FATEMEH BOOTORABI (AHMAD)

Production and Characterization of Carbonic Anhydrase VII

Acetaldehyde-derived modifications and comparison to the other cytosolic isozymes

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ACADEMIC DISSERTATION
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``*In the name of God*``

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The most important thing I have learned from science is: falling nine times and getting up ten, even though the world may write me down a failure.

To Adnan: for your support, encourage, and patience

To mom: for helping me get to where I am today

And to the loving memory of my father

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following papers, which are referred to in the text by the Roman numerals I-IV:

I Bootorabi F, Jänis J, Valjakka J, Isoniemi S, Vainiotalo P, Vullo D, Supuran CT, Waheed A, Sly WS, Niemelä O, Parkkila S. (2008): Modification of carbonic anhydrase II with acetaldehyde, the first metabolite of ethanol, leads to decreased enzyme activity. BMC Biochem 27:9-32.

II Bootorabi F, Jänis J, Smith E, Waheed A, Kukkurainen S, Hytönen V, Valjakka J, Supuran CT, Vullo D, Sly WS, Parkkila S. (2010): Analysis of a shortened form of human carbonic anhydrase VII expressed *in vitro* compared to the full-length enzyme. Biochimie 92(8):1072-80.

III Bootorabi F, Haapasalo J, Smith E, Haapasalo H, Parkkila S. (2011): Carbonic anhydrase VII – a potential prognostic marker in gliomas. Health, in press.

IV Bootorabi F, Jänis J, Hytönen V, Valjakka J, Kuuslahti M, Vullo D, Niemelä O, Supuran CT, Parkkila S. (2010): Acetaldehyde-derived modifications on cytosolic human carbonic anhydrases. Submitted for publication.

ABBREVIATIONS

ADH	Alcohol dehydrogenase
AIP	Autoimmune pancreatitis
CA	Carbonic anhydrase
Car 5	Carbonic anhydrase 5 (Gene/mRNA)
Car3	Carbonic anhydrase 3 (Gene/mRNA)
Car3$^{-/-}$	Carbonic anhydrase III knockout
CARP	Carbonic anhydrase-related protein
cDNA	Complementary deoxyribonucleic acid
CTL	Cytotoxic T lymphocytes
ClC-5	Chloride channel
DTT	Dithiothreitol
ECL	Electrochemiluminescence
E.coli	*Escherichia coli*
ER	Endoplasmic reticulum
ESI	Electrospray ionization
FA	Fatty acids
FT-ICR	Fourier transform ion cyclotron resonance
GPI	Glycosylphosphatidylinositol
GSH	Glutathione
GST	Glutathione S-transferase
HIF	Hypoxia inducible factor
IPTG	Isopropyl-b-D-thiogalactopyranoside
kDa	Kilo Dalton
L-FABP	Liver fatty-acid-binding protein
MEOS	Microsomal ethanol-oxidizing system
MG	Myasthenia gravis
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide dehydrogenase
NEM	N-ethylmaleimide
NPE	Non-pigmented ciliary epithelium
n.s.	not significant
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
RPL	Recurrent pregnancy losses
pSS	Primary Sjögren´s syndrome
PT	Proximal tubule
RA	Rheumatoid arthritis
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulfate

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SI	Staining index
SS	Sjogren's syndrome
UCTD	Undifferentiated connective tissue disease
UTR	Untranslated region

ABSTRACT

Carbonic anhydrase (CA) enzymes are ubiquitous metalloenzymes which are able to maintain acid-base homeostasis by catalyzing the reversible conversion of carbon dioxide to bicarbonate ions and protons. This family of enzymes plays an important role for the survival of the cell and whole organism by contributing to various physiological functions. The α-CA family consists of fifteen isozymes of which thirteen are expressed and have catalytically active forms in mammals. The CA isozymes have shown unique expression patterns, kinetic and inhibitory properties, as well as several different subcellular localizations: five isozymes are cytosolic, five are membrane-associated, two are mitochondrial, and one is a secretory form.

One major aim of our study was to produce and characterize human CA VII, a cytosolic isozyme. Two potential variants of CA VII were discovered based on GeneBank data. The investigations on the CA VII expression in the human cerebrum and hippocampus showed positive mRNA signals for both variants. The shorter variant turned out to be unstable and was not detectable in any murine tissues at the protein level. The full-length CA VII was expressed in the liver, colon, and muscle. In addition to certain normal tissues, CA VII was detected in gliomas. Our results indicated that CA VII expression became higher in high-grade tumors, suggesting an important role for this enzyme in tumor metabolism. This result led us to conclude that CA VII might serve as a novel potential biomarker of poor prognosis in diffuse astrocytomas.

The other main goal was to investigate and compare acetaldehyde-derived modifications in five cytosolic CAs, including CA I, II, III, VII, and XIII. Acetaldehyde, the first metabolite of ethanol, has been shown to be a toxic compound in the body, because of its capablity to form stable and unstable adducts with various proteins and cellular components. The acetaldehyde binding can result in functional and structural changes in proteins, which can further lead to harmful effects, such as autoimmune and carcinogenic processes.

Human CA II, the most active enzyme among the CA gene family, showed a significant decrease in the enzyme activity levels when treated with high concentrations of acetaldehyde. The other isozymes showed milder changes in the activity levels after acetaldehyde treatment. This difference can be explained by the unique structural features of CA II isozyme as compared to the other enzyme forms. CA II contains the highest number of reactive lysine residues on the surface, which makes it a more sensitive target for acetaldehyde binding and adduct formation.

The data obtained in the present studies will hopefully help to better understand the mechanisms of acetaldehyde adduct formation at the molecular level. This information may also deepen our insight into the pathogenesis of alcohol-related injuries in different organs.

