases (Proescholdt et al. 2005). Another recent study indicated that CA XII is a marker of poor prognosis in the diffusely infiltrating astrocytomas (Haapasalo et al. 2008). This study experimentally confirmed that the *CA12* mRNA is alternatively spliced and thus produces two CA XII isoforms. The shorter isoform lacks eleven amino acid residues that may affect the oligomerization state of the enzyme. In tumors, the role of CA XII together with CA IX has been proposed to maintain neutrality of the intracellular pH and to acidify the extracellular milieu, which is known to contribute to tumor growth and metastasis (Ivanov et al. 2001). This hypothesis is supported experimentally by a report that revealed that the CA inhibitor acetazolamide suppressed the invasion of renal cancer cells *in vitro* (Parkkila et al. 2000b).

The most recently characterized member of the CA isozyme family is the transmembrane enzyme **CA XIV**. This enzyme was characterized independently by two research groups in 1999, with one group reporting the human isozyme (Fujikawa-Adachi et al. 1999b) and the second group characterizing the mouse isozyme (Mori et al. 1999). CA XIV was found to be widely expressed in several human and mouse tissues, such as the kidney, heart, brain, skeletal muscle, liver, lung and intestinal tissues (Fujikawa-Adachi et al. 1999b, Mori et al. 1999). The expression of this enzyme has been studied in greater detail for several of these organs.

In the mouse kidney, CA XIV is produced most abundantly in the cortex region, where it is restricted to the apical plasma membrane of the S1 and S2 segments in the proximal tubules (Kaunisto et al. 2002). CA XIV is also expressed in the initial portion of the thin descending limbs of Henle. The presence of the enzyme in the proximal tubules suggests that it may perform an important role in the reabsorption of bicarbonate, and, therefore, together with CA IV, it is likely to account for the majority of the CA activity required for the acidification of urine. In the mouse liver, CA XIV is expressed on the plasma membrane of hepatocytes (Parkkila et al. 2002). Both the apical (canalicular) and basolateral (sinusoidal) membrane domains have been shown to express CA XIV. In the rat epididymis, CA XIV is expressed together with isozymes II, III, IV and XII (Kaunisto et al. 1999, Hermo et al. 2005). In both skeletal and cardiac muscle, CA XIV localizes to the longitudinal sarcoplasmic reticulum and to the sarcolemmal membrane (Scheibe et al. 2006, Wetzel et al. 2007).

CA XIV is expressed on neuronal membranes and axons in both the mouse and human brain (Parkkila et al. 2001). The most abundant expression has been observed on the large neuronal bodies and axons in the anterolateral part of the pons and medulla oblongata. Other tissues known to express CA XIV include the hippocampus, corpus callosum, cerebellar white matter and peduncles, pyramidal tract and choroid plexus. In the mouse brain, the molecular layer of the cerebral cortex and granular layer of the cerebellum were also found to express CA XIV. These observations suggested that CA XIV may be responsible for the extracellular CA activity known to contribute to the alkaline shift after synaptic transmission (Chen and Chesler 1992). It was later realized that both CA IV and CA XIV participate in this extracellular buffering. This realization was due to the observation that, while CA IV or XIV knock-out mice failed to show any phenotype in this context, the CA IV/XIV double knock-out mice displayed a delay in the alkaline shift (Shah et al. 2005).

The expression of CA XIV in the mouse retina has been described in two publications (Nagelhus et al. 2005, Ochrietor et al. 2005). The expression was highest on the apical surface of the pigmented epithelial cells in the retina, corresponding to the microvilli of the epithelium, but it was also found on the basal surface. CA XIV is also expressed in the Müller glial cells. Recently, studies using CA IV and CA XIV single and double knock-out mice demonstrated that CA XIV deficiency causes a functional defect in the retinal light response, although CA IV can partly compensate for the loss of CA XIV (Ogilvie et al. 2007).

2.3.4 Membrane-bound isozyme IV

CA IV has been the only member of the CA family reported to be attached to the cell membrane with a GPI-anchor (Zhu and Sly 1990, Waheed et al. 1992). This enzyme has been shown to perform important functions in several tissues. It is expressed at the luminal side of the alveolar capillary endothelium in adult rats (Fleming et al. 1993). The presumed function of CA IV in the lung is to catalyze the dehydration of plasma HCO_3^- to CO_2 that can then readily diffuse across the capillary endothelial surface and pass out of the lungs through expiration (Zhu and Sly 1990). CA IV is also localized in the capillary endothelium of skeletal and heart muscle (Sender et al. 1994, Sender et al. 1998). In the skeletal muscle, CA IV has been detected in the sarcolemma and in the sarcoplasmic reticulum (Decker et al. 1996, Scheibe et al. 2006). The subcellular localization of CA IV has also been investigated in the heart, where it has been found in the sarcolemma as well as in special sarcolemmal structures, intercalated discs and T-tubules (Sender et al. 1998, Scheibe et al. 2006). In the gastrointestinal tract, CA IV has been observed in the submucosal capillary endothelium of all gastrointestinal regions, as well as in the mucosal epithelium of the distal small and large intestine, particularly in the colon, where it is thought to contribute to the ion and water balance (Fleming et al. 1995). CA IV may function in combination with CA II and various ion transporters in the acidification of bile via bicarbonate reabsorption, since its expression has been observed in the luminal plasma membrane of the gallbladder epithelium and also in the endothelium of the subepithelial capillaries (Parkkila et al. 1996). The expression of *CA4* mRNA has also been observed in the pancreas and salivary glands, while expression in the liver is significantly lower (Parkkila et al. 1996, Fujikawa-Adachi et al. 1999a). CA IV is expressed on the luminal surface of endothelial cells in cerebral capillaries and may be considered a cytochemical marker for the blood-brain barrier (Ghandour et al. 1992). CA IV in erythrocytes is thought to participate in the bicarbonate/chloride exchange by interacting with the anion exchanger 1 protein (AE1, also known as band 3) (Wistrand et al. 1999). CA IV has also been reported to function in the rat astrocytes (Svichar et al. 2006) and to be expressed in the mouse placenta (Rosen et al. 2001).

In the rodent kidney, CA IV is localized in the apical and basolateral plasma membranes of the proximal convoluted tubule and in the thick ascending limb of Henle, both of which are important for the reabsorption of HCO_3^- (Brown et al. 1990). In the proximal tubules, CA IV is abundantly expressed in the S2 segment. In humans, CA IV has been observed in the apical plasma membrane of cells within the cortical and medullary collecting ducts, presumably in type A intercalated cells (Lönnerholm and Wistrand 1991). Weaker expression was observed inside a number of collecting duct cells in addition to the basolateral plasma membrane of the proximal convoluted tubule. The basolateral subcellular localization of CA IV is exceptional because most GPI-anchored proteins are targeted to the apical membrane (Purkerson et al. 2007). The cytosolic CA II accounts for more than 95% of the renal CA activity and only 5% is membrane-associated (Purkerson and Schwartz 2007). Nevertheless, both apical and

basolateral plasma membrane CA activities have been confirmed to play important roles in the reabsorption of HCO_3^- in the kidney (Lucci et al. 1983, Sly et al. 1985a, Brechue et al. 1991, Tsuruoka et al. 2001). It has become evident that several CA isozymes contribute to the membrane-bound CA activity in normal renal physiology, including CAs IV and XIV on the luminal plasma membrane and isozymes IV and XII on the basolateral plasma membrane (Parkkila et al. 2000a, Kaunisto et al. 2002).

CA IV has shown limited expression in the eye. It is highly expressed in a specific uveal capillary bed, known as the choriocapillaris, and in the epithelial and fiber cells of the lens as well as in the capillaries of the ciliary processes (Hageman et al. 1991, Wistrand 1999). Despite its limited distribution in the eye tissues, CA IV has been associated with the RP17 form of retinitis pigmentosa (Rebello et al. 2004). It was shown that these patients possess an R14W mutation in the signal sequence of CA IV. This mutation in the *CA4* gene is hypothesized to lead to an accumulation of improperly folded CA IV in the endoplasmic reticulum of the endothelial cells in the choriocapillaris, inducing the unfolded protein response that eventually results in cell apoptosis. Initiation of this response may eventually lead to ischemia of the overlying retina. This hypothesis has been supported by the following two findings: (i) chemical chaperones can protect the COS-7 cells (a monkey kidney fibroblast cell line) transfected with the mutant CA IV from apoptosis, and (ii) the absence of CA IV in knock-out mice does not produce any notable phenotype (Bonapace et al. 2004, Ogilvie et al. 2007). However, an alternative hypothesis has been proposed to explain the role of CA IV in retinitis pigmentosa. The retina, from a metabolic perspective, is a highly active tissue, and CA IV, which localizes together with the $\text{Na}^+/\text{HCO}_3^-$ co-transporter 1 (NBC1) in the choriocapillaris, may efficiently remove the acid produced by metabolism (Yang et al. 2005). Thus, a mutation in CA IV would potentially cause an imbalance in the acid-base homeostasis in the retina. This alternative theory is supported by the observation that mutation of the signal sequence of CA IV had no effect on the secretion of the protein from the cells. Moreover, a separate mutation (R69H) in the mature CA IV has been linked to the disease (Yang et al. 2005, Alvarez et al. 2007). Future studies may reveal whether or not one of these hypotheses is correct.

CA IV has been suggested to form a so-called metabolon together with CA II and several anion exchange proteins. A metabolon is a complex of enzymes involved in a metabolic pathway which allows metabolites to efficiently move directly between active sites (Reithmeier 2001). CA II and IV have been shown experimentally to interact with several anion exchange proteins, such as $\text{Cl}^-/\text{HCO}_3^-$ exchangers, $\text{Na}^+/\text{HCO}_3^-$ cotransporters and Na^+/H^+ exchangers (Purkerson and Schwartz 2007). Thus, it has been proposed that in the erythrocytes and renal tissues, for instance, CAs II and IV may facilitate the rate of anion exchange across the plasma membrane either by providing the substrate for exchange or by removing the exchanged ions via CA catalysis, depending on the direction of the transport (Reithmeier 2001, McMurtrie et al. 2004, Purkerson and Schwartz 2007). Recently, however, the direct interaction between CA II and the SLC4 family of bicarbonate transporters, which has been observed in

several studies, was jeopardized by the results of a study indicating that the GST-tag used in the aforementioned interaction studies may interfere with the results (Piermarini et al. 2007). While this result does not necessarily exclude the existence of the metabolon, it suggests that the interaction between the CA II and the anion exchangers may not be direct.

As already mentioned in the context of CA XIV, the mouse models of the CA IV/XIV double knock-out revealed that both of these isozymes are likely to participate in the extracellular buffering process after synaptic transmission in the brain (Shah et al. 2005). The results from the double knock-out mice also indicated that these isozymes may be redundant in producing the normal retinal light response (Ogilvie et al. 2007). The CA IV knock-out mice were produced in smaller numbers than predicted, and this may result from either fetal or postnatal losses preferentially affecting females (Shah et al. 2005). Otherwise, CA IV knock-out mice appeared healthy, and no abnormalities were reported.

2.3.5 Membrane-bound isozyme IX

2.3.5.1 General and historical aspects

For historical reasons, CA IX is known by several names in the literature. In 1992, a new membrane-bound and tumor-associated protein was discovered, and it was given the name MN (Pastoreková et al. 1992). It was soon realized that this protein was a new member of the CA family, and it was designated as MN/CA IX (Pastorek et al. 1994, Opavský et al. 1996). In addition, another group in 1986 reported that their monoclonal antibody, G250, recognized some antigen in renal cell carcinomas (RCCs) (Oosterwijk et al. 1986). However, it was not until the year 2000 that the G250 antigen was actually confirmed to be CA IX (Grabmaier et al. 2000). Researchers in the Netherlands studying CA IX were some of the first to focus on the clinical use of the G250 antibody, while the Slovak and Czech groups were intensively studying the molecular and cellular biology of this antigen. Currently, CA IX is intensively studied by several research groups, and all of the mentioned names, as well as several combinations, are still used for CA IX in the literature.

CA IX in its mature form is a transmembrane protein that is composed of a so-called proteoglycan-like (PG) domain, which has similarity to the keratan sulfate-binding domain of the large proteoglycan aggrecan, a CA catalytic domain, a transmembrane region and a short cytoplasmic tail at the C-terminus. CA IX is the only member of the CA isozyme family bearing a PG domain together with the CA domain. (Pastoreková and Zavada 2004)

2.3.5.2 Expression in normal tissues

The expression of CA IX has been investigated thoroughly in the human and rat alimentary tracts by immunohistochemical methods. No expression has been observed in the human oral or esophageal epithelium. CA IX is expressed abundantly in the stomach. There it is present throughout the gastric mucosa, from the gastric pits to the deep gastric glands, and it is confined to the basolateral surface of the epithelial cells. Furthermore, all major cell types of the gastric epithelium produce CA IX. In the rat intestine, the epithelial cells of the duodenum and colon produce CA IX, while the jejunum and ileum do not. The basolateral membranes of the epithelial cells were found to be immunopositive throughout the colon, but in the distal segments, the production of CA IX was much lower. In the human intestine, the duodenum, jejunum, ileum and proximal colon are all positive segments for CA IX expression. Also in humans, the expression decreases towards the distal parts of the intestinal tract, and the expression in the ileum and proximal colon is somewhat focal. However, differences have been observed in CA IX distribution in the mucosal layer between humans and rats. In the rat colon, CA IX is most intensively expressed in the surface epithelium, while in the human colon, the expression is highest at the base of the crypts. (Pastoreková et al. 1997, Saarnio et al. 1998b)

Submandibular and parotid glands do not produce CA IX. A faint signal has been observed in the basolateral membrane of the epithelial cells in the human pancreatic ducts, while the corresponding rat tissue is negative. However, both human and rat bile ducts show a positive signal at the basolateral surface of the epithelial cells. In addition, the human gallbladder epithelium produces abundantly CA IX. (Pastoreková et al. 1997)

Weak expression of CA IX has been shown in the human male excurrent ducts (Karhumaa et al. 2001a). Specifically, it has been observed in the basolateral plasma membrane of the efferent duct epithelium, but the epididymal duct epithelium is negative. Some expression of CA IX has also been observed in the lining cells of the body cavity, rete testis, rete ovarii, surface coelomic epithelium, the ventricular linings of the central nervous system and the choroid plexus (Ivanov et al. 2001).

When a knock-out mouse model was generated to study the function of CA IX, the first paper also described the main sites of *Car9* expression. In this work, the distribution of *Car9* mRNA was studied in some normal mouse tissues by ribonuclease protection assay. The highest level of mRNA was observed in the stomach, and a moderate level of expression was found in the small intestine and colon; the kidney and brain showed very weak mRNA expression. The liver and spleen were negative tissues for the mRNA. A signal for CA IX was also present in the mouse embryo at embryonic day E18.5, while it was absent in the embryonic stem cells and E10.5 embryo. In the same study, immunohistochemical staining was performed on the mouse stomach with a CA IX antibody. The results showed that CA IX is highly expressed in the body region of the stomach but not in the forestomach. Positive staining for CA IX in the body region was distributed in all epithelial cells, but its intensity was

strongest at the basolateral surfaces of mature glandular and superficial pit cells. In addition, CA IX was not found in the stomachs of E15.5 and E17.5 embryos, but weak expression was observed in the stomach of a newborn mouse. (Ortova Gut et al. 2002)

2.3.5.3 Expression in neoplastic tissues

The key feature of CA IX is that its expression in normal tissues is quite limited, whereas it is highly overexpressed in numerous tumors, in which it usually indicates a poor prognosis for the patient. An intense overexpression of CA IX was first observed in renal cell carcinomas (RCCs), especially of the clear cell type (Oosterwijk et al. 1986, McKiernan et al. 1997). In contrast to several other cancers, there is evidence that decreased levels of CA IX are a predictor of poor survival for RCC patients (Bui et al. 2003, Sandlund et al. 2007). CA IX is expressed in carcinomas of the ovary, endometrium and uterine cervix but not in normal tissues from the corresponding organs (Závada et al. 1993). More than 90% of dysplastic or malignant cervical tissues show immunoreactivity to CA IX (Liao et al. 1994). Most cases of carcinomas of the uterine cervix are moderately or strongly positive for CA IX, with the expression being most prominent in hypoxic regions (Loncaster et al. 2001, Hynninen et al. 2006). CA IX is absent from normal breast tissue, whereas in breast cancers the overexpression has been associated with high-grade, steroid receptor-negative cancers and tumor necrosis as well as the expression of ErbB2 (Chia et al. 2001, Bartosová et al. 2002, Span et al. 2003, Hussain et al. 2007, Trastour et al. 2007). For the patient, the expression of CA IX predicts a higher relapse rate as well as poorer overall survival.