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1. INTRODUCTION

Carbonic anhydrases (CAs) form a broad family of zinc-containing metalloenzymes which catalyze the reversible hydration of carbon dioxide $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. They participate in several physiological processes, such as the maintenance of pH homeostasis, respiration, bone resorption, gluconeogenesis, renal acidification, ion transport, and formation of cerebrospinal fluid and gastric acid (Maren 1960, Hewett-Emmett and Tashian 1996, Lehtonen et al. 2004, Parkkila and Parkkila 1996, Sly and Hu 1995). There are several evolutionally unrelated or related gene families (α, β, δ, γ, ε, and ζ). All the characterized CA enzymes in mammals belong to the α-CA family, which includes thirteen enzymatically active members: CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV, and XV (Hewett-Emmett and Tashian 1996, Hilvo et al. 2008, Supuran 2008). Most tissues of the human body contain at least one α-CA isozyme. Each isozyme is uniquely distributed with specific inhibition and activity profiles. They also have characteristic subcellular localizations: five isozymes are cytosolic, two mitochondrial, four membrane-bound, and one is a secretory form.

Although CA VII was discovered 20 years ago (Montgomery et al. 1991), it had not been thoroughly characterized. Our present studies were initiated after we discovered information on two forms of human CA VII mRNA in the GenBank. We expressed, purified and characterized human CA VII long and short form recombinant proteins. Corresponding proteins were named CA VII (the full-length enzyme, 266 residues) and the shortened form (CA VII-s, 210 residues). The CA VII enzyme is expressed in several organs, including the brain (Halmi et al. 2006, Lakkis et al. 1997), but in previous publications CA VII has never been investigated in astrocytomas, nor has it been determined in any other tumor categories. Therefore, we decided to study its expression in gliomas and to investigate its possible role as a biomarker of gliomas.

Acetaldehyde, the first metabolite of ethanol, can generate noncovalent and covalent modifications with various proteins and cellular constitutes. It is known that such modifications can lead to physiochemical changes in the properties of proteins and disturb their normal functions. Such modifications can also induce adverse immunological responses (Nicholls et al. 1992, Sorrell and Tuma 1987, Worrall et al. 1993). Since all cytosolic CAs – especially CA II – contain several lysine residues, potentially capable of binding acetaldehyde, these enzymes became interesting target proteins to structurally investigate the acetaldehyde adduct formation. First, we studied CA II, which is the most active CA isozyme, is widely expressed, and its structure has been well defined. In parallel to studies on CA VII and CA II, we cloned, expressed and purified the other cytosolic CAs, including human CA I and III. CA XIII enzyme was recently produced and characterized by our research group (Hilvo et al. 2008, Lehtonen et al. 2004). Availability of all cytosolic human CAs allowed us to continue our investigations on the generation and significance of acetaldehyde adducts on CAs. The obtained information will hopefully help to understand the mechanisms of acetaldehyde adduct formation at the molecular level. A better understanding of the molecular principles in adduct formation may further deepen our insight into the pathogenesis of alcohol-related injuries in various organs, such as the liver and brain.

2. REVIEW OF THE LITERATURE

2.1 Carbonic anhydrases

2.1.1 General aspects of carbonic anhydrase isozymes

Carbonic anhydrases are zinc-containing metalloenzymes that catalyse the reversible hydration of carbon dioxide, $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. CAs were first identified in the late 1920s when a red blood cell substance was found to catalyze the interconversion of carbon dioxide and bicarbonate in haemolyzed blood (Henriques 1928). Some years later this substance was purified from red blood cells, found to be an enzyme, and named carbonic anhydrase (Carter 1972, Edsall 1968, Meldrum and Roughton 1933). The CAs consist of α, β, δ, γ, ε, and ζ gene families, which were originally considered evolutionary unrelated (Hewett-Emmett and Tashian 1996). α-CAs are expressed in the animal kingdom, plants, green algae (Chlamydomonas), some eubacteria and also in some viruses (Chegwidden and Carter 2000, Hewett-Emmett and Tashian 1996). β-CAs have been described in plants, eubacteria and algae, and the γ-CAs in archae, some eubacteria and plants. The plant model organism, Arabidopsis, has homologues of all three families (Hewett-Emmett and Tashian 1996). The CAs are ubiquitous enzymes which have distinct cellular localizations, tissue distributions and functions in living systems (Table 1, Fig. 1), with remarkable biological functions. It is conceivable that CAs are present in all living organisms and they have been placed among the most efficient enzymes known (Tashian 1989, Zimmerman and Ferry 2008). α-CAs are involved in several biological processes, such as regulation of pH homeostasis, respiration, gluconeogenesis, ion transport, bone resorption, renal acidification, and formation of cerebrospinal fluid and gastric acid. So far, 13 active isozymes have been identified in mammals: five cytoplasmic (CA I, CA II, CA III, CA VII, and CA XIII), five membrane-associated (CA IV, CA IX, CA XII, CA XIV, and CA XV), two mitochondrial (CA VA and CA VB), and one secreted form (CA VI), (Hewett-Emmett 2000, Hewett-Emmett and Tashian 1996, Karhumaa et al. 2001b, Lehtonen et al. 2004, Parkkila et al. 1996, Sly and Hu 1995). In addition, the nuclear protein NonO/p54^{nrp} (Karhumaa et al. 2000b), which is a non-classical form, was shown to have CA activity and to be present in Leydig cells and uterine epithelium (Fig. 1). There are also three carbonic anhydrase-related proteins (CARPs), which belong to the α-CA enzyme family but lack the CA catalytic activity due to missing histidine residues in the active site cavity (Tashian et al. 2000).

Table 1. Kinetic properties and subcellular localizations of human CAs. Table has been modified from (Hilvo et al. 2008)

Isoenzyme	k_{cat} (s^{-1})	k_{cat}/k_M $(M^{-1}s^{-1})$	*Subcellular localization*
HCA I	2.0×10^5	5.0×10^7	Cytosolic
HCA II	1.4×10^6	1.5×10^8	Cytosolic
HCA III	1.3×10^4	2.5×10^5	Cytosolic
HCA IV	1.1×10^6	5.1×10^7	GPI-anchored
HCA VA	2.9×10^5	2.9×10^7	Mitochondrial
HCA VB	9.5×10^5	9.8×10^7	Mitochondrial
HCA VI	3.4×10^5	4.9×10^7	Secreted
HCA VII	9.5×10^5	8.3×10^7	Cytosolic
HCA IX	1.1×10^6	1.5×10^8	Transmembrane
HCA XII	4.2×10^5	3.5×10^7	Transmembrane
MCA XIII	1.5×10^5	1.1×10^7	Cytosolic
HCA XIV	3.1×10^5	3.9×10^7	Transmembrane
MCA XV	4.7×10^5	3.3×10^7	GPI-anchored