Several reports have indicated that CA IX is overexpressed in the majority of non-small cell lung cancer cases, and its expression is associated with tumor aggressiveness, hypoxia, necrosis, other tumor-related genes and processes, such as angiogenesis, and eventually a poor prognosis for the patient (Vermylen et al. 1999, Giatromanolaki et al. 2001, Swinson et al. 2003, Kim et al. 2005, Kon-no et al. 2006). Normal squamous epithelium has only minimal CA IX expression, but it is overexpressed in all esophageal squamous cell carcinomas and the majority of esophageal adenocarcinomas (Turner et al. 1997, Driessen et al. 2006). In the latter of these cases, it has been shown to correlate with a poor prognosis. In head and neck squamous cell carcinomas, CA IX expression correlates with tumor necrosis, higher microvessel density and advanced disease stage (Beasley et al. 2001). The expression of CA IX has been reported to correlate with increased malignancy grades in several brain tumors, such as astrocytic and oligodendroglial gliomas, hemangioblastomas and meningiomas (Proescholdt et al. 2005, Haapasalo et al. 2006, Korkolopoulou et al. 2007, Järvelä et al. 2008). Other tumors reported to abnormally express CA IX include colorectal tumors, bladder carcinomas, soft tissue carcinomas, pancreatic tumors, mesotheliomas, testis and germ cell tumors as well as some squamous and basal

cell carcinomas of the skin (Saarnio et al. 1998a, Kivelä et al. 2000b, Ivanov et al. 2001, Turner et al. 2002, Måseide et al. 2004, Niemelä et al. 2007).

Interestingly, tumors originating from CA IX positive tissues tend to have decreased expression of the enzyme. In gastric adenomas, CA IX expression decreases as the dysplasia grade increases (Leppilampi et al. 2003). In well differentiated adenocarcinomas, CA IX expression is as high as in the normal mucosa, while in less differentiated carcinomas, the expression declines. Similarly, hepatobiliary epithelial tumors show decreasing levels of CA IX with increasing grades of dysplasia and carcinoma (Saarnio et al. 2001).

2.3.5.4 Regulation of expression

The abnormal expression of CA IX in tumors has raised questions about the mechanisms that cause the differential expression of the *CA9* gene. The most important gene region regulating expression is a hypoxia-responsive element (HRE), which is located on the antisense strand at a nucleotide position of -10/-3 relative to the transcription start site (Wykoff et al. 2000). The HRE is recognized by the hypoxia inducible factor-1 (HIF-1). HIF-1 is a transcription factor that is composed of two subunits. The α -subunit resides in the cytosol and is stable only under hypoxic conditions, while the β -subunit is constitutively expressed and resides in the nucleus (Pastoreková and Pastorek 2004). At normal oxygen levels (normoxia), prolyl-4-hydroxylases (PHDs) hydroxylate specific proline residues of HIF-1 α . The von Hippel-Lindau (VHL) tumor suppressor protein binds hydroxylated HIF-1 α and targets it for degradation to the ubiquitin-proteasome system. However, under hypoxic conditions HIF-1 α is not hydroxylated because PHDs are inactive in the absence of oxygen. This leads to the stabilization of HIF-1 α , which is then translocated to the nucleus, where it dimerizes with the constitutively expressed β -subunit and forms an active transcription factor, HIF. Subsequently, HIF activates transcription of hypoxia-regulated genes, such as genes related to angiogenesis, cell proliferation and survival, glucose metabolism and pH (Semenza 2001). The VHL/HIF-pathway is illustrated in Figure 2.

The regulation of CA IX by the VHL/HIF pathway explains why CA IX is overexpressed especially in clear cell renal cell carcinomas (ccRCCs). Germline mutations of the *VHL* gene in humans cause a hereditary cancer syndrome called von Hippel-Lindau disease (Kondo and Kaelin 2001). One frequently occurring cancer among these patients is ccRCC, and the loss of heterozygosity for the *VHL* tumor suppressor gene has been observed in 98% of ccRCC samples (Gnarra et al. 1994). Thus, the frequent absence of a functional *VHL* gene induces the transcription of *CA9*.

In addition to the HRE, other regions and transcription factors influence the transcription of *CA9*. Analyses of the *CA9* promoter have revealed five regulatory regions designated as PR1–5 (protected regions 1–5), where cis-acting regulatory proteins can bind. PR1 and PR2 have been found to be important elements for the activation of the transcription, while PR4 acts as a silencer

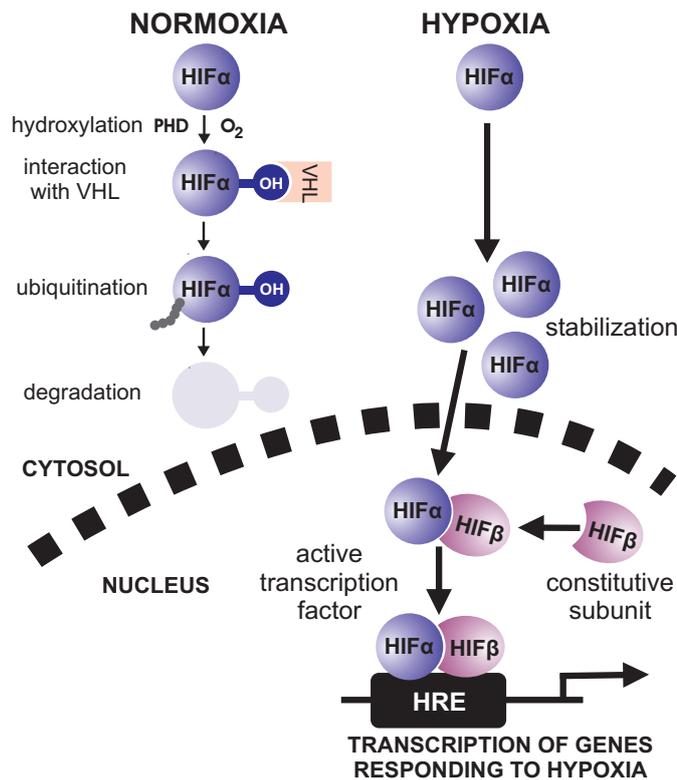


Figure 2. Schematic illustration of the VHL/HIF-pathway. The mechanism is explained in detail in the text. The figure is adapted from (Pastoreková and Pastorek 2004).

(Kaluz et al. 1999). PR5, which has a high sequence identity to PR1, also affects the transcriptional activity of *CA9*, but not as much as PR1, since the distance from PR5 to the transcription start site is greater (Kaluz et al. 2003). The SP1/SP3 (specificity protein 1/3) complex binds to these PR1 and PR5 regions, and the AP1 (activator protein 1) transcription factor binds to the PR2 position. The synergy achieved by the binding of the SP1 and AP1 transcription factors to the PR1 and PR2, respectively, is necessary for the transcriptional activity of *CA9* (Kaluzová et al. 2001, Kaluz et al. 2003).

The expression of *CA9* is also regulated by methylation of CpG dinucleotides in the promoter region. Hypomethylation of the promoter has been shown to induce the expression of *CA9* in RCC cell lines (Cho et al. 2000). It has also been revealed that in RCCs with VHL mutations, the expression of *CA9* does not occur without hypomethylation of the promoter, particularly at CpG sites -74 and -6 relative to the transcriptional start site (Ashida et al. 2002). Investigations regarding non-RCC cell lines have shown that methylation at nucleotide position -74 down-regulates the expression of *CA9* in cells at high densities, but not under conditions of hypoxia or sparse cell densities (Jakubicková et al. 2005).

CA IX is not confined only to the hypoxic regions in the tumors, suggesting that there might be some other pathways that regulate the expression of the gene. Indeed, it has been shown that the expression of CA IX is induced by high cell density, and this regulation is associated with increased phosphatidylinositol 3'-kinase (PI3K) activity (Kaluz et al. 2002). This mechanism is different from the HIF-1 mediated regulation but is still HIF-1 interrelated. This regulation occurs under mild hypoxia, and minimal HIF-1 activity is needed for the upregulation of CA IX by high cell densities. Furthermore, the expression of CA IX is also controlled by the mitogen-activated protein kinase (MAPK) pathway, which

controls the *CA9* promoter by both HIF-1-dependent and independent signal, and is also interrelated with the PI3K pathway and the SP1 transcription factor (Kopacek et al. 2005).

In summary, hypoxia and subsequent HIF-1 activation are the most important factors regulating the expression of *CA9*, but other pathways also contribute to the regulation of the gene. Based on the experimental results, a detailed model has been proposed for the assembly of the transcription factors to the minimal *CA9* promoter. It has been shown that SP1/SP3 complex is needed under all conditions for the expression of the *CA9* gene. Under high cell density and mild hypoxia, there could be some additional transcription factor that complexes SP1/SP3 with HIF-1 and causes the transcription to occur even without a concomitant increase in the levels of the HIF-1 α subunit. However, under conditions of true hypoxia and low cell density, transcription is driven primarily by enhanced activity of HIF-1. The maximal transcriptional activity is achieved under conditions of true hypoxia and high cell densities, when both the HIF-1 activity and a possible additional transcription factor can contribute to the induction of the transcription. (Kaluz et al. 2003)

2.3.5.5 Functional role

CA IX is an exceptional member of the CA family. This is because, in addition to the CA domain, it also contains a PG domain. For this reason, it is logical that CA IX has been proposed to contribute to both pH regulation and cell-to-cell adhesion processes (Pastorekova and Zavada 2004).

As mentioned earlier, CA IX has quite a limited distribution in normal tissues, and its expression pattern suggests that it participates in the regulation of the acid-base balance at the basolateral surfaces of the gastrointestinal tract epithelia. CA IX has been shown to functionally and physically interact with the SLC4 family of Cl⁻/HCO₃⁻ anion exchange proteins, AE1, AE2 and AE3. Furthermore, CA IX colocalizes with AE2 at the basolateral plasma membrane of parietal cells. Thus, these proteins are likely to contribute to the formation of the bicarbonate transport metabolon. The parietal cells of the gastric mucosa actively secrete H⁺ into the gastric lumen, accompanied by Cl⁻ movement through Cl⁻ channels. To sustain acid secretion, parietal cells must replace the HCl lost at the apical surface, and two coupled processes may perform this task. At the parietal cell's basolateral surface, CO₂ diffuses into the cell from adjacent capillaries. CA II catalyzes CO₂ conversion, thereby producing HCO₃⁻ and H⁺ in order to supply the parietal cells with protons. In addition, Cl⁻/HCO₃⁻ exchangers, present in the basolateral membrane, extrude excess HCO₃⁻ while importing Cl⁻, thus regulating the balance of these ions in the cell. The basolaterally localized CA IX may then catalyze the conversion of extruded HCO₃⁻, and thereby contribute to gastric acid secretion by forming the AE2-CA II-CA IX bicarbonate transport metabolon. (Morgan et al. 2007)

In contrast to normal tissues, CA IX is highly expressed in numerous tumors, particularly under hypoxic conditions. Therefore, CA IX might play an important

role in pH regulation in malignant processes. Because of their rapid growth, tumors commonly experience hypoxia, since they initially have no extensive capillary network to supply tumor cells with oxygen. As a result, cancer cells lying more than 100 to 200 μm from the nearest capillaries depend on anaerobic glycolysis for much of their energy production (Nelson and Cox 2005). This anaerobic tumor metabolism generates excesses of acidic products, such as lactic acid and H^+ that have to be extruded from the cell interior in order to maintain the neutral intracellular pH. In addition to lactic acid, CO_2 is also a significant source of acidity in tumors. These processes result in low extracellular pH which is a common feature for solid tumors. A hypothetical model consisting of a bicarbonate transport metabolon has been constructed to predict the role of CA IX in this process (Figure 3). CA IX is likely to convert CO_2 to HCO_3^- and H^+ at the extracellular side of the cell membrane, thereby providing the bicarbonate for the anion exchanger (AE) to transport into the cytosol (Pastorekova and Zavada 2004). Thus, the protons produced by CA IX could facilitate the acidosis of the tumor microenvironment. In the cytosol, CA II could contribute to HCO_3^- dehydration back to CO_2 , which then would leave the cell by diffusion. This dehydration would consume H^+ and thereby help to neutralize the intracellular pH, which promotes the growth of the tumor cells. CA IX has been experimentally confirmed to acidify the extracellular microenvironment of the tumor cells under hypoxic conditions (Svastová et al. 2004). In the same study, it was also shown that some sulfonamide inhibitors bind CA IX only under hypoxic conditions, suggesting that the activity and/or conformation of this enzyme may be altered in hypoxia. The correlation of CA IX with a poor prognosis in several cancers is understandable, because the acidic extracellular environment is known to induce production of growth factors, increase genomic instability, perturb cell-to-cell adhesion and facilitate tumor spread and metastasis (Pastorekova and Zavada 2004).

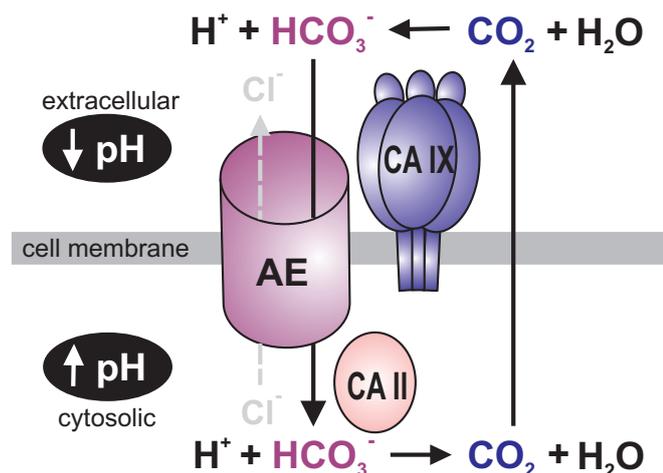


Figure 3. Proposed hypothesis of the role of CA IX in the pH regulation in hypoxic tumors. The model is based on the formation of a bicarbonate transport metabolon composed of an anion exchanger (AE) and CAs II and IX (see text for details). The figure is adapted from (Pastorekova and Zavada 2004).

In addition to pH regulation, CA IX has been indicated to participate in the cell adhesion and proliferation processes that are important in both normal and malignant tissues. Initial evidence indicating that the PG domain of CA IX is related to cell adhesion came from a study showing that CA IX can mediate attachment of cells to non-adhesive solid support, and this phenomenon is blocked by a M75 antibody that binds the PG domain (Závada et al. 2000). Later experiments with MDCK (Madin-Darby canine kidney) epithelial cells revealed that CA IX reduced E-cadherin-mediated cell-cell adhesion by interacting with β -catenin (Svastová et al. 2003). β -catenin plays a role in the formation of adherence junctions between epithelial cells by connecting E-cadherin to α -catenin and then to the cytoskeleton. In normal tissues, this property of CA IX is likely to be important in the intestinal mucosa, where it is located in the crypt enterocytes that possess the greatest proliferative capacity (Saarnio et al. 1998b, Svastová et al. 2003). In these cells, CA IX may decrease cell-to-cell adhesion in order to support production and integration of new cells. In tumors, CA IX can cause diminished intercellular adhesion, which is known to be related to tumor invasion and metastasis (Svastová et al. 2003, Pastorekova and Závada 2004). Further evidence showing the contribution of CA IX to cell proliferation came from CA IX knock-out mice, which showed gastric hyperplasia of the glandular epithelium with numerous cysts (Ortova Gut et al. 2002). The most pronounced hyperplasia was observed in the corpus region of the stomach. The first changes were observed in the newborn animals, and the hyperplasia became prominent at the end of gastric morphogenesis in four-week-old mice. Loss of CA IX led to overproduction of mucus-secreting pit cells and depletion of pepsinogen-producing chief cells. The absolute number of parietal cells was increased in the CA IX deficient animals, although the proportion of these cells was reduced compared with the gastric epithelium of the wild-type mice. However, the CA IX knock-out mice had normal gastric pH, acid secretion and serum gastrin levels.

Participation of CA IX in cell signaling has generated a great deal of speculation in the literature. The first supporting evidence came when it was revealed that the intracellular domain of CA IX contains a tyrosine residue whose phosphorylation can interact with the regulatory subunit of PI3K, which contributes to the activation of the serine/threonine protein kinase Akt (Dorai et al. 2005). In the study, a model of a positive feedback loop was presented illustrating the involvement of CA IX in the cell signaling pathways. In summary, CA IX has been shown to be a unique enzyme which probably contributes to pH regulation, cell adhesion, cell proliferation and cell signaling.

2.4 Biochemical properties of carbonic anhydrase isozymes

A summary of the catalytic activities presented below can be found in Table 6, in section 6.4.

2.4.1 Cytosolic isozymes (I, II, III, VII and XIII)

The biochemical properties of cytosolic isozymes have been studied using recombinant proteins produced in the bacterial expression systems or, in some cases, native proteins that have been obtained directly from human tissues.

The catalytic activity of CA I has been measured for protein purified from human erythrocytes and for protein produced in *Escherichia coli* (*E. coli*). In both cases, the k_{cat} was measured to be $2.0 \times 10^5 \text{ s}^{-1}$, and the protein produced in the bacteria showed a $k_{\text{cat}}/k_{\text{M}} = 5.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Khalifah 1971, Nishimori et al. 2007a). Thus, CA I has moderately low catalytic activity. The first x-ray crystal structure of CA I was refined to a 2.2 Å resolution (Kannan et al. 1975). The best structure for CA I in terms of resolution is the enzyme complexed with an inhibitor at 1.55 Å (Jude et al. 2006). In addition, the structure of CA I complexed with bicarbonate, other inhibitors and activators and the structure of the so-called Michigan 1 mutant have been reported (Kumar and Kannan 1994, Ferraroni et al. 2002, Temperini et al. 2006).

The activity of CA II has also been measured for native and bacterially produced proteins, and this isozyme seems to show the highest catalytic activity with $k_{\text{cat}} = 1.4 \times 10^6 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{M}} = 1.5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ (Khalifah 1971, Nishimori et al. 2007a). CA II was the first isozyme whose three-dimensional structure was solved in 1972. Although it was done 36 years ago, a high 2.0 Å resolution was reached (Liljas et al. 1972). Since then, numerous structures of CA II and its mutants, as well as complexes with inhibitors, have been reported, some of them at very high resolution.

CA III is an isozyme possessing the lowest catalytic activity, and, as already mentioned in section 2.3.1, its physiological function is possibly not related to the CA activity at all. For bacterially produced CA III, the k_{cat} has been reported to be $1.3 \times 10^4 \text{ s}^{-1}$ (Nishimori et al. 2007b) and the $k_{\text{cat}}/k_{\text{M}} = 2.5 - 3.0 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (Tu et al. 1994, Nishimori et al. 2007b). The crystal structure has been reported for human CA III as well as for its site-directed mutants (Duda et al. 2005, Elder et al. 2007). As mentioned earlier, the two sulfhydryl groups of CA III (Cys 183 and 188) can be conjugated to the tripeptide glutathione (GSH) through a disulfide bond. In fact, the crystal structure of S-glutathiolated CA III has been obtained for native protein extracted from rat liver (Mallis et al. 2000).

CA VII is a high-activity enzyme with a $k_{\text{cat}} = 9.5 \times 10^5 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{M}} = 8.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for the human (Vullo et al. 2005b) and $k_{\text{cat}}/k_{\text{M}} = 8.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for murine enzyme (Earnhardt et al. 1998). No crystal structure has been reported for this isozyme.

CA XIII is the latest member that has been characterized, and its activity appears to be the second lowest of the mammalian isozyme family. The activity values are as follows: $k_{\text{cat}} = 1.5 \times 10^5 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{M}} = 1.1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for the human enzyme and $k_{\text{cat}} = 8.3 \times 10^4 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{M}} = 4.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ for the mouse enzyme (IV). The crystal structure has recently been solved for the human enzyme at a 1.60 Å resolution (Di Fiore et al. in press).

2.4.2 Mitochondrial and secretory isozymes (VA, VB and VI)

The mitochondrial CA isozymes VA and VB are nuclear-encoded proteins. Therefore, they contain the cleavable mitochondrial leader sequence at their N-terminus that guides their translocation to the organelle (Shah et al. 2000). The ubiquitously expressed CA VB possesses higher catalytic activity ($k_{\text{cat}} = 9.5 \times 10^5 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{M}} = 9.8 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$) than the VA isozyme ($k_{\text{cat}} = 2.9 \times 10^5 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{M}} = 2.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$) (Franchi et al. 2003, Nishimori et al. 2005a). The crystal structure has been reported for mouse CA VA, as well as for its site-directed mutants (Boriack-Sjodin et al. 1995, Heck et al. 1996, Jude et al. 2002), while the three-dimensional structures of isozyme VB or human VA have not yet been obtained.

The only secreted isozyme, CA VI, has been purified from human saliva and milk, and its molecular weight was determined to be 42 kDa. Thus, the molecular mass of CA VI is greater than that for the cytosolic enzymes; this results from the glycosylations of CA VI (Murakami and Sly 1987, Karhumaa et al. 2001b). CA VI has three potential N-linked glycosylation sites, and two of these have been reported to contain N-linked complex-type glycans; but no O-linked glycosylations have been found (Murakami and Sly 1987). CA VI is one of those rare glycoproteins that bear N-linked or O-linked oligosaccharides that are modified with β 1,4-linked N-acetylgalactosamine (GalNAc) rather than β 1,4-linked galactose (Gal) (Miller et al. 2008). In fact, it has been demonstrated for bovine CA VI that differential expression of certain sulfotransferases in parotid and submaxillary glands results in unique glycosylation patterns of CA VI (Hooper et al. 1995). For example, the glycosylation of CA VI is terminated with GalNAc-4-SO₄ in the submandibular gland and with GalNAc without sulfation in the parotid gland. Recently a carboxy-terminal sequence was discovered that targets the bovine CA VI for the addition of the GalNAc (Miller et al. 2008). Like the membrane-bound isozymes, CA VI contains a cleavable signal peptide and an intramolecular disulfide bond (Jiang et al. 1996). The catalytic activity of human CA VI produced in the bacteria is moderate, with $k_{\text{cat}} = 3.4 \times 10^5 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{M}} = 4.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Nishimori et al. 2007a). No crystal structure has been reported for the CA VI isozyme.

2.4.3 Membrane-bound isozymes (IV, IX, XII, XIV and XV)

All the characterized membrane-bound isozymes contain a signal peptide at their N-terminus that directs them to the secretory pathway in the cell. The membrane-bound isozymes IV, XII and XIV contain a disulfide bond in the same position of their three-dimensional structure, and this bond helps to stabilize a polypeptide loop in the active site containing Thr 199 (Whittington et al. 2004). The loop promotes efficient catalysis by orienting the nucleophilic zinc-bound solvent molecule through a hydrogen bonding interaction. The biochemical properties of isozymes IV, XII and XIV have been studied using protein produced in mammalian cell expression systems.

Human CA IV purified from lung or expressed in COS-7 cells has an apparent molecular mass of 35 kDa and seems to contain no N- or O-linked glycosylations (Zhu and Sly 1990, Okuyama et al. 1992). However, CA IV from other species, such as rat and cow, are N-glycosylated (Whitney and Briggles 1982, Waheed et al. 1992). CA IV has been the only membrane-bound CA isozyme reported that does not contain transmembrane domain but is linked to the membrane by a GPI-anchor. CA IV has an N-terminal signal sequence and a C-terminal hydrophobic domain; this domain is cleaved, and the GPI-anchor is then attached to the Ser 266 residue (Okuyama et al. 1995). CA IV has two intramolecular disulfide bonds, which are crucial to maintaining the enzyme in an active state (Waheed et al. 1996). In addition, the disulfide bonds enable the enzyme to maintain its catalytic activity in the presence of sodium dodecyl sulfate (SDS) detergent, a unique property among the CA isozymes (Whitney and Briggles 1982, Waheed et al. 1996). The crystal structures of human CA IV produced in COS-7 cells and mouse CA IV produced in *E. coli* have been resolved at a 2.80 Å resolution (Stams et al. 1996, Stams et al. 1998). In both proteins, the C-terminus is surrounded by electropositive surface potential, which is likely to stabilize the association of CA IV with the negatively charged phospholipid headgroups of the membrane. The activity for CO₂ hydration of human CA IV is high, with a $k_{\text{cat}} = 1.1 \times 10^6 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{M}} = 5.1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Baird et al. 1997, Stams and Christianson 2000).

The full-length CA XII produced in COS-7 cells migrates as a 43- to 44-kDa double band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which is collapsed to 39 kDa after PNGase F treatment, indicating that CA XII has two N-linked glycosylations (Türeci et al. 1998). Catalytic activity has been measured for the enzyme produced in both CHO (Chinese hamster ovary) cells and bacteria, and these have yielded quite similar values for $k_{\text{cat}} = 4.0 - 4.2 \times 10^5 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{M}} = 3.4 - 3.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, making CA XII a moderately active isozyme (Ulmasov et al. 2000, Vullo et al. 2005a). The crystal structure of the extracellular domain of CA XII produced in *E. coli* has been reported at a 1.55 Å resolution and with acetazolamide at a 1.50 Å resolution. CA XII contains one intramolecular disulfide bond and forms dimers through non-covalent interactions (Whittington et al. 2001). The transmembrane domain of CA XII contains GXXXG and GXXXS motifs that are considered to be important for the native enzyme (Whittington et al. 2001). Recently, an alternatively spliced isoform of CA XII where the GXXXG motif is missing was identified (Haapasalo et al. 2008). Therefore, the oligomerization of this isoform may be different compared to the longer form of CA XII.

In the first publications considering CA XIV, the human and mouse enzymes were produced in COS-7 cells, and the human enzyme was suggested to possess relatively low catalytic activity (Fujikawa-Adachi et al. 1999b, Mori et al. 1999). Later, the mouse enzyme produced in CHO cells indicated that this isozyme contains a very high catalytic activity; but in contrast to CA IV, it is not resistant to SDS (Whittington et al. 2004). The kinetic parameters have been reported for human CA XIV produced in a bacterial expression system, showing moderate values of $k_{\text{cat}} = 3.1 \times 10^5 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{M}} = 3.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Nishimori et al.

2005b). The extracellular domain of the murine enzyme produced in CHO cells migrates as a 44-kDa polypeptide on SDS-PAGE, and the observed increase in molecular mass compared to the theoretical one calculated from the amino acid sequence is explained by an N-linked glycosylation. The crystal structure of this truncated mouse enzyme was reported at a 2.81 Å resolution. The structure revealed a disulfide bond identical to the one characterized in isozymes IV and XII, as well as a single N-linked glycosylation site having a pentasaccharide core that is commonly found in this type of glycosylation (Whittington et al. 2004). In contrast to CAs IV and XII, CA XIV likely functions as a monomer. Another study revealed the structure of dCA II, which is an α -CA present in an exceptionally salt-tolerant unicellular green alga, *Dunaliella salina* (Premkumar et al. 2005). It was shown that mouse CA XIV and halotolerant dCA II shared several properties, such as a predominantly negative surface potential. Indeed, it was proven that murine CA XIV possesses an outstanding salt tolerance, which may be important for its function in the kidney.

CA IX, in addition to the CA domain, contains an N-terminal proteoglycan-like (PG) domain that shows significant similarity with the keratan sulfate attachment domain of the large human aggregating proteoglycan, aggrecan (Opavský et al. 1996). However, the presence of glycosaminoglycans on the PG domain of CA IX has not been confirmed experimentally. The native CA IX extracted from HeLa cells migrates as a 58/54 kDa double band on reducing SDS-PAGE and as a 153 kDa band on non-reducing SDS-PAGE (Pastoreková et al. 1992, Pastorek et al. 1994). This has indicated that CA IX is likely to form disulfide-bonded trimers. CA IX has been shown to contain an N-linked glycosylation with oligosaccharide residues containing mannose sugars (Pastorek et al. 1994). The activity of CA IX has been measured for the bacterially produced protein that contains only the CA domain of CA IX (Wingo et al. 2001). Stopped-flow spectrophotometric results indicate the enzymatic activity values to be $k_{\text{cat}} = 3.8 \times 10^5 \text{ s}^{-1}$ and $k_{\text{cat}}/k_{\text{M}} = 2.4 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, while the ^{18}O exchange method revealed a slightly higher activity with a $k_{\text{cat}}/k_{\text{M}} = 5.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. Thus, these results indicate that the activity of the CA catalytic domain is moderate. There have been no reports examining the catalytic activity of the whole extracellular part of CA IX or of enzyme that was purified from eukaryotic cells.

Mammals may possess still another CA isozyme, CA XV, the cDNA sequence of which was submitted to the National Center for Biotechnology Information (NCBI) by Hewett-Emmett and Shimmin in 2000 (GenBank[®] accession no AF231122). However, there have been no publications considering the expression, function or biochemical properties of this potential CA isozyme.

3. AIMS OF THE STUDY

The aims of this study were to

- 1) investigate whether isozyme XV would represent a new member of the mammalian CA isozyme family (II),
- 2) study the expression of CA IX and CA XV in mouse tissues (I, II),
- 3) produce recombinant human CA IX and mouse CA XV proteins and investigate their biochemical properties, such as catalytic activity and post-translational modifications (II–IV).

4. MATERIALS AND METHODS

4.1 Sequence analyses of carbonic anhydrase XV (II)

Sequence analyses were performed to identify species that possibly encode active CA XV. The analyses were performed in collaboration with Dr. Martti Tolvanen from Dr. Mauno Vihinen's group (Institute of Medical Technology, University of Tampere, Tampere, Finland). In the procedure, mouse *Car15* was used as an initial query sequence. Subsequently, BLAT (Kent 2002) searches were performed on all selected genomes using the sequences found in the previous step as new query sequences. The obtained genomic sequences were translated in three frames. The translations were aligned with known sequences, and the exon locations were identified. The gene models were confirmed and fine-tuned using GeneWise (Birney et al. 2004). Finally, the best transcripts and protein sequences were assembled manually.

The following sequences were obtained from the Ensembl Genome browser (<http://www.ensembl.org>): *Mus musculus* (ENSMUSP00000012152) and *Rattus norvegicus* (ENSRNOP00000000312). The UCSC Genome Browser (<http://genome.ucsc.edu>) showed two mRNAs for *CA15* of *Gallus gallus*; of these two, accession number BX929589 was selected and translated into protein because it was in closest accordance with the other species. For *Danio rerio*, the sequence was constructed from an EST sequence (CO960501) and manually from the genome. The sequence for *Tetraodon nigroviridis* was also obtained from the UCSC Genome Browser, Genscan Gene Prediction GSCT00001777001. The *CA15* gene of *Fugu rubripes* was constructed manually using the information from the Ensembl Genome Browser (SINFRUP00000165581 and SINFRUP00000175429). The *CA15* gene of *Canis familiaris* was constructed manually from the UCSC Genome Browser from region chr26:32,168,776–32,171,692. The CA XV amino acid sequence of *Xenopus tropicalis* was constructed manually using EST sequences (BX734706 and AL890846). The alignment for sequences from all of the species was constructed with T-Coffee version 2.11 (Notredame et al. 2000).

For *Homo sapiens* and *Pan troglodytes*, there were no mRNA or EST sequences representing *CA15* in the Ensembl Genome Browser. However, the human genome showed three copies of *CA15* located on chromosome 22q11.21 at positions 17,393,598–17,396,941, 18,860,411–18,863,739 and 20,034,808–20,038,136. These will be referred to as human *CA15* gene candidates 1, 2 and 3, respectively. The genome of *Pan troglodytes* contained two gene candidates on chromosome 23 at positions 17,310,504–17,312,161 and 19,990,225–

19,993,434. These are referred to as gene candidates 1 and 3, respectively. The second gene candidate, which should be syntenic with the human gene candidate 2, was obviously in a sequencing gap of the genome.

Phylogenetic analysis was performed by Dr. Mauno Vihinen's group and the procedure is explained in detail in (II).

4.2 Expression of carbonic anhydrase IX and XV isozymes in mouse tissues (I, II)

4.2.1 mRNA expression studies (I, II)

The reverse transcription-polymerase chain reaction (RT-PCR) method was used to reveal mouse tissues that express *Car9* and/or *Car15* mRNAs. The expression studies were performed using a commercial cDNA kit purchased from BD Biosciences (Palo Alto, CA, USA). The mouse MTC™ panel I contained first-strand cDNA preparations produced from polyadenylated RNAs isolated from twelve different murine tissues. TRIZOL® reagent (Invitrogen, Carlsbad, CA) was used to isolate mRNA from some mouse tissues that were absent from the cDNA panel or to confirm the result of the panel cDNAs. For *Car9* studies, these tissues were the stomach, gut, muscle and kidney, and, for *Car15* studies, the stomach, duodenum, jejunum, ileum, colon and blood were included. These procedures, as well as other animal procedures in the present study, were conducted according to the principles of the Declaration of Helsinki and approved by the institutional animal care committee (University of Tampere, Tampere, Finland). Reverse transcription to cDNA was performed with Mo-MuLV reverse transcriptase (Finnzymes, Espoo, Finland) using random primers (500 µg/ml).