H=human
M=mouse

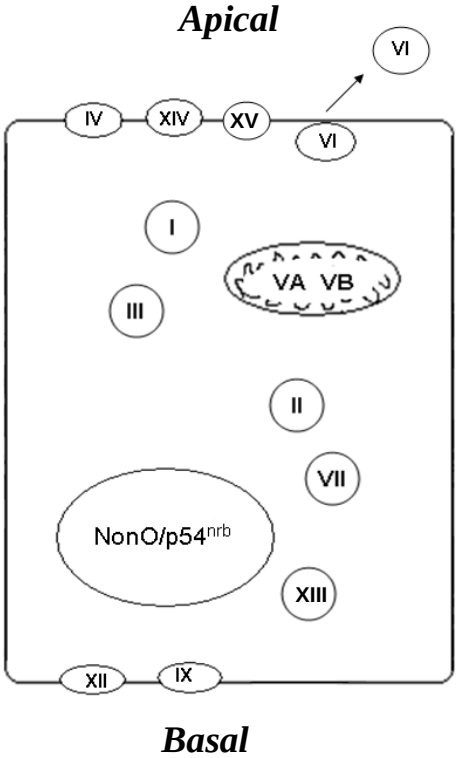

Fig. 1. Subcellular localization of the active CA isozymes in a schematic mammalian cell model. CA I, II, III, VII, and XIII are cytoplasmic isozymes, CA VA and VB are mitochondrial, CA IV, IX, XII, XIV, and XV are membrane-bound, and CA VI is secreted from the cells. NonO/p54^{nrb} is a nuclear non-classical form.

2.1.2 Cytosolic carbonic anhydrase isozymes

2.1.2.1 Carbonic anhydrase isozyme I

CA I is one of the well-characterized cytoplasmic CA isozymes and shows quite low enzymatic activity compared to several other CAs (Bundy 1977, Lindskog et al. 1984). The *CA1* gene has been cloned from a human genomic cDNA library, and its transcription seems to be controlled by two promoters, which produce erythroid and non-erythroid transcripts (Brady et al. 1991, Tashian 1989). Although CA I is one of the most abundant proteins in mammalian erythrocytes, it is not expressed in the red cells of certain species, e.g., ruminants and felids, and no hematological abnormalities have emerged in CA I deficient humans (Tashian 1992, Tashian et al. 1971, Tashian et al. 1980). Therefore, its exact physiological role still remains unclear. CA I is also expressed in the capillary and corneal endothelium and lens of eye (Wistrand et al. 1986), intestinal esophageal epithelium (Christie et al. 1997, Lonnerholm, 1985), placenta and foetal membranes (Muhlhauser et al. 1994), adipose tissue, and endocrine α-cells which are so far the only cell type in the human alimentary tract (Parkkila et al. 1994) that specifically express CA I but not CA II. A defective form of CA I was reported as a possible cause for an inherited type of renal tubular acidosis (Tashian et al. 1980), but even this phenotypic change has remained a single observation without further confirmation. So far there are no convincing reports showing phenotypic changes in CA I deficient individuals, suggesting that the other CA isozymes probably compensate for the missing CA I function. A recent study showed that several patients with recurrent pregnancy losses (RPL) had positive anti-CA I autoantibody levels as well as anti-CA II autoantibodies (Karahan et al. 2009). Although this observation was certainly very interesting, it does not provide any direct explanations for the underlying pathophysiological mechanisms in RPL.
During the last decades there has been a lot of interest in investigating the structural differences between various CAs, to see if they contributed to the different catalytic activities of the isozymes. Site-directed mutagenesis has been used to change one amino acid residue (Phe-91 to Asn) considered non-essential for catalysis of CA I. This single amino acid change led to a steady 16% increase of the CA I catalytic activity over the wild type enzyme. This effect may be due to the bigger hydrophobic pocket in the mutant enzyme compared to the wild type, which probably led to reorganization of the solvent molecules present in the cavity and to a diverse proton transfer pathway in the mutant enzyme. The mutant CA I was not only a better catalyst for the physiologic reaction, but in many cases also showed higher affinity (2.6–15.9 times) for sulfonamide/sulfamate inhibitors. (Kockar et al. 2010)

2.1.2.2 Carbonic anhydrase isozyme II

CA II, is a cytoplasmic enzyme which is distributed more widely in different tissues than the other members of the α-CA family (Parkkila 2000). It was originally found and purified from erythrocytes where it catalyzes the reversible hydration of CO_2 (Meldrum and Roughton 1933, Meldrum and Roughton 1932). The Human *CA2* gene is 17 kb long, and is located on chromosome 8 like the *CA1* and *CA3* genes, and encodes a 30 kDa protein (Nakai et al. 1987, Tashian 1989). Importantly, CA II is a highly active isozyme, and is therefore essential for several physiological functions as one of the most efficient enzymes ever known (Khalifah 1971,

Sanyal and Maren 1981, Wistrand 1981). CA II is present in almost every human tissue or organ (Parkkila 2000, Tashian 1992). In the human alimentary tract, it contributes to bicarbonate secretion in the exocrine glands, such as the salivary and pancreatic glands (Parkkila et al. 1990, Parkkila and Parkkila 1996). It is produced by squamous epithelial cells of the esophagus, where it probably protects the esophageal mucosa from the excess acidity that is occasionally leaked from the stomach through the lower esophageal sphincter (Parkkila et al. 1994). In the stomach, the surface epithelial and parietal cells express high levels of CA II. In addition to its well-defined function in the production of gastric acid by the parietal cells, it also plays a pivotal role in the maintenance of acid-base homeostasis on the surface of the gastric mucosa by catalyzing the production of bicarbonate from the surface epithelial cells (Davenport 1939, Davenport and Fisher 1938, Kumpulainen 1984, O'Brien et al. 1977, Parkkila and Parkkila 1996, Parkkila et al. 1994). CA II is also expressed in the surface epithelial cells of the colon, duodenum, jejunum, ileum, caecum, and rectum (Sasaki et al. 1993). The duodenal CA II was recently demonstrated to be necessary for prostaglandin E2-induced bicarbonate secretion (Leppilampi et al. 2005). Prostaglandin E2 is one of the major stimulants of bicarbonate secretion in the gastrointestinal canal. Its effect was completely abolished in CA II deficient mice, which was the first experimental proof of a gastrointestinal phenotype in CA II deficient mice. In the liver, CA II has been demonstrated in the hepatocytes and the epithelium of the bile ducts (Carter et al. 1989, Parkkila et al. 1994, Spicer et al. 1982). The role of CA II in the bile ducts is probably linked to production of bicarbonate for alkalization of bile. In the pancreas, immunohistochemical staining has shown an intensive positive signal for CA II in the epithelial duct cells (Kumpulainen 1984, Kumpulainen and Jalovaara 1981, Spicer et al. 1982). In addition to erythrocytes and gastrointestinal tract organs, CA II is expressed in several other tissues. In the brain, it is present in both oligodendrocytes and the choroid plexus epithelium (Cammer and Brion 2000, Kumpulainen and Korhonen 1982, Kumpulainen et al. 1985, Maren 1967, Parkkila et al. 1994). In the choroid plexus, it participates in the formation of cerebrospinal fluid. It has also been demonstrated in the human adrenal and pituitary glands (AK Parkkila et al. 1993, Parkkila and Parkkila 1996), human placenta, foetal membranes (Muhlhauser et al. 1994), reproductive tract (Ichihara et al. 1997, Kaunisto et al. 1990, Parkkila et al. 1991), and alveolar type 2 pneumocytes (Fleming et al. 1994). In the kidney, CA II is also expressed in the renal tubular cells and intercalated cells of the collective ducts, where it contributes to urinary acidification (Kumpulainen 1984, Sly and Hu 1995, Wahlstrand and Wistrand 1980, Wistrand 1980). CA II is important for the bone physiology. It is expressed in the bone resorbing cells, osteoclasts, where it catalyzes the acidification of the resorption lacuna, and therefore, stimulates the local demineralization process leading to bone resorption (Lehenkari et al. 1998, Vaananen 1984).

CA II deficiency is a rare inherited syndrome with *CA2* gene mutations as the primary defect (Sly et al. 1983, Sly and Hu 1995, Sly et al. 1985). As outlined in the previous chapter, CA II participates in the control of several important functions. Therefore, CA II deficiency can result in several clinical signs and symptoms, including osteopetrosis, renal tubular acidosis, cerebral calcification, growth failure, dental malocclusions, and mental retardation (Strisciuglio et al. 1990). Twenty-three mutations in *CA2* gene have been reported to lead to this severe and rare disease (Shah et al. 2004). Even though CA II is the most abundant isozyme in the alimentary tract, no gastrointestinal symptoms have been reported so far in CA II-deficient patients. As described in the previous chapter, a recent study with CA II-deficient mice showed that these animals lacked the duodenal bicarbonate secretory response to prostaglandin E2 (Leppilampi et

al. 2005). This finding warrants further investigations in CA II-deficient patients to determine the possible gastrointestinal phenotype in these subjects.

2.1.2.3 Carbonic anhydrase isozyme III

CA III is cytosolic 30 kDa protein which has several unique features compared to the all other α-CA isozymes. Its overall protein structure is fairly similar to CA II, but it has very low catalytic activity and is resistant to acetazolamide and several other sulfonamide inhibitors (Engberg et al. 1985, Sly and Hu 1995, Tashian 1989). CA III has a well-defined binding site for bicarbonate in spite of the low activity (Eriksson and Liljas 1993). Threonine-199 is a hydrogen bond acceptor for the zinc-bound water, and Phe-198 forms a part of the hydrophobic side of the active-site cavity of CA. The presence of Phe-198 and absence of His-65, the latter being an essential residue for the proton shuttle, are the main reasons for the low activity of CA III. Replacement of Phe-198 by site-directed mutagenesis markedly increased the CA III activity (Chen et al. 1993, LoGrasso et al. 1993, Tu et al. 1989).
CA III is highly expressed in type I fibers of the skeletal muscle (Register et al. 1978, Vaananen et al. 1985), and lower levels have also been demonstrated in both human and rat livers (Carter et al. 1989, Kelly et al. 1991). CA III is also expressed in the epithelium of the salivary gland ducts, colon, bronchi, and male genital tract as well as in adipocytes (Spicer et al. 1990, Stanton et al. 1991), Mice lacking CA III, had normal amounts and distribution of fat, despite the fact that it constitutes about 30% of the soluble protein in adipocytes (Kim et al. 2004). In addition to the tissues mentioned before, CA III has been observed in the human urinary bladder, uterus, myoephithelial cells, cardiac muscle, and lung (Jeffery et al. 1980, Vaananen and Autio-Harmainen 1987). CA III is a serum marker for rhabdomyolysis (Syrjala et al. 1990) and can be also considered a potential biomarker for other muscle damage. Serum myoglobin/CA III ratio has been suggested as a marker of reperfusion after myocardial infarction (Vaananen et al. 1990, Vuotikka et al. 2003). The first published data on *Car3*$^{-/-}$ knockout mice failed to demonstrate any functional or morphological abnormalities linked to the CA III deficiency (Kim et al. 2004). More recently, it was shown that *Car3*$^{-/-}$ mice have an impaired mitochondrial ATP synthesis that was demonstrated in gastrocnemius muscle (Liu et al. 2007). Previous studies have shown that CA III plays a role in protecting cells by functioning as an oxyradical scavenger from oxidative damage (Raisanen et al. 1999). S-Glutathiolation of CA III occurs rapidly in hepatocytes under oxidative stress, which reveals covalent adducts on Cys-183 and Cys-188 residues (Mallis et al. 2000). Electrostatic charge and steric contacts at each site of modification inversely correlate with the relative rates of reactivity of these cysteines toward glutathione (GSH). Diffuse electron density associated with the GSH adducts suggests a lack of preferred bonding interactions between CA III and the glutathionyl moieties. Therefore, the GSH adducts are available for binding by a protein capable of reducing this mixed disulfide. These properties show that CA III has an important function in the protection/recovery process from the damaging effects of oxidative agents.
Myasthenia gravis (MG) is an autoimmune disease and a recent report showed that the level of CA III is specifically insufficient in the skeletal muscle of MG patients (Du et al. 2009). Another recent study showed that CA III enzymes were detectable in the urine of ClC-5-deficient mice, a well-established model of human Dent's disease (Gailly et al. 2008). Similarly, CA III was measured in the urine of a patient with Dent's disease, and also in the cultured proximal tubule