To study *Car9* mRNA expression in the mouse, three primer pairs were used in PCR. Sequences of the primers are listed in Table 1 (IXF1/IXR1, IXF2/IXR2 and IXF3/IXR3). The primer pair used to study the expression of *Car15* can be also found in Table 1 (XVF1/XVR1). The control PCR reaction was performed with primers for mouse β-actin (BACTF1/BACTR1). The primers were from Sigma Genosys (Cambridge, UK), and all of the other reagents were from BD Biosciences, except for the dNTP mix, which was from Finnzymes (Espoo, Finland). Five nanograms of cDNA was used as a template in the reaction. The PCR reaction was carried out in a thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, Foster City, CA), and the protocol for *Car9* mRNA studies consisted of a 94°C denaturation step for 1 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 90 s, followed by a final extension at 72°C for 3 min. The control reaction was the same, except that the annealing temperature was 60°C and the extension lasted for 40 s. For *Car15* mRNA analysis, a touchdown protocol was performed: it consisted of a 94°C denaturation step for 1 min, followed by 3

cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 68°C for 90 s, followed by 4 cycles where the annealing temperature was 58°C, followed by 26 cycles of annealing at 56°C and a final extension at 68°C for 3 min.

The human MTC™ Panels I and II were used to study the expression of *CA15* mRNA in fifteen human tissues. Sequence analyses revealed that the human genome contains three copies of *CA15* genes that have most likely become pseudogenes. In order to study this experimentally, primers were designed for each of them (HXVF1/HXVR1, HXVF1/HXVR2, HXVF2/HXVR3), and, additionally, one primer pair was designed to recognize all of them (HXVF2/HXVR4; see Table 1). For human mRNA analysis, the PCR reaction was the same as for *Car9*, except that 33 cycles were used and the annealing temperature was 54°C.

The PCR results were analyzed using a 1.2% agarose gel containing 0.1 µg/ml ethidium bromide with a DNA standard (100 bp DNA Ladder, New England Biolabs, Beverly, MA). For *Car9* mRNA studies, the PCR products from the mouse muscle and kidney were sequenced in order to confirm the presence of the correct amplification product. The 609 and 713 bp PCR products obtained for *Car15* were sequenced in order to confirm their identity and to reveal possible nonspecific binding of primers. The PCR products were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Poole, UK). For *Car9* PCR products, the sequencing was performed in both directions using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready

Table 1. Primer sequences that were used in the RT-PCR studies of the *Car9*, *Car15* and *CA15* genes.

Primer	Sequence	Product (bp)
IXF1	5'-GCTCCAAGATTGAGATC-3'	873
IXR1	5'-TCTGCCTGCATAGTAAGA-3'	
IXF2	5'-GGAGGCCTGGCAGTTTTGGCT-3'	565
IXR2	5'-CTCCAGTTTCTGTCTCTGCC-3'	
IXF3	5'-CTGGGCGTGGATGTTCTGCC-3'	675
IXR3	5'-CGAGATTTCTTCCAAATGGGACAG-3'	
XVF1	5'-TACCTGGTGCTACGACTC-3'	609
XVR1	5'-TATCGGTAGTACCGCAAG-3'	
BACTF1	5'-GTTGGCATAGAGGTCTTTACG-3'	206
BACTR1	5'-GCCGCATCCTCTTCCCTCCCT-3'	
HXVF1	5'-TCTATAGGTGCCTGGTGC-3'	372
HXVR1	5'-GTGCATCTCCATAGCCTG-3'	
HXVF1	see above	298
HXVR2	5'-CTCTCTCCAGCATGGCAT-3'	
HXVF2	5'-CTTCCGAGGCTATGACTC-3'	380
HXVR3	5'-AGATTCACTGAGAGCCCTG-3'	
HXVF2	see above	444 (1) ^a / 416 (2) ^a /
HXVR4	5'-AGTAGCTCGAGGTGTTGG-3'	

^aThe numbers refer to human *CA15* gene candidates 1, 2 and 3.

Reactions Kit version 2.0, and an ABI PRISM Genetic Analyser instrument (310, Applied Biosystems) (I). Before sequencing, the *Car15* PCR products were ligated (T4 ligase, Invitrogen) into the pGEM-T Easy Vector System I (Promega, Madison, WI), and the vectors were transformed into TOP10 cells (Invitrogen), according to the manufacturer's instructions. The plasmids were purified using the Qiagen Spin Miniprep Kit (Hilden, Germany), and sequencing was carried out in both directions using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reactions Kit version 3.1 (Applied Biosystems) (II–IV). Sequencing was performed with an ABI PRISM Genetic Analyser 9100 instrument (Applied Biosystems).

For *Car15* mRNA, *in situ* hybridization was performed for mouse tissues as described previously (Heikinheimo et al. 1994).

4.2.2 Western blotting (I, III)

Western blot analysis was performed in order to study the expression of CA IX. Samples of the stomach, colon, duodenum, jejunum, ileum, brain, cerebellum, heart, liver, kidney, pancreas, lung, spleen, thymus, psoas muscle, testis and epididymis were obtained from adult Balb/c mice. The tissue samples were homogenized in PBS, and 50 µg of protein from each sample was analyzed by SDS-PAGE (NuPAGE 10% Bis-Tris, Invitrogen) under reducing conditions.

The separated proteins were transferred electrophoretically from the gel to polyvinylidene fluoride (PVDF) membrane (Macherey-Nagel, Düren, Germany) in a Novex XCell II blot module (Invitrogen). The membranes were blocked with cow colostrum whey (Biotop Oy, Oulu, Finland) diluted 1:10 in TBST buffer (20 mM Tris, 500 mM NaCl, 0.3% Tween-20, pH 7.5) for 25 min. The membranes were then incubated with primary antibody diluted 1:5000 in TBST buffer for 1 h and washed in TBST buffer five times for 5 min. The production of polyclonal rabbit antibody against the recombinant mouse CA IX has previously been described (Ortova Gut et al. 2002). Normal rabbit serum was used as a negative control. The secondary antibody was horseradish peroxidase (HRP)-labeled donkey anti-rabbit IgG, and the rest of the procedure was performed according to the instructions of the manufacturer of the ECL detection system (Amersham Biosciences).

4.2.3 Immunohistochemistry (I)

The expression of CA IX was investigated by immunohistochemical staining. Immunoperoxidase staining for mouse tissues was performed using the peroxidase-antiperoxidase (PAP) complex method. The polyclonal rabbit antibody against the recombinant mouse CA IX was used to detect CA IX in the tissues. Normal rabbit serum was used in the control stainings.

Tissue specimens from the brain, stomach, duodenum, jejunum, ileum, colon, liver, psoas muscle, kidney, heart, lung, pancreas, spleen, thymus, testis and

epididymis were obtained from two adult Balb/c mice. The specimens were fixed in Carnoy's fluid (absolute ethanol + chloroform + glacial acetic acid in 6:3:1 ratio) at 4°C for 20 h. The samples were then dehydrated and embedded in paraffin, and 4- μ m sections were cut and placed on microscope slides.

The immunostaining was performed according to the following protocol: (i) incubation in 3% H₂O₂ in methanol for 5 min, (ii) treatment with undiluted cow colostrum whey (Biotop) for 30 min and rinsing in PBS (phosphate buffered saline), (iii) incubation with primary antibody diluted 1:100 in 1% bovine serum albumin (BSA) in PBS, (iv) treatment with undiluted cow colostrum whey for 30 min and rinsing in PBS, (v) incubation with secondary antibody (swine anti-rabbit IgG, DAKO, Glostrup, Denmark) diluted 1:100 in 1% BSA for 1 h, (vi) incubation with PAP complex (PAP Rabbit, DAKO) diluted 1:100 in PBS for 30 min, (vii) incubation in DAB solution (6 mg of 3,3'-diaminobenzidine tetrahydrochloride [Sigma, St. Louis, MO] in 10 ml PBS plus 3.3 μ l 30% H₂O₂) for 2 min 30 s, (viii) counterstaining with Mayer's hematoxylin solution (Sigma). The sections were washed in PBS for 5 min after step (i), 3 times for 10 min after steps (iii) and (v) and four times for 5 min after step (vi). All of the incubations and washes were performed at 25°C, and all the washes were carried out in an orbital shaker. Finally, the sections were mounted in Entellan Neu (Merck, Darmstadt, Germany) and then examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany).

4.3 Production of recombinant human carbonic anhydrase IX and mouse XV isozymes using the baculovirus-insect cell expression system (III, IV)

4.3.1 Construction of recombinant baculoviruses (III, IV)

Two constructs encoding recombinant human CA IX and one construct encoding recombinant mouse CA XV were made. One CA IX construct contained the proteoglycan (PG) and CA catalytic (CA) domains (PG+CA form) and the other had only the CA domain (CA form). The amino acid sequences for human CA IX and mouse CA XV, as well as for the recombinant proteins, are shown in Figure 5 (section 5.3.2). The purpose was to construct truncated recombinant proteins targeted to the secretory pathway in the cell that could be purified directly from the insect cell medium. For this purpose, the C-terminal transmembrane domain and the intracytoplasmic tail were excluded from the recombinant CA IX proteins and the C-terminal GPI-anchor signal peptide was excluded from the recombinant CA XV sequence. Each recombinant protein contained its native signal sequence, whose codon usage had been optimized for *Spodoptera frugiperda* (Sf), eight histidines for protein purification as well as a thrombin cleavage site for polyhistidine tag removal. The cDNA sequences were constructed by SES-PCR (stepwise elongation of sequence-PCR). For cloning,

BglIII and HindIII restriction enzyme sites were added to the 5' and 3' ends, respectively. The PCR reactions were carried out using Phusion polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's instructions. The PCR program used to obtain the first CA9 PCR product as well as the product encoding the PG+CA form of CA IX consisted of a 98°C denaturation step for 2 min, followed by 33 cycles of denaturation at 98°C for 10 s, annealing at 65°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 8 min. In the first PCR reaction (P1), the template was the full-length human CA9 cDNA in the pOTB7 vector (IMAGE Clone 4865275, Geneservice Ltd, Cambridge, UK). Table 2 provides the primer sequences used in the PCR reactions, and Table 3 lists the details of the reactions that were used to construct the sequences that encode both the PG+CA and CA form of CA IX as well as CA XV. In some reactions, indicated in Table 3, 10% dimethyl sulfoxide

Table 2. Primers used in the SES-PCR reactions.

Primer	Isozyme	Sequence
F1	IX	5'-CTGCTGCTTCTGATGCCTGTCC-3'
F2	IX	5'-CACCACCATCACCACCATCACCACCTGGTGCCCCGTGGT TCCCAGAGGTTGCCCGGATGCAG-3'
F3	IX	5'-CTGTCCCTGCTGCTGCTGATGCCCGTGACCCCCAGCG TCTGCACCACCATCACCACCATCAC-3'
F4	IX	5'-CCCCTGCTGATCCCCGCTCCCGCTCCCGGTCTGACCGT GCAGCTGCTGCTGTCCCTGCTGCTGCTGATG-3'
F5	IX	5'-GGCCAGATCTATGGCTCCCCTGTGCCCTCCCCCTGGCT GCCCTGCTGATCCCCGCTCCC-3' (BglIII site underlined)
F6	IX	5'-CACCCCCAGAGTCATTGGCGCTATGGAGGC-3'
F7	IX	5'-CAGCTGCTGCTGTCCCTGCTGCTGCTGATGCCCGTGAC CCCCAGAGTCATTGGCGCTAT-3'
F8	XV	5'-ATGTGGGCCCTGGACTTCTT-3'
F9	XV	5'-CACCACCATCACCACCATCACCACCTGGTGCCCCGTGGT TCCCAGGTGGACTCCAGTGGTACCTGGTGC-3'
F10	XV	5'-TTCCTGCTGATCCAGCTGGCTGCTCAGGTGACTCCTCC GGTACCCACCACCATCACCACCATCAC-3'
F11	XV	5'-GGCCAGATCTATGTGGGCTCTGGACTTCTGCTGTCTT CCTGCTGATCCAGCTGGCT-3' (BglIII site underlined)
R1	IX	5'-CCTCTGGCTGGCTTCTCACATTCT-3'
R2	IX	5'-TTCGCCAAGCTTTTAGTCACCAGCAGCCAGGCAGGAATT CAGCTGGACT-3' (HindIII site underlined)
R3	IX	5'-ATGGTGGTGGGAACCACGGGGCACCAGAGGGAAGGAG GCCTCAATCACTCGCCATT-3'
R4	IX	5'-TTCGCCAAGCTTTTAGTGGTGGTGGTGGTGGTGGTGGT GGAACCACGGGGCACCAG-3' (HindIII site underlined)
R5	XV	5'-CTAGGGACCCTGCCAGAGTC-3'
R6	XV	5'-TTCGCCAAGCTTTTATGATCGGACGGATGCTTCGGGAGA GGCTGATATC-3' (HindIII site underlined)

Table 3. Details of SES-PCR reactions.

Protein	Reaction	Template	Primers	Annealing T (°C)	DMSO
CA IX	P1	Vector	F1+R1	65	-
CA IX (PG+CA)	P2	P1	F2+R2	50	-
CA IX (PG+CA)	P3	P2	F3+R2	55	-
CA IX (PG+CA)	P4	P3	F4+R2	45	+
CA IX (PG+CA)	P5	P4	F5+R2	50	+
CA IX (CA)	P6	P1	F6+R3	50	-
CA IX (CA)	P7	P6	F7+R3	50	-
CA IX (CA)	P8	P7	F4+R3	50	+
CA IX (CA)	P9	P8	F5+R4	45	+
CA XV	P10	Vector	F8+R5	60	-
CA XV	P11	P10	F9+R6	45	-
CA XV	P12	P11	F10+R6	45	-
CA XV	P13	P12	F11+R6	45	-

(DMSO) was used as an additive. For the PG+CA form, the PCR reactions (P2–P5) were as described above. For the CA form (PCR reactions P6–P9), the initial denaturation step lasted for 2 min, the extension step for 30 s and the final extension for 7 min. For CA XV, the PCR reactions were P10–P13, as indicated in Table 3. The template for the first PCR reaction (P10) was full-length *Car15* cDNA in the pME18S-FL3 vector (IMAGE Clone 1908347, Geneservice Ltd), and the PCR reaction consisted of a 98°C denaturation step for 2 min, followed by 33 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. For PCR reactions P11–P13, the protocol consisted of a 98°C denaturation step for 4 min, followed by 33 cycles of denaturation at 98°C for 10 s, annealing at 45°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 8 min.

The recombinant baculoviruses encoding the CA IX and XV recombinant proteins were generated according to the instructions of the Bac-To-Bac[®] Baculovirus expression system (Invitrogen, Carlsbad, CA). The final PCR products were digested with BglII and HindIII enzymes, and the pFastBac1 vector was digested with BamHI and HindIII (BglII and BamHI produce the same overhangs). Ligation of the PCR products into the vector was performed using T4 ligase (Invitrogen), and then the recombinant plasmids were transformed into TOP10 cells (Invitrogen). In order to confirm the correct coding sequence for the recombinant proteins, the plasmids were sequenced as described in section 4.2.1 for *Car15* PCR product. The expression cassettes were transferred from the donor vectors (pFastBac1) to the baculovirus genomes by site-directed transposition by transforming the DH10Bac cells, which contain the baculovirus shuttle vector (bacmid) and a helper plasmid that produces the proteins needed for the transposition, with the donor vector. Transformation was performed by the following method: a streak of DH10Bac bacteria was resuspended to 100 µl of 100 mM CaCl₂ and incubated on ice for 15 min. After

the incubation, 100 ng of plasmid was added to the suspension, and the cells were incubated on ice for 30 min. Heat shock was performed at 37°C for 2 min, and then 450 µl of SOC medium (Invitrogen) was added. The cells were grown at 37°C for 4 h in an orbital shaker. The cells were centrifuged at 6000g (20°C, 1 min), resuspended to 100 µl with SOC medium and then spread on Luria-Bertani (LB) plates, which were prepared according to the Bac-To-Bac[®] instructions. Recombinant bacmid selection and transfection of the insect cells were carried out according to the manufacturer's instructions (Invitrogen).

4.3.2 Production, purification and preparation of recombinant enzymes (III, IV)

The Sf9 insect cells (derived from pupal ovarian tissue of *Spodoptera frugiperda*, Invitrogen) were maintained in HyQ SFX-Insect serum free cell culture medium (HyClone, Logan, UT) at 27°C in an orbital shaker. The cells were centrifuged (2000g, 20°C, 5 min) 72 h after infection, and the medium was collected for protein purification. The purification was performed with the Probond[™] Purification System (Invitrogen). The native binding, wash and elution buffers were made according to the manufacturer's instructions. The purification procedure per 100 ml of insect cell medium was as follows: 1000 ml of native binding buffer and 7 ml of the nickel-chelating resin were added to the medium, and the His-tagged protein was allowed to bind to the resin on a magnetic stirrer at 25°C for 1 h. The resin was washed with 500 ml of binding buffer, and the protein was eluted with elution buffer containing 250 mM imidazole.