(PT) cell lines exposed to oxidative stress, indicating that lack of PT ClC-5 in mice and men are associated with CA III induction, increased cell proliferation, and oxidative stress. Interestingly, CA III has also been observed as a novel target for autoantibodies in autoimmune diseases (Robert-Pachot et al. 2007).

2.1.2.4 Carbonic anhydrase isozyme VII

CA VII has been considered one of the most evolutionarily conserved active isozymes amongst the mammalian α-CAs (Lakkis et al. 1997). It is one of the cytosolic CAs. The *CA7* gene located on chromosome 16, was originally identified and characterized from a human genomic library (Montgomery et al. 1991). The human *CA7* gene is 10 kb long and contains seven exons and six introns (Hewett-Emmett and Tashian 1996). CA VII mRNA has been observed in the human salivary gland (Montgomery et al. 1991), mouse hippocampus and cerebellum (Lakkis et al. 1996, Lakkis et al. 1997), and rat lung (Ling B et al. 1994). The catalytic activity of human CA VII is considered moderate with k_{cat}/K_M being 8.3 x 10^7 $M^{-1}s^{-1}$ (Vullo et al. 2005). A truncation mutant of CA VII, in which 23 residues from the amino-terminal end were deleted, had its rate constant for intramolecular proton transfer decreased by an order of magnitude with no change in k_{cat}/K_M. The inhibition profile of CA VII is rather different compared to the other cytosolic isozymes, providing a possibility for the design of more selective inhibitors (Vullo et al. 2005). The recent study has pointed to CA VII as a major developmental switch that governs the electrophysiological behavior of CA1 pyramidal neurons. The major output pathway of the hippocampus under both physiological and pathophysiological conditions showed a crucial role for the developmental expression of intrapyramidal CA VII activity in regulating integrative functions, long-term plasticity and susceptibility to epileptogenesis (Ruusuvuori et al. 2004).

2.1.2.5 Carbonic anhydrase isozyme XIII

CA XIII is a cytosolic CA isozyme that was most recently identified and characterized (Lehtonen et al. 2004). It was identified in the human thymus, small intestine, spleen, prostate, ovary, colon, and testis. In the mouse, positive tissues included the spleen, lung, kidney, heart, brain, skeletal muscle, and testis. The wide expression in several tissues suggested that it probably plays an important role in pH regulation, and therefore, its inhibition by specific sulfonamides could potentially lead to novel therapeutic applications (Lehtonen et al. 2004) (Parkkila et al. 2006) Another recent study showed that CA XIII can be activated by some amino acids and amines, such as D-amino acids (His, Phe, and Trp), serotonin, and 4-(2-aminoethyl)-morpholine, whereas some L-amino acids, dopamine, histamine, and 1-(2-aminoethyl)-piperazine, were weaker activators. The first investigations on CA XIII in cancer tissues indicated that the expression of CA XIII is down-regulated in tumor cells compared to the normal tissue (Kummola et al. 2005). It was proposed that the down-regulation of cytosolic CA I, II and XIII in colorectal cancer may result from reduced levels of a common transcription factor.

2.1.3 Other carbonic anhydrases

2.1.3.1 Membrane-associated and secretory isozymes (IV, VI, IX, XII, XIV, and XV)

CA VI is the only secretory isozyme among the CA family. It was first identified in the ovine parotid gland (Fernley et al. 1979), and purified from human and rat saliva (Feldstein and Silverman 1984, Kadoya et al. 1987, Murakami and Sly 1987). The human CA VI cDNA sequence was reported 20 years ago (Aldred et al. 1991) and the amino acid sequence in sheep a little earlier (Fernley et al. 1988). Later studies suggested that CA VI secretion into saliva is under the control of the autonomic nervous system (Parkkila et al. 1995). The secretion strictly follows a circadian periodicity that is comparable to amylase secretion. The catalytic domain of CA VI is highly homologous to four other ´´extracellular`` CAs (CA IV, CA IX, CA XII, and CA XIV), which are membrane-associated proteins with an extracellular CA domain (Fujikawa-Adachi et al. 1999a, Mori et al. 1999). A recent study showed that CA VI is expressed in the mammary gland and high concentrations of CA VI can be measured in human milk. It was proposed that it may be an important factor for normal growth and development of the infant alimentary tract (Karhumaa et al. 2001b). Surprisingly, CA VI seems to play a unique role in the regulation of taste. It was shown that a taste-linked salivary factor, named gustin, is in fact identical to CA VI (Thatcher et al. 1998). Inhibition of gustin/CA VI synthesis was associated with the development of taste bud abnormalities, and consequently, with the loss of taste function (Henkin et al. 1999). The role of CA VI in saliva has turned out to be a challenging question. In contrast to many predictions, it has been shown that salivary CA VI concentration does not correlate directly with the actual pH and buffering capacity of human saliva (Kivela et al. 1997, Kivela et al. 1999). Interestingly, low salivary CA VI concentrations have been associated with increased caries prevalence, particularly in subjects with neglected oral hygiene (Kivela et al. 1999). The presence of the active CA VI enzyme in the human enamel pellicle suggests that it may accelerate the removal of acid by functioning locally in the pellicle layer on dental surfaces (Leinonen et al. 1999). Secretory CA VI levels in the saliva have been reported to be lower in patients with verified esophagitis, gastric ulcer, or duodenal ulcer than in control patients with nonacid peptic diseases (Parkkila et al. 1997). It was also demonstrated that CA VI probably retains its activity in the harsh environment of the gastric lumen. In the upper alimentary canal, CA VI may neutralize the acid by catalyzing the formation of carbon dioxide and water from protons and biocarbonate ions secreted by the epithelial cells. Based on these findings it was suggested that drugs supplemented with CA VI may prove beneficial in treating acid-peptic diseases (Parkkila et al. 1997). Another form of CA VI (type b) has been identified as a stress-inducible form, which may participate in intracellular processes induced by stress, including apoptosis (Sok et al. 1999). In tear fluid, CA VI is presumed to have a role in the maintenance of acid/base balance on the surface of the eye (Ogawa et al. 2002).

Because the previous information has been both scattered and descriptive, the recently described knockout mouse model of CA VI deficiency will be an important tool to further investigate the exact physiological roles of CA VI (Pan et al. 2011). The first report recently published showed quite minor phenotypic changes in the morphology of the upper gastrointestinal tract and salivary glands. cDNA microarray studies demonstrated that a total of 94, 56, and 127 genes were either up- or down-regulated in the submandibular gland, stomach, and duodenum of *Car6*$^{-/-}$ mice, respectively. The functional clustering of differentially expressed genes revealed a

number of altered biological processes, including e.g. catabolism, immunity, and oxidative stress.

CA IV is one of the four membrane-associated CAs, and was first identified from bovine lung microsomes (Whitney and Briggle 1982) and then from the human kidney (Wistrand and Knuuttila 1989). The *CA4* gene is located on chromosome 17 (Okuyama et al. 1993, Okuyama et al. 1992). The molecular mass of the human CA IV protein is 35 kDa, and the enzyme is anchored to the plasma membrane by a phosphatidylinositol-glycan link (Zhu and Sly 1990). Human CA IV has shown prominent location on the apical surface of the epithelial cells in the colon and also in the subepithelial capillary endothelium of all segments of the gastrointestinal canal (Fleming et al. 1995). It has been also observed in the human gallbladder and biliary epithelial cells where it probably works together with cytoplasmic CA II in acidification of the gallbladder bile (Parkkila et al. 1996). CA IV has been detected in the rat brain, skeletal muscle, and heart (Ghandour et al. 1992, Sender et al. 1994, Waheed et al. 1992). High expression of CA IV has been reported in the human male reproductive tract (Kaunisto et al. 1995, Parkkila et al. 1993). In the epididymal duct, it may facilitate reabsorption of bicarbonate from the sperm fluid, which is important for the quiescence of the sperm within this particular segment of the reproductive tract. Serum antibodies to CA II were already reported in patients with autoimmune pancreatitis (AIP) and Sjogren's syndrome (SS), but new investigation among three CAs (IV, IX, and XII) showed that CA IV may be another target antigen for autoimmune processes, that is commonly expressed in epithelial cells of specific tissues affected in idiopathic chronic pancreatitis, including AIP and its related diseases (Nishimori et al. 2005). CA IV is expressed in the endothelial cells of the choriocapillaries of the eye (Hageman et al. 1991). Interestingly, recent studies have shown that RP17 form of retinitis pigmentosa is associated with a mutation in the *CA4* gene, resulting in accumulation of unfolded protein in the endoplasmic reticulum (ER), which then leads to ER stress (Datta et al. 2009). The ER stress, in turn, induces apoptosis in the endothelial cells of the choriocapillaris and results in ischemia in the overlying retina, and finally leads to the autosomal dominant form of retinitis pigmentosa. The unfolded protein response and apoptosis in a large fraction of transfected COS-7 cells expressing the mutant but not wild-type CA IV showed further evidence that impaired pH regulation may underlie photoreceptor degeneration in RP17 (Alvarez et al. 2007, Bonapace et al. 2004, Rebello et al. 2004). In the kidney, GPI-anchored CA IV resides in the basolateral plasma membrane of the proximal tubule epithelium where it may facilitate HCO_3^- reabsorption via association with kNBC1 transporter protein (Purkerson et al. 2007). The apical CA IV is one of the luminal CAs responsible for mediating bicarbonate reabsorption in the proximal tubule and also in the thick ascending limb of Henle (Brown et al. 1990, Purkerson et al. 2007, Tsuruoka et al. 2001).

CA IX was originally described as a 54-58 kDa tumor-associated antigen named MN and observed in the cervical carcinoma cell line HeLa (Pastorekova et al. 1992). Human cDNA for the CA IX (MN) protein was cloned and sequenced by Pastorek's group (Pastorek et al. 1994), and two years later they reported a more detailed characterization of the *MN/CA9* gene, including some corrections to the original sequence published (Opavsky et al. 1996). The MN/CA IX protein molecule has been shown to contain a signal peptide, proteoglycan domain, CA domain, transmembrane segment, and short intracellular tail (Pastorek et al. 1994). Later, the protein was named CA IX, and the term MN withdrawn from the nomenclature (Hewett-Emmett and Tashian 1996). The human *CA9* gene has been mapped to chromosome 17q21 by fluorescence *in situ*