The purified CA form of CA IX and CA XV were exchanged into 50 mM Tris-HCl, pH 7.5 (Sigma-Aldrich, St. Louis, MO) using Amicon Ultra 10 kDa cut-off centrifugal filter devices (Millipore, Carrigtwohill, Ireland). The purified PG+CA form of CA IX was exchanged into 50 mM NaH₂PO₄, 100 mM NaCl, pH 8.0 (using 30 kDa cut-off centrifugal filter devices) because the PG domain was shown to be unstable in Tris-based buffers (data not shown). To remove the His-tag from the PG+CA form, the recombinant protein was treated with 60 µl of 50% thrombin slurry (Thrombin CleanCleave KIT, Sigma-Aldrich) per 1 mg of protein with gentle shaking at 25°C for 3 h. CA XV was treated with 460 µl of 50% thrombin slurry per 1 mg of protein with gentle shaking at 25°C for 27 h. The thrombin slurry was removed from the protein solution by filtration, and the His-tag was removed by washing, using a centrifugal filter device. The His-tag could not be removed from the CA form of CA IX, although thrombin from three different manufacturers (Novagen, Sigma-Aldrich and Amersham Biosciences), a large enzyme excess and long incubation times were applied. Thus, the CA form containing the His-tag at C-terminus was used in the further analyses. Protein concentration was measured at three different dilutions by the DC Protein Assay (Bio-Rad, Hercules, CA) and the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL), and the mean value of these results was used as the final concentration.

The purified recombinant proteins were analyzed by SDS-PAGE followed by either treatment with the Colloidal Blue Staining Kit (Invitrogen) or Western blotting. SDS-PAGE was performed using 5% (stacking) and 10% (separating) polyacrylamide gels under reducing conditions according to Laemmli (Laemmli 1970). Western blot analysis was performed as described in section 4.2.2. Anti-human CA IX serum was raised by Innovagen AB (Lund, Sweden) in rabbits against the CA form of CA IX. The M75 antibody (Pastoreková et al. 1992) at a dilution of 1:200 was used to detect the PG+CA form of CA IX. The polyclonal human CA IX antibody (at a dilution of 1:1000) recognized both recombinant CA IX proteins. Mouse CA IV antibody (II) (at a dilution of 1:1000) appeared to cross-react with CA XV and was, therefore, utilized to detect the CA XV recombinant enzyme. The HRP-labeled secondary antibodies were sheep anti-mouse IgG for M75 and donkey anti-rabbit IgG for CA IX and IV polyclonal antibodies.

4.4 Biochemical characterization of human carbonic anhydrase IX and mouse XV isozymes (II–IV)

4.4.1 Post-translational modification and oligomerization studies (II, III)

The prediction of N-glycosylation sites for mouse CA XV was performed using the NetNGlyc 1.0 Server with the default parameters (<http://www.cbs.dtu.dk/services/NetNGlyc>). In order to study the subcellular localization, GPI-anchoring and glycosylation status of CA XV, the full-length mouse enzyme was expressed in COS-7 cells in the laboratory of Dr. William S. Sly (Saint Louis University School of Medicine, Saint Louis, MO). The detailed procedures of the experiments are explained in (II). The structural prediction for mouse CA XV was performed in the research group of Dr. Mauno Vihinen as described in (II).

The oligomerization of CA IX was studied using several techniques, and, apart from the thermal shift analyses, the details of these experiments are described in (III). Size exclusion chromatography (SEC) experiments were provided by Dr. Simona Maria Monti and Dr. Giuseppina De Simone (Institute of Biostructures and Bioimages, National Research Council, Naples, Italy). The SEC followed by light scattering experiment was performed by Dr. Ewa Foltastogniew (Biophysics Resource of Keck Facility at Yale University, New Haven, CT).

Thermal shift analyses combined with SDS-PAGE were performed by Dr. Lina Baranauskiene in the research group of Dr. Daumantas Matulis (Laboratory of Biothermodynamics and Drug Design, Institute of Biotechnology, Vilnius, Lithuania). Thermal shift assays were performed as reported previously (Pantoliano et al. 2001, Todd and Salemme 2003, Lo et al. 2004), using the

iCycler iQ Real Time Detection System (Bio-Rad), which was originally designed for PCR. Protein unfolding was monitored by measuring fluorescence of the solvatochromic fluorescent dye, Dapoxyl™ sulfonic acid sodium salt. A temperature increment of 1°C/min was applied. Samples (10 µl final volume) contained 10–20 µM protein, 50 mM phosphate buffer, pH 7.0, 50 mM NaCl and 100 µM Dapoxyl™ sulfonate (Invitrogen), and were covered with 2.5 µl of silicone oil (DC 200, Fluka). The reaction was performed in 96-well iCycler iQ PCR plates. In order to study the covalent oligomerization of CA IX, 7 µg of both recombinant protein samples were incubated at 25°C for 2 h, with different amounts of dithiotreitol (DTT); these samples were then analyzed in parallel by thermal shift assay and SDS-PAGE (details for SDS-PAGE described in [III]).

The post-translational modifications of CA IX recombinant enzymes were investigated by mass spectrometric analyses in the research group of Dr. Andrea Scaloni (Proteomics and Mass Spectrometry Laboratory, ISPAAM, National Research Council, Naples, Italy), and the experiments are described in (III). In addition to the enzymes produced in the baculovirus-insect cell expression system, a sample of recombinant human CA IX protein produced in the murine myeloma cell line NS0 was purchased from R&D Systems (Minneapolis, MN) and investigated using mass spectrometric techniques.

4.4.2 Carbonic anhydrase activity and inhibition assays (II–IV)

For the bacterially produced CA XV, whose production is described in detail in (II), CA activity was measured by the procedure of Maren, described previously (Maren 1960, Sundaram et al. 1986), and the activity measurements were carried out in the laboratory of Dr. William S. Sly.

For the other recombinant proteins used in the study, CO₂ hydration activities as well as inhibition constants for acetazolamide were studied using a stopped-flow instrument. The experiments were performed in the laboratory of Dr. Claudiu Supuran (Bioinorganic Chemistry Laboratory, University of Florence, Sesto Fiorentino [Florence], Italy), and the details of the experiments have been described in (III, IV). The studies were performed for all three recombinant enzymes obtained from the baculovirus-insect cell expression system and also for a CA domain of CA IX produced in the *E. coli* expression system (Zat'ovicová et al. 2003, Özensoy et al. 2005). The measurements for the CA IX recombinant proteins produced in the baculovirus-insect cell expression system were also carried out in the presence of 50 µM ZnCl₂, 50 µM MgCl₂, 50 µM CoCl₂ or 50 µM MnSO₄. In the presence of 50 µM ZnCl₂, the measurements were also performed for isozymes I, II, IV, XII, XIV and XV (III).

5. RESULTS

5.1 Sequence analyses of carbonic anhydrase XV (II)

The sequence for the mouse CA XV isozyme was deposited into the NCBI database by Drs. Hewett-Emmett and Shimmin in 2000. The aim was to explore whether CA XV would represent a new member of the CA isozyme family. Mouse CA XV was taken as an initial query for sequence analyses, which were performed in order to investigate those species that could possess an active *CA15* gene in their genome. In the genome databases, candidate genes possibly encoding a functional CA XV enzyme could be found in or constructed manually from eight species, including mouse (*Mus musculus*), rat (*Rattus norvegicus*), dog (*Canis familiaris*), chicken (*Gallus gallus*), pipid frog (*Xenopus tropicalis*) and three fish species (*Danio rerio*, *Tetraodon nigroviridis* and *Fugu rubripes*). The predicted amino acid sequences for CA XV seemed to have high sequence similarity among these species, and each protein appeared to contain the three conserved histidine residues found in all functional CAs. Therefore, the sequence analyses indicated that this enzyme is likely to possess CA catalytic activity and is not a CA-RP. The exon structure of these genes was also practically identical, reflecting the conservation of this enzyme in evolution all the way from fish to mammals. (II, Fig. 1)

Surprisingly, the human genome showed three and the chimpanzee genome two potential copies of the *CA15* gene. There was a sequencing gap in the region where the third copy of the potential gene syntenic to the human version could reside, and, therefore, chimpanzees may also have three *CA15* gene copies. However, it was concluded that all of these gene copies have become pseudogenes because they all shared the following disrupting features, each of which would alone probably make this gene non-functional (II, Fig. 2 and Supplementary Figs. 1 and 2):

- a frameshift in the beginning of exon 2 (extra GT-dinucleotide);
- a frameshift in the end of exon 3 (extra G/A nucleotide; no sequence information was available for chimpanzee gene candidate 1 at this site);
- exon 4 is split into two parts (A and B) and the beginning of the intron after exon 4A has a GA instead of the GT-dinucleotide that is essential in introns;
- an insertion (9 bp) in exon 4B that disrupts the active site;
- a GAGT-insertion in exon 5, which causes a frameshift in a region that is highly conserved in all functional CAs;

- exon 8 is split by an insertion of an AluY repeat sequence, also duplicating 17 bp of the exon sequence.

In addition, each copy also showed unique point mutations and frame shifts (II, Supplementary Fig. 1). Considering all of these disrupting features and the fact that no mRNA or EST sequences could be found in the genome databases, it became evident that humans and chimpanzees do not possess a functional CA XV. More recent analyses have revealed that *CA15* has become a pseudogene also in rhesus monkey (*Macaca mulatta*). One of the critical histidine residues is mutated from histidine to asparagine as in humans and chimpanzees, and the gene has frameshifts and other disrupting mutations. Furthermore, there seems to be a deletion that eliminates exons 5 to 7 and part of exon 8. Only the His to Asn causing point mutation and three minor point mutations are conserved between all of these primate *CA15* pseudogenes (data not shown; personal communications with Dr. Martti Tolvanen).

Phylogenetic analysis was performed for the mouse CA isozymes in order to reveal the position of CA XV in the phylogenetic tree. The results indicated that CA XV is most closely related to the extracellular, GPI-anchored CA IV isozyme (II, Fig. 3).

5.2 Expression of carbonic anhydrase IX and XV isozymes in mouse tissues (I, II)

The mouse is one of the most important model organisms in biomedical research. The expression of CA IX has not been investigated thoroughly in the mouse tissues, and the goal of this study was to fill in this gap of information. The expression of CA XV was also investigated in mice because the sequence analyses revealed that CA XV has become non-functional in humans. The expression of both isozymes was investigated at the mRNA level using RT-PCR. In addition, *in situ* hybridization was performed in mouse tissues for the *Car15* mRNA. The expression of CA IX was investigated also at the protein level using Western blotting and immunohistochemistry. In order to have experimental evidence that *CA15* is a pseudogene in humans, RT-PCR was used to investigate human tissues in order to see if any of the three human gene candidates were expressed.

The distribution of isozymes IX and XV in mouse tissues has been summarized in Table 4. It is evident that both of these isozymes have quite limited distributions in normal tissues. CA IX is mainly located in the gastrointestinal tract and CA XV is mainly expressed in the kidney.

The most intensive expression of CA IX was observed in the stomach, where it was found in the basolateral plasma membrane of the surface epithelial cells, chief cells and parietal cells. Moderate expression was found in the colon, where the signal was found in the plasma membrane of the enterocytes and the strongest reaction was confined to the surface epithelial cuff region. In the small intestine, the expression was generally weak. Interestingly, in the Western blot,

Table 4. Expression of CA IX and XV in mouse tissues.^a

Organ	CA IX			CA XV	
	RT-PCR ^b	WB ^c	IHC ^c	RT-PCR ^b	ISH ^b
Stomach	+	+++	+++	-	-
Duodenum	+	-	+	-	-
Jejunum	+	-	+	-	ND
Ileum	+	+	+	-	ND
Colon	+	++	++	-	-
Liver	+	+	-	-	-
Pancreas	ND	++	+	ND	-
Spleen	+	+/-	-	-	-
Thymus	ND	+	-	ND	ND
Lung	+	-	-	-	ND
Skeletal muscle	+	-	+/-	-	-
Heart	-	-	-	-	-
Brain	+	-	++	+/-	+
Cerebellum	ND	-	++	ND	ND
Epididymis	ND	+	+	ND	ND
Testis	-	+/-	+	+/-	ND
Kidney	+	+/-	-	+	+
7-day embryo	-	ND	ND	+/-	ND
11-day embryo	-	ND	ND	-	ND
15-day embryo	-	ND	ND	-	ND
17-day embryo	+	ND	ND	+/-	ND

^aabbreviations: WB = Western blotting, IHC = immunohistochemistry, ISH = *in situ* hybridization, ND = not done.

^bscores in RT-PCR and ISH: +, strong reaction; +/-, weak reaction; -, no reaction.

^cscores in WB and IHC: +++, strong reaction; ++, moderate reaction; +, weak reaction; +/-, very weak reaction; -, no reaction.

all of the investigated intestinal tissues showed a smaller polypeptide (~37 kDa) compared to full-length CA IX. A moderate level of expression was observed in the pancreas, where the acinar cells revealed a diffuse staining, which was probably caused by rapid protein degradation in the tissue samples. Both pancreas and stomach tissues showed an additional ~30 kDa band in the Western blot. The additional polypeptides observed in the intestinal tissues as well as in the stomach and pancreas may represent alternatively spliced isoforms of CA IX. Some neuronal axons in the brain and Purkinje cells in the cerebellum appeared to produce CA IX. Faint expression was observed in the epithelial cells of the epididymis and in mature spermatozoa located in the seminiferous tubules. In the embryos, expression was investigated only at the mRNA level, and it became prominent in the embryos at day 17. (I, Figs. 1, 2 and 4–6)

A discrepancy was observed between the mRNA and protein levels in the skeletal muscle and kidney. In both cases, RT-PCR showed a very strong band whose sequence was confirmed by DNA sequencing. In contrast, at the protein level, expression was barely detectable, and only weak immunoreactions were

observed in some fibers of the skeletal muscle. To exclude the possibility of inter-animal differences, RT-PCR and Western blot were performed in parallel from the same tissue samples, which were obtained from two mice. The results were in agreement with the earlier findings, i.e. the skeletal muscle and kidney seem to produce high levels of *Car9* mRNA that is not translated into protein. (I, Figs. 1, 2, 3 and 5)

The strongest expression of *Car15* mRNA was found in the kidney and weaker bands in the RT-PCR were observed in the brain, testes, 7-day-old embryo and 17-day-old embryo. *In situ* hybridization indicated that the expression of *Car15* mRNA is higher in the renal cortex and lower in the medulla. In the RT-PCR, the testis showed an additional band that was 104 bp longer than expected. Sequencing reactions were performed in order to confirm the correct sequence of the band observed in the kidney and to investigate the exceptional band observed in the testis. The longer PCR product in the testis appeared to represent a *Car15* splicing variant, which contains a longer second exon (with 104 nt of the following intron included). This causes a frameshift and ultimately a stop codon near the beginning of the third exon, and, therefore, this splicing variant is not able to encode a functional CA XV protein. (II, Figs. 5 and 6 and Table 1)

The possible expression of the three human *CA15* gene candidates was investigated in fifteen human tissues, including those that were observed to be positive in the mouse by RT-PCR. One primer pair should have recognized all three copies, but all the tissues remained negative with these primers. In addition, the gene-specific primers for the three candidate genes were used to investigate the possible expression in the kidney, brain and heart, and these results were also negative. Thus, the RT-PCR provided experimental confirmation for the results of the sequence analyses, indicating that *CA15* has become a pseudogene and is not expressed in humans. (II, data not shown)

5.3 Biochemical properties of human carbonic anhydrase IX and mouse XV isozymes (II–IV, unpublished results)

5.3.1 CA XV is GPI-anchored to the cell membrane (II)

Because humans were not shown to produce the CA XV isozyme, the mouse enzyme was investigated to study the biochemical properties of the newest member of the CA family. According to the phylogenetic analysis, CA XV was shown to be most closely related to the GPI-anchored isozyme IV, and, therefore, these enzymes were produced in parallel in COS-7 cells to study their subcellular localization. The results showed that both of these proteins were associated with the membrane fraction, indicating that CA XV also localizes to the cell membrane (II, Figs. 7A and 7B). Furthermore, it was found that treatment with PI-PLC (phosphoinositide-specific phospholipase C) released the

majority of the CA IV and XV proteins from the membrane, showing that both of these enzymes are GPI-anchored to the cell membrane (II, Fig. 8B). In conclusion, CA XV appears to be a second GPI-anchored member of the CA isozyme family.