hybridization (Ivanov et al. 1998), however, radiation hybrid mapping localized it to chromosome 9p13-p12 (http://www.ncbi.nlm.nih.gov/gene/768).
CA IX has strong relevance to human oncogenesis; its expression has been identified in several human cancer cell lines, but not in normal counterparts (Zavada et al. 1993). Immunohistochemical studies have revealed a few locations of normal tissues where CA IX is present. Indeed, it has distinctive distribution pattern in normal tissues compared to the other CAs, being most intense in the basolateral surfaces of the gastric epithelial cells and the enterocytes of duodenum and jejunum (Pastorekova et al. 1997, Saarnio et al. 1998). It is also expressed in some other human gastrointestinal tissues, such as the epithelial cells of the ileum, colon, pancreatic ducts, and esophagus (Pastorekova et al. 1997). In addition to the gastrointestinal tract, CA IX is expressed in a few other tissues, including occasional cells of the uterine cervix (Liao et al. 1994, Pastorek et al. 1994).
Von Hippel Lindau disease was the first hereditary cancer syndrome where overexpression of CA IX was reported (Ivanov et al. 2001, Ivanov et al. 1998). The overexpression was found to be due to a mutant von Hippel Lindau protein, which fails to polyubiquitinate a specific transcription factor, named hypoxia inducible factor (HIF)-1α, under normoxic conditions, whereby HIF-1α escapes the normal proteosomal degradation (Maxwell 2003). HIF-1α is a key factor which can bind to the promoter region of *CA9* gene, inducing its expression (Wykoff et al. 2000).
The presence of CA IX usually indicates a poor prognosis for the patient with the exception of renal cancer (Bartosova et al. 2002, Hynninen et al. 2006, Liao et al. 1994, McKiernan et al. 1997, Vermylen et al. 1999). CA IX clearly colocalizes with regions of tumor hypoxia, and its expression is also induced in tumor cells when cultured under hypoxia (Beasley et al. 2001, Ivanov et al. 2001, Wykoff et al. 2000). Due to its hypoxic regulation, CA IX has been considered a potential biomarker not only for differential diagnostics and prognosis but also for determination of tumor hypoxia (Olive et al. 2001, Saarnio et al. 1998). Ectopic expression is an important hallmark of CA IX. Thus, CA IX is most abundant in tumors that originate from CA IX-negative tissues. Because the normal gastric mucosa contains the highest levels of CA IX among normal tissues, it was not surprising that gastric carcinomas showed relatively low expression (Leppilampi et al. 2003). However, a subgroup of gastric cancers retains CA IX expression in cancer cells at the invasion front (Chen et al. 2005). CA IX expression is strongly associated with tumor progression and indicates a poor prognosis for patients with lung adenocarcinoma, and overexpression has also been reported in several different cancers, such as breast, biliary, colon, and head and neck squamous cell carcinomas (Beasley et al. 2001, Chia et al. 2001, Hoogsteen et al. 2005, Hussain et al. 2007, Kon-no et al. 2006, Saarnio et al. 2001, Saarnio et al. 1998, Span et al. 2003, Swinson et al. 2003, Vermylen et al. 1999). Recent result showed that although prostate cancer can be a hypoxic tumor, it does not express CA IX (Smyth et al. 2010). Although the CA IX expression levels are negligible in the normal human brain, it is highly expressed in malignant gliomas (Haapasalo et al. 2006, Jarvela et al. 2008). In these tumors, the expression shows a significant correlation with tumor cell hypoxia and also correlates with poor prognosis.

CA XII is another transmembrane cancer-associated isozyme. It was cloned and characterized in 1998 when it was first identified in human renal cancer (Tureci et al. 1998). The *CA12* gene has been mapped to chromosome 15 (Ivanov et al. 1998). When the three dimensional structure of the extracellular catalytic domain of human CA XII was defined, it was found that two CA XII

monomers associate to form an isologous dimer (Whittington et al. 2001). The extracellular CA domain shows 30-42% homology with known human CAs, contains all three Zn-binding histidine residues found in active CAs, and also contains two potential sites for asparagine glycosylation. When expressed in COS-7 cells, the cDNA produced an enzymatically active 43- to 44-kDa protein in the cell membranes; this can reduce to 39 kDa by PNGase F digestion, which is consistent with the removal of two oligosccharide chains (Tureci et al. 1998). Similar to the proposed function of CA IX, CA XII may be another enzyme which can participate in acidifying the microenvironment of cancer cells, providing a mechanism for CA XII to augment tumor invasiveness (Barathova et al. 2008). The high expression of CA XII in cancer also suggests that it may serve as a potential novel target for chemotherapeutic agents (Ulmasov et al. 2000).

CA12 mRNA has been demonstrated in various tissues, including the pancreas, ovary, colon, lung, prostate, testis, kidney, and brain (Ivanov et al. 1998, Tureci et al. 1998). CA XII expression in both mouse and human endometrium suggests a role for this isozyme in reproductive physiology (Hynninen et al. 2004, Karhumaa et al. 2000a). In the female reproductive tract, CA XII may be functionally linked to the pH-dependent events that precede fertilization. As already suggested by the northern blotting results (Ivanov et al. 2001, Tureci et al. 1998), CA XII was shown to be highly expressed in the male reproductive tract (Karhumaa et al. 2001a). Co-expression of CA XII with a water channel protein, aquaporin-1, in the same efferent duct epithelial cells suggests their functional involvement in ion transport and concentration processes of testicular fluid (Karhumaa et al. 2001a). The cellular and subcellular distribution of CA XII along the rodent and human nephron and collecting duct suggests that it may be one of the key enzymes involved in normal renal physiology, particularly in the regulation of water and pH homeostasis (Kyllonen et al. 2003, Parkkila et al. 2000). In the eye, CA XII was localized primarily to the non-pigmented ciliary epithelium (NPE) with the highest expression observed during the embryonic eye development (Liao et al. 2003). NPE cells from a glaucoma patient showed a five-fold increase in the *CA12* mRNA levels, suggesting that CA XII may be involved in aqueous humour production. During the mouse embryonic development, the CA XII-positive tissues included the brain, where the most prominent staining was seen in the choroid plexus, and the stomach, pancreas, liver, and kidney (Kallio et al. 2006).

As already pointed out, CA XII is highly expressed in several cancers. Renal cell cancer was the first tumor type where overexpression of CA XII was reported, and the role in von Hippel-Lindau carcinogenesis was suggested (Parkkila et al. 2000, Tureci et al. 1998). In addition, CA XII-positive tumors include e.g. ovarian and colorectal cancers (Hynninen et al. 2006, Kivela et al. 2000). Because RNA sequence databases indicated that two isoforms of CA XII might exist in human tissues and alternatively spliced protein forms have been linked to aggressive behaviour of cancer cells, the presence of the two forms of CA XII was investigated in diffuse astrocytomas (Haapasalo et al. 2007a). Reverse transcription-polymerase chain reaction (RT-PCR) of tumor samples surprisingly revealed that CA XII present in diffuse astrocytomas is mainly encoded by a shorter mRNA variant. The expression of CA XII in astrocytomas was investigated by immunohistochemistry and the results were correlated with various clinicopathological and molecular factors. Most cases (98%) of diffusely infiltrating astrocytomas showed positive immunoreactions for CA XII. The expression correlated with poorer patient prognosis in univariate and multivariate survival analyses. From these results, it was concluded that CA XII is commonly expressed in diffuse astrocytomas, where it might

predict poor prognosis. The authors proposed that the absence of 11 amino acids in the short variant may affect the normal quaternary structure and biological function of CA XII.