5.3.2 Production of recombinant enzymes in the baculovirus-insect cell expression system (III, IV, unpublished results)

To investigate the biochemical properties of human CA IX and mouse CA XV in more detail, both of these isozymes were produced in the baculovirus-insect cell expression system. The native protein sequences as well as the produced recombinant proteins are shown in Figure 5. The figure also highlights the amino acid residues that are important in regard to the present study. For CA IX, two recombinant proteins were produced: one contained the full-length extracellular part (PG+CA form) and the other only the catalytic domain (CA form). Both proteins contained the native CA IX signal sequence, which directed the proteins to the secretory pathway but lacked the transmembrane and intracellular domains, thus causing the protein to accumulate in the cell culture medium. Also, CA XV was secreted to the cell culture medium, since the C-terminal GPI-anchoring signal was left out of the recombinant protein.

All of the recombinant isozymes were purified to homogeneity with a single purification step (CA IX: III, Fig. 2; CA XV: unpublished results, Fig. 4). The His-tag was removed from the PG+CA form of CA IX, while the tag removal failed for the CA form. For CA XV, the His-tag was successfully cleaved off (unpublished results, Fig. 4).

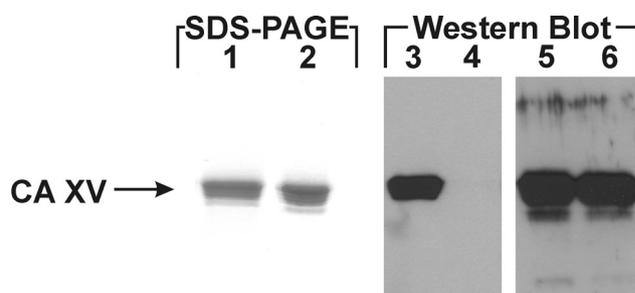


Figure 4. SDS-PAGE and Western blot of the purified recombinant CA XV protein. The coomassie-stained gel shows 2.4 μ g of protein after the His-tag removal under reducing (lane 1) and non-reducing (lane 2) conditions. 900 ng of protein was applied to the Western blot, indicating that the removal of the His-tag was successful (lanes 3 and 5 before and lanes 4 and 6 after the thrombin treatment). Lanes 3 and 4 were detected with His-tag antibody and the control lanes 5 and 6 with the CA IV antibody, which cross-reacts with CA XV.

CA IX

```

CAIX : MAPLCPSWLP1LLIPAPAPGLTVQLLSL*LLLVPH20-----QRLPRMQEDSP1LGGS*SGEDDPLGEE
CA+PG : MAPLCPSWLP1LLIPAPAPGLTVQLLSL*LLLMVPH20QRLHHHHHHHLVPRGSQRLPRMQEDSP1LGGS*SGEDDPLGEE
CA : MAPLCPSWLP1LLIPAPAPGLTVQLLSL*LLLMVPH20-----QRLPRMQEDSP1LGGS*SGEDDPLGEE

SIGNAL PEPTIDE

* 40 * 60 * 80 * 100
CAIX : DLPSEEDSPREEDPPGEEDLPGEEDLPGEEDLPV1PKPKSEEEGSLKLEDLP1VEAPGDPQEPQ*NNAHRDKEGDDQ100SHWRY
CA+PG : DLPSEEDSPREEDPPGEEDLPGEEDLPGEEDLPV1PKPKSEEEGSLKLEDLP1VEAPGDPQEPQ*NNAHRDKEGDDQ100SHWRY
CA : DLPSEEDSPREEDPPGEEDLPGEEDLPGEEDLPV1PKPKSEEEGSLKLEDLP1VEAPGDPQEPQ*NNAHRDKEGDDQ100SHWRY

PG DOMAIN

* 120 * 140 * 160 * 180
CAIX : GGDPPWPRVSPAC120AGRFQSPVDIRPQLAAFC140PALRPLELLGFQLPPLPELRLR*NNGH160SVQLTLP*PGLEMALGPGREYRAL
CA+PG : GGDPPWPRVSPAC120AGRFQSPVDIRPQLAAFC140PALRPLELLGFQLPPLPELRLR*NNGH160SVQLTLP*PGLEMALGPGREYRAL
CA : GGDPPWPRVSPAC120AGRFQSPVDIRPQLAAFC140PALRPLELLGFQLPPLPELRLR*NNGH160SVQLTLP*PGLEMALGPGREYRAL

* 200 * 220 * 240 * 260
CAIX : QL1HL1HWGAAGRPGSEHTVEGHRFP*AEI1HV1VHLSTAFARVDEALGRPGGLAVLAAFL*EEGPEENSAYEQLLSR*LREEI260ABEG
CA+PG : QL1HL1HWGAAGRPGSEHTVEGHRFP*AEI1HV1VHLSTAFARVDEALGRPGGLAVLAAFL*EEGPEENSAYEQLLSR*LREEI260ABEG
CA : QL1HL1HWGAAGRPGSEHTVEGHRFP*AEI1HV1VHLSTAFARVDEALGRPGGLAVLAAFL*EEGPEENSAYEQLLSR*LREEI260ABEG

CA DOMAIN

* 280 * 300 * 320 * 340
CAIX : SETQVPGLDISALLPSDFSR*YFQYEGSLTTPPC1CAQGV*IWTVFN1QTVMSAKQLHTLSDTLWGP*GDSRLQ*LNFRATQPLNG
CA+PG : SETQVPGLDISALLPSDFSR*YFQYEGSLTTPPC1CAQGV*IWTVFN1QTVMSAKQLHTLSDTLWGP*GDSRLQ*LNFRATQPLNG
CA : SETQVPGLDISALLPSDFSR*YFQYEGSLTTPPC1CAQGV*IWTVFN1QTVMSAKQLHTLSDTLWGP*GDSRLQ*LNFRATQPLNG

* 360 * 380 * 400 * 420
CAIX : RVIEASFPAGVDSSPRAAEPVQLNS*CLAAGDILALV*GLLFAVTSVAFLVQMR*RQHRRG*TGGVSYRPAEVAETGA
CA+PG : RVIEASFPAGVDSSPRAAEPVQLNS*CLAAGDILALV*GLLFAVTSVAFLVQMR*RQHRRG*TGGVSYRPAEVAETGA
CA : RVIEASFP1LVPRGSHHHHHHHH

TM IC

```

CA XV

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CAXV : MWALDFLLSFLLIQLAAQ-----VDS1SGTW*YDSQDPKCGPAHWKELAPAC1GGPTQSPINIDL40R
CA : MWALDFLLSFLLIQLAAQVDS1SGTHHHHHHHHLVPRGSQVDS1SGTW*YDSQDPKCGPAHWKELAPAC1GGPTQSPINIDL40R

SIGNAL PEPTIDE

* 60 * 80 * 100 * 120
CAXV : LVQRDYTLKPFIFQGYDSAPQDPVWLENDGHTVLLRVNS1Q1QNC1PAIRGAGLPSFEYRL1LQL1HF1HWGSPGHQ*SEHSLDE
CA : LVQRDYTLKPFIFQGYDSAPQDPVWLENDGHTVLLRVNS1Q1QNC1PAIRGAGLPSFEYRL1LQL1HF1HWGSPGHQ*SEHSLDE

* 140 * 160 * 180 * 200
CAXV : KHGSMEM1VMHMNTKYQSMEDARSQPDGFALAVLLVEEDRDNTN*FSAIVSGLK1NLSSPGVAVNLTSTFALASLLPSALR
CA : KHGSMEM1VMHMNTKYQSMEDARSQPDGFALAVLLVEEDRDNTN*FSAIVSGLK1NLSSPGVAVNLTSTFALASLLPSALR

CA DOMAIN

* 220 * 240 * 260 * 280
CAXV : LLRYRYSGSLTTPG1EPAVLWTVFENTVPIGHAQV1VQ1QAVLQTGPPGLHPRPLTNS*FRPQQPLGRRISASPEASVRS
CA : LLRYRYSGSLTTPG1EPAVLWTVFENTVPIGHAQV1VQ1QAVLQTGPPGLHPRPLTNS*FRPQQPLGRRISASPEASVRS

* 300
CAXV : SVSTLPCLHLALVGLGVGLRLWQGP
CA :

GPI SIGNAL SEQUENCE

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Figure 5. Sequences of human CA IX and mouse CA XV and the recombinant proteins that were used in the study. In both alignments, the histidine residues critical for CA activity are indicated in black. The glycosylation sites are shown in dark gray. The cysteine residues of the mature proteins are highlighted by light gray. For CA IX, the residues forming the intramolecular disulfide bond are underlined, and the residue forming the intermolecular disulfide bond is shown in bold (in addition, Cys 372 may also form an intermolecular disulfide bond, but this was not proven in the study). In the case of CA XV, the pairs forming the intramolecular disulfide bridges are indicated by underlining. The polyhistidine tag together with the thrombin recognition sequence is shown in italics. The numbering for CA IX is -37 relative to the SwissProt entry Q16790 and for CA XV -18 relative to the SwissProt entry Q99N23.

5.3.3 Disulfide bonds and oligomerization (II, III, unpublished results)

Thermal shift analysis is a method that has been used to investigate proteins for stability and ligand binding and has been applied previously to investigate the thermodynamic properties of CA isozymes I and II (Matulis et al. 2005). In the present study, the method was used to investigate the recombinant CA IX proteins and both of these proteins appeared to show a double transition in the assay, in contrast with the results obtained for CAs I and II. The first transition was observed approximately at 57°C for all of the investigated isozymes (I, II and IX), while for the PG+CA form, a second transition was observed at a higher temperature, approximately 74°C (unpublished results, Fig. 6). Since CA IX has been previously reported to form trimeric oligomers (Pastoreková et al. 1992), the involvement of oligomerization in the second transition was investigated. To this end, the recombinant proteins were incubated with various concentrations of DTT and subjected in parallel to SDS-PAGE under non-reducing conditions and to the thermal shift analysis (SDS-PAGE, III, Fig. 3; thermal shift analysis, unpublished results, Fig. 7). The results indicated that both of these recombinant proteins form higher oligomeric structures, in addition to monomers: the PG+CA form consisted of approximately 60% oligomers and 40% monomers and the CA form of 54% oligomers and 46% monomers. Increasing concentrations of DTT caused the disappearance of the oligomers with concomitant increase of the monomers both in the SDS-PAGE and thermal shift assay, thus indicating that monomers are held together by disulfide bonds. The molecular mass for these proteins was estimated from the SDS-PAGE, and, for the CA form, the result was clear, with the molecular masses corresponding to monomers and dimers (III, Fig. 3A). For the PG+CA form, the molecular mass of the monomer appeared to be slightly higher than expected, and the molecular mass of the oligomers corresponded roughly to that calculated for trimers (III, Figs. 3B and 4B).

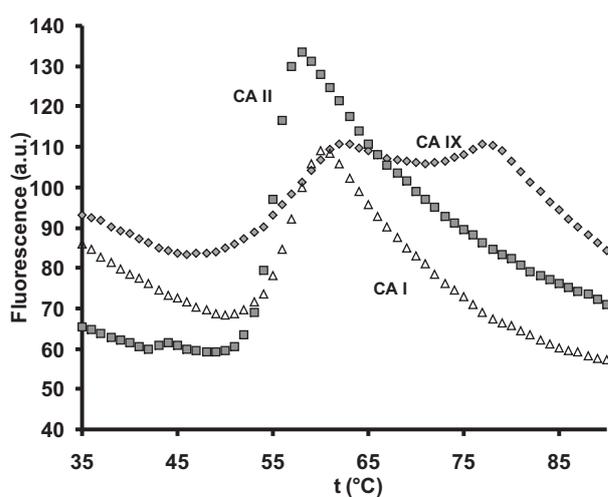


Figure 6. Thermal denaturation profiles of three carbonic anhydrase isozymes. The PG+CA form of CA IX (gray diamonds), CA I (open triangles) and CA II (gray squares). CAs I and II show a single transition, while CA IX shows two transitions.

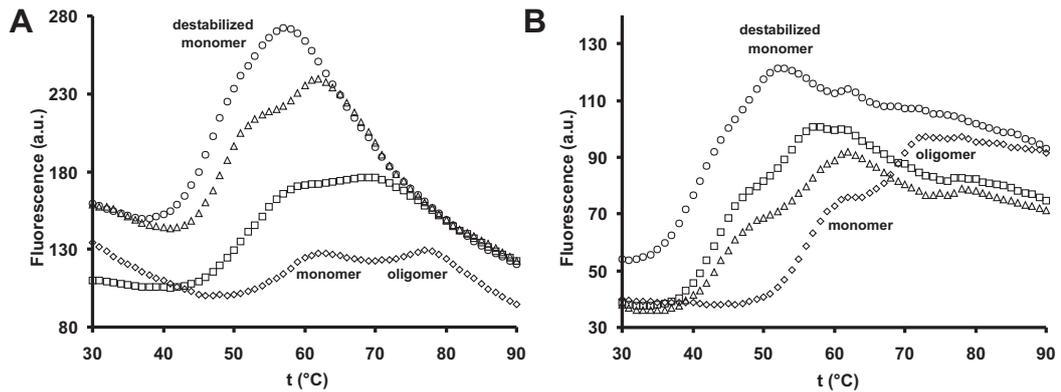


Figure 7. A and B show the thermal shift assay results for the PG+CA form and CA form, respectively. Experiments performed without, or with 0.25 mM, 1.0 mM and 10 mM DTT are represented by diamonds, squares, triangles and spheres, respectively. Equimolar amounts of recombinant proteins were applied to the assay, and the PG+CA form possessing greater molecular mass gave therefore higher fluorescence than the CA form. For both recombinant proteins, addition of DTT caused disappearance of the oligomeric transition with concomitant increase of the monomeric one. The monomeric transition in the presence of DTT occurred at lower temperature, because the reduction of the intramolecular disulfide bond destabilized the monomeric structure.

To investigate the oligomerization of CA IX further, size exclusion chromatography (SEC), was performed. For the CA form, the results of the SEC indicated that the protein exists as monomers and dimers (III, data not shown). For the PG+CA form, two peaks were also observed in the SEC. The first peak of higher molecular weight appeared to have disulfide-bonded oligomers, while the second peak did not contain disulfide-bonded protein species (III, Fig. 4). However, the molecular masses of the peaks were unexpected. The first peak showed a molecular mass of 334 kDa and the second peak a molecular mass of about 121 kDa (III, Supplementary Fig. 2). Because it was suspected that the molecular masses of the peaks of the PG+CA form were greatly exaggerated, still another approach was applied to investigate the oligomerization of the PG+CA form. SEC followed by light scattering experiments indicated clearly that the SEC exaggerated the size of the PG+CA form by several fold, and, like the CA form, the PG+CA form also exists as monomers and dimers (III, Fig. 4A). Thus, it was concluded that the PG domain of the PG+CA form causes the protein to behave unexpectedly in the SDS-PAGE and SEC experiments and that these are not suitable methods to determine the molecular mass of the full-length CA IX. In summary, when all of these results were taken together, it was concluded that both recombinant proteins existed as monomers and disulfide-bonded dimers and the dimers were shown to possess higher thermodynamic stability.

DTT was also shown to destabilize the monomeric forms of both proteins in the thermal shift assay (unpublished results, Fig. 7), and this could be explained by an intramolecular disulfide bond. Indeed, mass spectrometric analyses confirmed that both of these CA IX proteins showed a disulfide bond between residues Cys 119 and Cys 299 (III). With this result, it became clear that the intermolecular disulfide bonds formed both by the PG+CA and CA forms were

due to the presence of Cys 137 because the CA form, while missing one C-terminal cysteine present in the PG+CA form (Cys 372), still formed covalently linked dimers. The cysteines forming intra- and intermolecular disulfide bridges are highlighted in Figure 5.

Because the structure of CA XV is not available, a computer-based homology model was constructed for the murine enzyme. Among other things, the model was used to estimate the possible intra- or intermolecular disulfide bonds of the enzyme. Mouse CA XV contains seven cysteine residues, of which two are likely to form the same intramolecular disulfide bond as that characterized for CA IX (Cys 28 and Cys 217 in Fig. 5). CA IV has been reported to contain an additional stabilizing disulfide bond at the N-terminus (Waheed et al. 1996), and it is likely that the two cysteine residues found also at the N-terminus of CA XV form a disulfide bridge (Cys 8 and Cys 16 in Fig. 5). The structural prediction confirmed that the distance between the cysteine pairs is compatible with disulfide bond formation. In addition, the distance between the Cys 81 and Cys 85 residues was also shown to be compatible with the formation of a disulfide bond (II, Fig. 4C; amino acid numbering in this text according to Fig. 5). The seventh cysteine residue (Cys 288) resides in the C-terminal GPI-anchor signal sequence, which is cleaved off in the mature protein. Thus, it is likely that CA XV contains three intramolecular disulfide bonds that stabilize the structure. Moreover, the existence of the intermolecular disulfide bonds was excluded experimentally because the protein preparations from the insect cell expression system showed identical results in both reducing and non-reducing SDS-PAGE (Fig. 4). However, it is still possible that CA XV forms non-covalently linked oligomers, as it is highly similar to CA IV, which has been reported to form non-covalently linked dimers.

5.3.4 *N- and O-linked glycosylations (II, III)*

The prediction of possible N-glycosylation sites for mouse CA XV with the NetNGlyc 1.0 Server indicated that the enzyme has three potential glycosylated asparagines residues (II) (Asn 166, Asn 176 and Asn 185 in Fig. 5). The constructed homology model also showed that these residues are located on the surface of the molecule, thus supporting the prediction (II, Fig. 4D). CA XV protein produced in the COS-7 cells showed one major polypeptide of 36 kDa and three minor polypeptides of 34, 32 and 29 kDa, respectively. After EndoH treatment, all of the polypeptides collapsed to 29 kDa, thus providing experimental evidence that all of the three potential glycosylation sites may possess N-linked glycans. Parallel experiments with CA IV showed that this closely related enzyme has two N-linked glycans (II, Fig. 8A). Mass spectrometric analyses have revealed that the recombinant mouse CA XV produced by the baculovirus-insect cell expression system also possesses three N-linked glycans (unpublished results, data not shown).

The post-translational modifications of both CA IX recombinant proteins were investigated by mass spectrometric analysis. In both cases, the only

modification was an N-linked glycosylation on residue Asn 309 (see Fig. 5). Both of these recombinant proteins showed nearly identical, high-mannose type glycosylation, with a pentasaccharide core (2 x N-acetylglucosamine [GlcNAc] + 3 x mannose [Man]), and an additional 3–6 mannose residues (III, Fig. 5A).

The post-translational modifications of recombinant human CA IX produced in a mammalian expression system (murine NS0 myeloma cell line) was also analyzed by mass spectrometric analysis. Also in this protein, the N-linked glycosylation contained a pentasaccharide core which had an additional 1-4 mannose residues or GlcNAc-Gal (N-acetylglucosamine-galactose) together with 1-3 mannose residues (III, Fig. 5B). Furthermore, the protein exhibited an O-linked glycosylation on Thr78 (III, Fig. 6; Fig. 5). In this position, a keratan sulfate unit (GlcNAc-Gal), together with N-acetyl-neuraminic acid (NeuAc) and N-glycolyl-neuraminic acid (NeuGc)-containing glycans, was observed. The mass spectrometric analyses showed a complex pattern of peaks that may result either from real glycopeptide heterogeneity or the well-known partial loss of carbohydrate groups, especially NeuAc and NeuGc, during the MALDI-TOF-MS analysis (Holland et al. 2004). Satellite signals ($\Delta m = +80$ Da) were also detected in the spectra, and they are likely to represent O-linked glycans bearing a sulfate moiety (III, Fig. 6).

5.3.5 Catalytic activity and inhibition (II–IV)

To date, the catalytic activity of CA IX has been measured only for the catalytic domain produced in *E. coli* (Wingo et al. 2001). The kinetic measurements were performed for both CA IX recombinant proteins produced in the insect cell expression system as well as for the bacterially produced CA IX catalytic domain (Table 5). It was found that the catalytic domain produced in *E. coli* and Sf9 cells showed nearly identical values, both in catalytic activity and inhibition with acetazolamide (AZA). However, the catalytic activity of the PG+CA recombinant protein was three times higher, showing a k_{cat}/K_M value identical to that measured for CA II, which has been thought to possess the highest catalytic activity of the CA isozymes (Khalifah 1971, Nishimori et al. 2007a). In addition, AZA seemed to more effectively inhibit the PG+CA form.

Because it was not known initially whether the insect cell preparations contained enough Zn^{2+} to occupy all of the active sites of the recombinant proteins, 50 μM $ZnCl_2$ was added to the assay buffer. For the CA form, the activity was increased approximately ten-fold, and for the PG+CA form, more than twenty-fold (Table 5). Surprisingly, the K_I of AZA for the PG+CA form was increased considerably with the addition of extra zinc ions. However, addition of $ZnCl_2$ to the CA XV activity measurements caused an inhibition of the catalytic activity (III, Table 2). This argues against the lack of Zn^{2+} ions in the insect cell expression system. Furthermore, control experiments with isozymes I, II, IV, XII and XIV showed that addition of $ZnCl_2$ also inhibits their activity and excluded the possibility that the $ZnCl_2$ would cause some artificial acceleration of the activity in the assay (III, Table 2). Therefore, it appears that

the enhancement of activity with ZnCl₂ is a unique property of isozyme IX. The increased activity led us to study whether some other metal ions (MgCl₂, CoCl₂ and MnSO₄) could also enhance the catalytic activity (Table 5). They had no effect on the CA form (data not shown) but surprisingly seemed to activate the PG+CA form, and MnSO₄ raised the activity to the same level as addition of ZnCl₂. However, the other metal ions raised the K_I of acetazolamide only by three-fold.

For CA XV, the activity was first measured for a recombinant protein produced in *E. coli* (II, Fig. 10). The activity appeared to be 5.3 units/mg of enzyme, comparable to that of the low activity isozyme III (for comparison, the high activity isozymes II and IV show activities between 2000–3000 units/mg [Karhumaa et al. 2000b]). However, the protein produced in the baculovirus-insect cell expression system possessed moderate catalytic activity (Table 5), comparable to isozymes XII and XIV. The K_I for AZA was found to be very close to that of human CA IV (74 nM) (IV, Table 1).

Table 5. Catalytic activities and inhibition constants of acetazolamide (AZA) for human CA IX and mouse CA XV.

Enzyme	Ions	k _{cat} (s ⁻¹)	K _M (mM)	k _{cat} /k _M (M ⁻¹ s ⁻¹)	K _I AZA (nM)
IX (CA, <i>E. coli</i>)	-	3.8 × 10 ⁵	6.9	5.5 × 10 ⁷	25
IX (CA, Sf9)	-	3.8 × 10 ⁵	7.0	5.4 × 10 ⁷	24
IX (CA, Sf9)	ZnCl ₂	4.2 × 10 ⁶	7.5	5.6 × 10 ⁸	9
IX (PG+CA, Sf9)	-	1.1 × 10 ⁶	7.5	1.5 × 10 ⁸	16
IX (PG+CA, Sf9)	ZnCl ₂	2.5 × 10 ⁷	7.3	3.4 × 10 ⁹	2325
IX (PG+CA, Sf9)	MgCl ₂	1.1 × 10 ⁷	7.3	1.5 × 10 ⁹	55
IX (PG+CA, Sf9)	CoCl ₂	1.2 × 10 ⁷	7.3	1.5 × 10 ⁹	56
IX (PG+CA, Sf9)	MnSO ₄	2.5 × 10 ⁷	7.3	3.4 × 10 ⁹	41
XV (Sf9)	-	4.7 × 10 ⁵	14.2	3.3 × 10 ⁷	72

6. DISCUSSION

6.1 CA XV is not functional in humans

Mammals have been reported to possess twelve active members of the CA isozyme family: CAs I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII and XIV (Lehtonen et al. 2004). In addition to these isozymes, a sequence for another isozyme, mouse CA XV, was found in the databases. One of the main goals of the present study was to investigate whether CA XV is a functional isozyme in some species and would thus represent a new member of this protein family.

The first aim was to locate the gene (*CA15*) encoding the CA XV protein in the human genome. Surprisingly, three genes were found in the human genome that showed very high sequence identity to the mouse *Car15* gene. However, it was determined that these three genes had acquired mutations, insertions and frameshifts that destroyed their ability to encode functional CA XV enzyme. It was concluded that all three gene copies of human *CA15* have become non-processed pseudogenes.

This finding led to extensive sequence analyses in order to identify the species that possess a gene encoding functional CA XV. The strategy was to obtain the sequence for CA XV from as many species as possible; the limiting factor was the availability of genome sequences. For some species, mRNA or EST sequences could be found to support the functionality of the *CA15* gene, but for other species, the gene models had to be manually constructed. A sequence coding for a potentially active CA XV was found in eight species that belonged to different taxonomic groups: three fish species, frog, chicken, dog, mouse and rat. Only in humans and chimpanzees has the gene become a pseudogene. Additional sequencing information revealed later that a sequence for CA XV is also found in the opossum (*Monodelphis domestica*) and that CA XV is inactive in the rhesus monkey.

Therefore, it appears that CA XV was highly conserved during the evolution of vertebrates and non-primate mammals, but lost its function in primates. Since the rhesus monkey also has a *CA15* pseudogene, it appears that the inactivation of the gene occurred before the divergence of the apes and the old world monkeys. Thus, in terms of the evolutionary timescale, the inactivation of the gene has occurred quite recently. Due to the presence of deletions in the rhesus monkey genome eliminating exons 5–7 and a part of exon 8, the original disrupting mutation in the *CA15* gene cannot be determined definitively. However, since the mutation in the active site changing one of its critical

histidine residues to asparagine is shared with all the primates investigated, this mutation might well represent the first one to destroy the function of CA XV.

In conclusion, CA XV appears to be a new member of the CA isozyme family, although it is the first isozyme discovered that is not expressed in all mammals.

6.2 Expression of isozymes IX and XV in mouse tissues

6.2.1 Carbonic anhydrase IX

The expression of CA IX has been previously investigated in human tissues and in the rat alimentary tract (Pastoreková et al. 1997), but prior to the present study, there were few data on the distribution of CA IX in mouse tissues. Therefore, the purpose was to investigate the expression of CA IX also in other murine tissues than the alimentary tract.

It appears that CA IX has a very limited distribution in murine tissues. As has been observed in humans and rats, the highest expression in mice occurred in the gastric mucosa. This result is consistent with the fact that the only phenotype for CA IX knock-out mice was reported in the stomach (Ortova Gut et al. 2002). Based on this and previous results, it is evident that rodents and humans have different cellular localizations of CA IX in the colon. In the mouse, CA IX expression was observed in the surface epithelial cuff region, but in humans, the expression of CA IX increases from the surface toward the base of the crypts (Pastoreková et al. 1997). This difference in distribution may reflect different functional roles for CA IX in the two species. The small intestine showed relatively weak CA IX expression in mouse. In the present study, CA IX was also found in the pancreas, which is consistent with previous observations for human CA IX expression (Pastoreková et al. 1997). Some neuronal axons also showed a positive staining for CA IX. Recently, both CA IV and CA XIV have been reported to participate in the extracellular CA activity that is known to be involved in the alkaline shift after synaptic transmission (Shah et al. 2005), so it is possible that CA IX also contributes to this process in mice.

Interestingly, Western blot analyses revealed a strong band of approximately 37 kDa in all the intestinal tissues and a band of about 30 kDa in the stomach and pancreas. These bands could represent alternatively spliced isoforms of CA IX, since they are smaller than the expected size for the full-length protein. Recently, a splicing variant was reported for humans (Barathova et al. 2008), so it is possible that mice may also produce alternatively spliced forms of CA IX. Further studies are warranted to identify additional alternatively spliced isoforms of CA IX in humans and rodents and to understand their physiological importance.

The present study also revealed surprising results concerning the CA IX mRNA and protein levels in the kidney and skeletal muscle. These tissues

showed strong mRNA expression, while only occasional fibers in the skeletal muscle were positive, and the kidney appeared to be completely negative in the immunohistochemical staining. These results imply that, at least in mice, post-transcriptional mechanisms may exist to regulate CA IX expression. The expression of CA IX is known to be induced under hypoxic conditions (Wykoff et al. 2000), and it is conceivable that in the muscle, a lack of oxygen may occasionally occur, at least locally. This could potentially cause the upregulation of *Car9* mRNA. The possible post-transcriptional regulation of CA IX is beyond the scope of the present study, but it would be an interesting topic for future studies, especially if it represents a general regulatory mechanism that is also applicable to human CA IX.

6.2.2 Carbonic anhydrase XV

The expression of CA XV seems to be even more limited than that of CA IX. In mouse tissues, the expression of *Car15* mRNA was mainly localized in the kidney, especially in the cortex region. A positive signal was also observed in the brain. In the testis, RT-PCR results showed a faint signal for the expected PCR product and a strong signal for an additional band, which by sequencing was confirmed to be a splicing variant of *Car15* mRNA. This splicing variant appeared to contain a frameshift which creates a premature STOP codon for the encoding sequence of CA XV. Since the premature STOP codon is found at the beginning of the third exon, the splicing variant does not encode an enzyme possessing the active site. Consequently, it is difficult to interpret the biological significance of this alternatively spliced mRNA.

Since antibodies specific for mouse CA XV were not available during the present study, the cellular distribution of CA XV could not be determined by immunohistochemistry. In the Western blot analyses, an antibody for mouse CA IV, which seems to cross-react with CA XV, was used. In fact, this observation raises the possibility that the cross-reaction also occurred in the previous studies that investigated the expression of rodent CA IV. Therefore, it is possible that the previous results represent the expression of both isozymes IV and XV. This possibility has to be taken into account especially in the kidney, where the expression of CA XV seems to be highest.

In humans, the expression of any of the gene candidates for *CA15* could not be observed by RT-PCR, although several tissues, including those with a high expression of *Car15* in mice, were studied. This gave experimental confirmation to the sequence analyses results which indicated that CA XV is not produced in humans. This inter-species difference raises the interesting question of why CA XV is not necessary in humans, in spite of its high conservation during evolution. The simplest explanation is that the other human CA isozymes have filled the role of this enzyme. CA IV, for example, is a good candidate for compensating the lack of CA XV, because it is the other GPI-anchored isozyme of the family and also shows other biochemical properties similar to those of CA XV.

The inter-species difference must also be taken into account, when the results in the knock-out animals are extrapolated to human physiology. The CA IV/XIV double knock-out mice have shown that these enzymes are important for buffering the extracellular space in the brain, as well as for the retinal light response (Shah et al. 2005, Ogilvie et al. 2007). It has been reported that these isozymes are functionally redundant, and in both cases the phenotype was mainly observed only when both isozymes were missing. However, it is possible that CAs IV and XIV have even more crucial roles in human physiology, since CA XV could compensate for the loss of either or both of these isozymes in mice but not in humans. In particular, a positive signal for *Car15* mRNA was found in the brain, indicating that this isozyme may contribute to the buffering of the extracellular space.

A CA XV-specific antibody would be a valuable reagent for increasing our understanding of the roles of the different isozymes, because it would make possible detailed localization studies of CA XV in mouse tissues. Such studies could also shed light on the role of CA IV mutations in retinitis pigmentosa. There are currently two hypotheses for the association between CA IV and retinitis pigmentosa. The first hypothesis proposes that mutations in CA IV impair its function in pH regulation in the retina and causes retinitis pigmentosa. The second hypothesis proposes that it is the unfolded protein response resulting from improperly folded CA IV molecules that causes the disease. The finding that a lack of CA IV does not cause disease in the knock-out animals appears to support the second hypothesis (Ogilvie et al. 2007). However, the possible expression of CA XV in the absence of CA IV could be compensating for the loss of isozyme IV, resulting in the lack of disease phenotype in the CA IV knock-out animals. If this is the case, the first hypothesis is favored. Ultimately, CA XV single or CA IV/XV double knock-out mice are necessary for gaining a better understanding of this particular issue, as well as of the general role of these GPI-anchored enzymes in both mouse and human physiologies.

6.3 Biochemical properties of human carbonic anhydrase IX and mouse XV isozymes

6.3.1 Disulfide bonds and oligomerization

Previously, it was reported that the native CA IX from human tissues forms disulfide-bonded trimers (Pastoreková et al. 1992). In the present study, two recombinant human CA IX proteins were produced for biochemical analyses: a PG+CA form that contains both the PG and CA domains, and a CA form that contains only the CA domain. It was found that both recombinant proteins formed disulfide-bonded dimers. The CA form is unable to form covalently-linked trimers, since the protein contains only the catalytic domain of CA IX common to all CA isozymes and lacks one cysteine residue at the C-terminal part

of the CA domain. The PG+CA form, however, contains all the extracellular cysteine residues and is theoretically able to form trimers; this was supported by the results from the SDS-PAGE analyses. In SEC experiments, the molecular masses obtained for the monomers and oligomers were so surprising that another technique, light scattering, was used to further study the oligomerization of CA IX. In light scattering, the non-globular shape of a protein does not affect its molecular mass determination. This technique is considered as preferential for the absolute determination of the size of a protein and its oligomers, because a calibration curve of standard proteins is not needed (Folta-Stogniew and Williams 1999). The light scattering experiments confirmed that both the SDS-PAGE and SEC had provided biased results for the PG+CA form of CA IX, suggesting that the PG domain, which is most likely non-globular in shape, greatly affects the migration of the protein in SDS-PAGE and SEC analyses. It was also determined that the PG+CA form of CA IX is comprised of a mixture of monomers and dimers. These results raise interesting speculations about the oligomerization of CA IX. If native CA IX existed as trimers in the cells, the transmembrane domain and/or the intracytoplasmic tail would assist in the trimerization. However, it has to be taken into account that the trimerization of CA IX has previously been proven only by SDS-PAGE, which, in the present experiments, misleadingly suggested trimerization of the protein that contained the PG domain. Therefore, it is at present not possible to make any final conclusions about the oligomerization status of native CA IX *in vivo*. Further experiments are required to resolve this issue.

The intramolecular disulfide bond of CA IX seems to be conserved in all the secretory or membrane-bound isozymes, since it has been previously reported for isozymes IV, VI, XII and XIV (Jiang et al. 1996, Waheed et al. 1996, Whittington et al. 2001, Whittington et al. 2004). However, the intermolecular disulfide bonded dimerization is a unique feature of isozyme IX. A detailed x-ray crystallographic structure of isozyme IX might reveal why the covalent dimerization is required for proper function of the CA and/or PG domain. In fact, intensive efforts have been made to solve the three-dimensional structure of CA IX, but its crystallization has proven to be very challenging (data not shown).

For CA XV, the present study did not provide detailed experimental evidence regarding its disulfide bonding or oligomerization. However, the structural prediction for the mouse isozyme indicated that it is likely to possess three intramolecular disulfide bonds. Two of these bonds are in positions similar to those of isozyme IV, and the cysteines of the third pair are in close proximity to each other and thus capable of forming a bridge. All the cysteine residues are also located inside the molecule, a state consistent with the ability to form intramolecular bonds. Furthermore, the SDS-PAGE under reducing and non-reducing conditions showed only monomers of CA XV, excluding the possibility of intermolecular disulfide bonds. However, the SDS-PAGE cannot exclude the possibility that CA XV can form non-covalently linked oligomers, since the denaturing conditions in the electrophoresis would disrupt these non-covalent interactions. In fact, since CA IV forms non-covalent dimers and shares several properties with CA XV, it is probable that the latter also exists as a dimer.

6.3.2 Glycosylation

Both isozymes IX and XV were found to be N-glycosylated proteins. CA XV was predicted to contain three potential N-linked glycosylation sites, and all of these sites were experimentally confirmed to have N-linked oligosaccharides. CA IX was found to possess only one N-linked glycosylation site in the CA domain. This N-linked oligosaccharide was characterized by mass spectrometric analysis from both recombinant CA IX proteins produced in the baculovirus-insect cell expression system, as well as the protein obtained from mammalian cells. The proteins obtained from the insect cells contain high mannose-type glycan structures, while the proteins from the mammalian cells contain both high mannose and hybrid type-structures.

Other secretory or membrane-bound CA isozymes have also been reported to contain N-linked glycosylations. CAs VI and XII have been reported to have two N-linked glycosylations and CA XIV one (Murakami and Sly 1987, Türeci et al. 1998, Whittington et al. 2004). The results of the present study showed that CA IV also has two N-linked glycosylations. It is interesting to speculate about the function of these N-linked oligosaccharides, because it is quite difficult to imagine how they would have any direct effect on the catalytic activity of the CA isozymes. Nevertheless, the N-linked glycosylations may still be important for the proper formation of the membrane-bound or secretory CA isozymes. It has been observed that the N-linked glycans may have a kind of chaperone-like activity, i.e., they are important for the folding, oligomerization and stability of proteins (Mitra et al. 2006). Several studies have demonstrated that the removal of the glycan from the folded protein has no effect on its activity, but may alter its stability and folding kinetics. It has been suggested that the glycans can increase the solubility of the folding intermediates and prevent aggregation. It is possible that the N-linked glycosylations may assist in the folding of the membrane-bound CA isozymes. Moreover, together with disulfide bonds, they may provide stability for the secretory or membrane-bound enzymes that have to retain their catalytic activity in harsh environments. An extreme example is CA VI, which has been suggested to maintain its catalytic activity even in the gastric lumen (Parkkila et al. 1997).

The PG domain of CA IX produced in the mammalian cells was found to contain an O-linked glycosylation. This O-linked glycosylation contained NeuAc- and NeuGc-containing O-linked glycans with and without sulfate moieties. The detected oligosaccharides resemble the keratan sulfate unit that has previously been described in other proteins involved in cell adhesion and tumor progression (Funderburgh 2000). In CD44, which is a protein implicated in cell motility, tumor metastasis and lymphocyte activation, the O-linked glycosylation modulates the adhesion to the extracellular matrix component, hyaluronan (Takahashi et al. 1996). Interestingly, it has been observed that the structure of the O-linked glycosylation of CD44, and other proteins, changes during the malignant transformation (Maiti et al. 1998, Hakomori 2002, Gasbarri et al. 2003). Thus far, the PG domain of CA IX has been predicted only by sequence similarity, and the present study provides the first experimental evidence that the

PG domain of CA IX actually contains an O-linked glycosylation site. However, further studies are warranted to identify the proteins with which the PG domain of CA IX may interact, and its functional role in cell adhesion and proliferation. It would also be of great interest to investigate whether the structure of the O-linked glycosylation differs under certain circumstances, such as malignant transformation.

6.3.3 Catalytic activity and inhibition

To date, the catalytic activity of CA IX has been measured for only the CA domain produced in a bacterial expression system. This kind of protein was used as a reference while measuring the catalytic activity and inhibition constant value for acetazolamide (AZA) for the recombinant CA IX proteins produced in the insect cells. It was found that the catalytic domain produced in bacteria or insect cells showed practically identical results in their CO₂ hydration activity and inhibition. However, the PG+CA form of CA IX showed an activity three times higher than that of the CA form and a $k_{\text{cat}}/K_{\text{M}}$ value identical with that of CA II. The PG+CA form was also inhibited slightly more effectively by AZA. These results suggest that the PG domain of CA IX can also modulate the CO₂ hydration activity of CA IX.

Initially, it was not known whether the insect cell protein preparations contained enough Zn²⁺ ions to occupy all the active sites of the CA IX enzyme molecules. However, experiments performed with CA XV indicated that the system contains enough Zn²⁺ to fill all the active sites. Nevertheless, during the experiments, it was found that addition of ZnCl₂ increased the catalytic activity of the CA form of CA IX by ten-fold and of the PG+CA form by more than twenty-fold. Most notably, the $k_{\text{cat}}/K_{\text{M}}$ of the PG+CA form became $3.4 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, which is by far the highest value ever measured for any CA isozyme. Control experiments with other isozymes indicated that the increase in catalytic activity with ZnCl₂ is a unique feature for CA IX. The effect of other metal ions on the catalytic activity was also investigated. These metal ions did not have any effect on the CA form, whereas they increased the activity of the PG+CA form. For example, the addition of MnSO₄ raised the activity to the same level as that observed with the addition of ZnCl₂. In contrast to the other metal ions, ZnCl₂ significantly affected the K_{I} of acetazolamide for the PG+CA form.

How could these results be interpreted? First, it seems that the effect of Zn²⁺ is directed to both the CA and PG domains, because it was observed in both recombinant proteins, but more so in the PG+CA form. Second, the other metal ions clearly affect only the PG domain, since they had no effect on the activity of the CA form. One possible explanation for this behavior might be based on the fact that the PG domain contains acidic amino acid repeats, and the positively charged ions might relieve the electrostatic repulsion and thereby stabilize the PG domain, consequently stabilizing the whole protein. Our unpublished observations revealed that the PG domain is stable mainly in a phosphate-based

buffer that contains 100 mM NaCl. Changing the buffer to low-ionic Tris-HCl caused rapid degradation of the PG domain. Therefore, it seems plausible that the positively charged ions are needed to maintain the stability of the PG domain. Furthermore, it is notable that under physiological conditions, CA IX resides in the interstitial fluid, which contains a considerable amount of free ions, such as Na^+ in the order of 100 mM (Fogh-Andersen et al. 1995). Therefore, the measured activity of the PG+CA form with the metal additives may be a more accurate representation of the catalytic efficiency of this enzyme under physiological conditions. However, only detailed structural analyses of the CA and PG domains of CA IX could provide the final explanation of the results obtained in the present study.

The activity of CA XV produced in the bacteria was found to be very low and in the same range as that of CA III. However, the protein produced in the insect cells showed completely different results, indicating that the activity of CA XV is comparable to that of the membrane-bound CAs XII and XIV. This discrepancy in the results can probably be explained by the fact that the recombinant enzyme produced in the bacteria was not properly folded. It appeared to form disulfide-linked dimers, a fact which, according to the structural modeling and the protein produced in the insect cells, most likely does not reflect the physiological conditions. The protein obtained from the insect cells was purified from the media; therefore, it had passed the secretory pathway and the cellular quality control mechanisms and was likely to be properly folded. In addition, the K_I value for AZA was measured from the protein produced in the insect cells and was found to be very similar to that of human CA IV, again reflecting the similarity of these enzymes.

6.4 The mammalian CA isozyme family

Currently, the mammalian genome databases do not show sequences for any uncharacterized CA isozymes. We have performed sequence analyses to search for new α -CA isozymes in the mouse and human genomes, but have not found sequences representing novel α -CA isozymes. Thus, it seems that CA XV may be the last mammalian α -CA to be characterized. If this is the case, then evolution has produced thirteen active mammalian CA isozymes, all of which are needed in most mammals and twelve in the physiology of primates.

At the time the present study was initiated, there were two human isozymes that had missing data regarding their CO_2 hydration activity: the catalytic activity had not been measured for CA IX containing the PG domain, and there was no activity data for human isozyme XIII. In addition, CA XV was a completely uncharacterized enzyme. In the present study, the activity and inhibition data for CA IX (with and without the PG domain) and mouse CA XV were elucidated. Furthermore, these data were also obtained for human CA XIII (IV, Table 1). Based on these results, it is now possible for the first time to present the CO_2 hydration activity for all human CA isozymes (I–XIV) as well as for isozyme

XV, which is represented by the mouse isoform. These data, as well as the K_I of AZA for all the isozymes, are shown in Table 6. Figure 8 shows a schematic drawing of the subcellular localization of all the mammalian CA isozymes that have been characterized. The catalytic efficiencies of all the isozymes are illustrated in Figure 8, where the color intensity reflects the level of catalytic activity for each isozyme. The alignment containing all the sequences can be found in IV (Fig. 1).

Table 6. Catalytic activity and inhibition constant of acetazolamide (AZA) for all human CAs and mouse CA XV.

CA ^a	k_{cat} (s ⁻¹)	K_M (mM)	k_{cat}/K_M (M ⁻¹ s ⁻¹)	K_I AZA (nM)	Reference
I	2.0×10^5	4.0	5.0×10^7	250	(Khalifah 1971, Nishimori et al. 2007a)
II	1.4×10^6	9.3	1.5×10^8	12	(Khalifah 1971, Nishimori et al. 2007a)
III	1.3×10^4	52.0	$2.5 - 3.0 \times 10^5$	240000	(Tu et al. 1994, Nishimori et al. 2007b)
IV	1.1×10^6	21.5	5.1×10^7	74	(Baird et al. 1997, Stams and Christianson 2000)
VA	2.9×10^5	10.0	2.9×10^7	63	(Franchi et al. 2003)
VB	9.5×10^5	9.7	9.8×10^7	54	(Nishimori et al. 2005a)
VI	3.4×10^5	6.9	4.9×10^7	11	(Nishimori et al. 2007a)
VII	9.5×10^5	11.4	8.3×10^7	2.5	(Vullo et al. 2005b)
IX ^b	1.1×10^6	7.5	1.5×10^8	16	(III)
XII	$4.0 - 4.2 \times 10^5$	12.0	$3.4 - 3.5 \times 10^7$	5.7	(Ulmasov et al. 2000, Vullo et al. 2005a)
XIII	1.5×10^5	13.8	1.1×10^7	16	(IV)
XIV	3.1×10^5	7.9	3.9×10^7	41	(Nishimori et al. 2005b)
XV	4.7×10^5	14.2	3.3×10^7	72	(IV)

^aThe enzymes are human isozymes, except for CA XV, which is a murine isozyme.

^bThe PG+CA form, i.e. CA IX containing both the PG and CA domains.

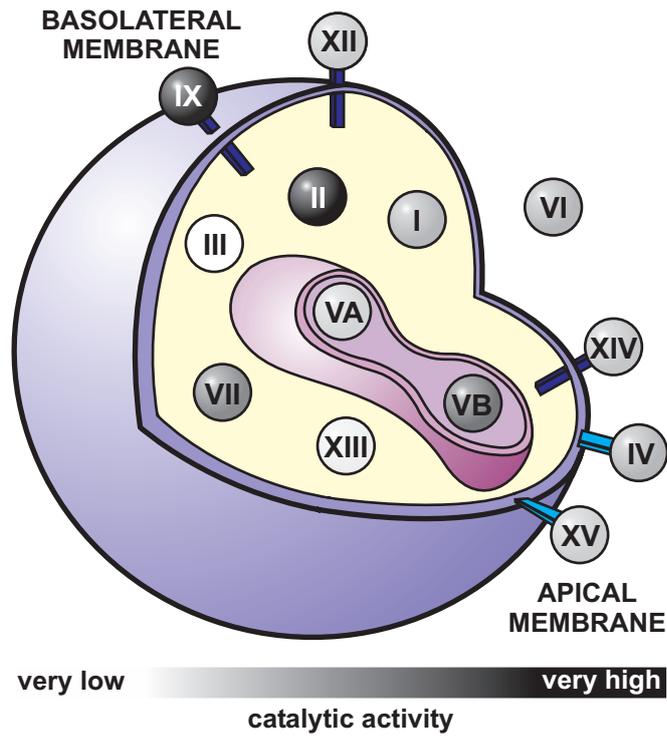


Figure 8. Subcellular localization and catalytic activity of mammalian CA isozymes. The intensity of the black color reflects the k_{cat}/K_M value of each isozyme.

7. SUMMARY AND CONCLUSIONS

In this study the expression and biochemical properties of membrane bound carbonic anhydrase isozymes IX and XV were investigated. CA XV was found to be a novel member of the mammalian α -CA isozyme family. It is the first isozyme found to be active in numerous vertebrates, but that has become inactive in the primates, such as humans and chimpanzees. The expression of CA XV and CA IX was investigated in mouse tissues. The results showed that these enzymes have a quite limited distribution in murine tissues. The most intense expression of CA IX was observed in the gastric mucosa, followed by the colonic enterocytes and pancreatic acini. The expression patterns in humans and mice seem to be quite similar, although subtle differences exist. A discrepancy was observed between mRNA and protein levels in the kidney and skeletal muscle, suggesting that the expression of CA IX may be regulated by post-transcriptional mechanisms. CA XV has an even more limited distribution, since the mRNA coding for the enzyme is mainly expressed in the kidney, especially in the cortex region. The brain, testis as well as 7-day-old and 17-day-old embryos also showed faint expression of the *Car15* mRNA. It was confirmed that humans do not express *CA15* mRNA, suggesting that some other isozyme, such as CA IV, has probably taken over the function of this protein in humans.

CA XV was found to share several biochemical properties with isozyme IV. Most notably, it is attached by a GPI-anchor to the cell membrane, as is CA IV. CA XV contains three N-linked glycosylations, and according to the structural modeling, three intramolecular disulfide bonds. The activity of the enzyme is moderate and comparable to those of isozymes XII and XIV. Two recombinant proteins were produced to investigate the biochemical properties of CA IX. It was found that both of these proteins formed monomers and disulfide-bonded dimers. Mass spectrometry experiments revealed that there is one intramolecular disulfide bond in the CA IX monomer, and the N-linked glycosylation is of the high-mannose type. Parallel experiments for the protein obtained from mammalian cells provided the first experimental evidence for an O-linked glycosylation of the PG domain. Finally, the catalytic activity of CA IX was measured for the first time for the full-length extracellular domain. The catalytic activity of this enzyme was as high as that of CA II, which has thus far been considered the most active CA isozyme. With certain metal additives, the catalytic activity became the highest ever measured for a CA isozyme. The results suggest that the PG domain may also affect the CO₂ hydration activity.

In conclusion, this work presents the characterization of CA XV, a new member of the mammalian CA isozyme family, and provides novel information on the expression and biochemical properties of CA IX.

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