CA XIV is another transmembrane isoenzyme with a molecular mass of 37 kDa. Structurally, it is closest to CA XII. Human and mouse cDNAs were reported in 1999, and the human *CA14* gene was mapped to chromosome 1 (Fujikawa-Adachi et al. 1999b, Mori et al. 1999). CA XIV was detected in the mouse kidney, and its mRNA was observed in the liver, skeletal muscle, lung, heart, and brain (Mori et al. 1999). Mouse CA XIV showed low activity and was sensitive to acetazolamide (Fujikawa-Adachi et al. 1999b). Northern blotting showed strong mRNA experession in all parts of the human brain and a weaker signal in the colon, small intestine, urinary bladder, and kidney (Fujikawa-Adachi et al. 1999b). In 2002, it was shown that luminal membrane-associated CA XIV is strongly expressed in the rodent nephron (Kaunisto et al. 2002). The distribution suggested that it is important in urinary acidification. In the same year, another study showed that CA XIV is also located in the hepatocyte plasma membrane, where it is at a strategic site to control pH regulation and ion transport between the hepatocytes, sinusoids, and bile canaliculi (Parkkila et al. 2002). CA XIV was found on neuronal membranes and axons in both the mouse and human brain (Parkkila et al. 2001). The positive areas included the hippocampus, corpus callosum, cerebellar white matter and peduncles, pyramidal tract, and choroid plexus. The distribution pattern of CA XIV led to a hypothesis that it has an important role in buffering the alkaline pH shifts that accompany neuronal activity in the brain. This hypothesis was later confirmed to be true by electrophysiological studies on *Car14*$^{-/-}$ knockout mice (Shah et al. 2005). In addition, expression of CA XIV has been described in the mouse retinal pigment epithelium (RPE), Muller cells, and astrocytes (Nagelhus et al. 2005, Ochrietor et al. 2005). CA XIV, which regulates extracellular pH and CO_2 levels, plays an important part in producing a normal retinal light response (Ogilvie et al. 2007). Skeletal muscle is also an important source of CA XIV expression (Scheibe et al. 2006, Wetzel et al. 2007). According to a recent report, the skeletal muscle plasma membrane contains several CA isozymes (Hallerdei et al. 2010). CA XIV, which is homogeneously distributed across the surface membrane of muscle fibers, plays an important role in lactic acid transport across this membrane. The first crystal structure of the extracellular domain of murine CA XIV was published at 2.8 A resolution and of an enzyme-acetazolamide complex at 2.9 A resolution (Whittington et al. 2004). The structure showed a monomeric glycoprotein with a topology similar to that of other mammalian CA isozymes.

CA XV is the most recently characterized isozyme of the α-CA family. The cDNA sequence of CA XV was submitted to the National Center for Biotechnology Information (NCBI) by Dr. David Hewett-Emmett in 2000. Recombinant mouse CA XV has shown low-activity, and it is anchored to plasma membrane via a GPI anchor in the same manner as CA IV. Importantly, CA XV is the first member of the α-CA gene family that is expressed in several species, but not in humans and chimpanzees, as it has become a non-processed pseudogene. (Hilvo et al. 2005)

2.1.3.2 Mitochondrial isozymes (VA and VB)

CA VA is a unique CA which is located in the mitochondrial matrix (Heck et al. 1994, Nagao et al. 1993). The gene for *CA5A* has been cloned from the human liver and mapped on chromosome

16, the same chromosome to which CA VII has previously been mapped (Nagao et al. 1993). Later, another mitochondrial CA was characterized (Fujikawa-Adachi et al. 1999c), and these two isozymes were termed as CA VA and VB, respectively. cDNA clones for CA VB were isolated from the human pancreas and salivary glands. CA VB was demonstrated in several organs, including the kidney, salivary glands, brain, heart, lung, spleen, intestine, testis, and spinal cord, but it was absent in the liver. CA VA mRNA expression was observed only in the liver and skeletal muscle. Different patterns of tissue-specific distribution suggest different physiological roles for the two mitochondrial isozymes. Both CA VA and CA VB are "low activity" enzymes, which are sensitive to acetazolamide (Fujikawa-Adachi et al. 1999c, Shah et al. 2000). It has been shown that the mitochondrial CA of the liver (CA VA) is involved in ureagenesis and gluconeogenesis (Dodgson 1991). The presence of CA VB in pancreatic β-cells has suggested a specific role in insulin secretion (Parkkila et al. 1998). In addition, CA VB may also participate in bicarbonate ion-induced GABA responses in the nervous system by regulating the bicarbonate homeostasis in neurons, and its inhibition could be the basis of some neurotropic effects of CA inhibitors (Ghandour et al. 2000).

2.2 Alcoholism and alcohol metabolism

Alcohol is the most frequently abused drug throughout the world (Angell and Kassirer 1994). Alcohol permeates all tissues of the body and affects most vital functions because it is a small molecule, soluble in both water and lipids (Adams et al. 1993). Until two decades ago, dietary deficiencies were considered to be the major reason why alcoholics developed liver disease. As the overall nutrition of the population improved, more emphasis was placed on secondary malnutrition. Direct hepatotoxic effects of ethanol were also established. The liver is the organ most severely affected by alcoholism. In some urban areas, cirrhosis (usually a complication of alcoholism) is the fourth most frequent cause of death amongst people between 25 to 64 years of age. (Byun and Jeong 2010, Lieber 1991, Lieber 1995)

Many of the metabolic and toxic effects of alcohol have been linked to its metabolism in the liver (Fig. 2). Ethanol is readily absorbed from the gastrointestinal tract. Only 2-10% of the absorbed alcohol is eliminated through the kidneys and lungs; the rest is oxidized in the body, principally in the liver (Lieber 2005). Except for the stomach, extrahepatic metabolism of ethanol plays a minor role (Lieber 2005). Unlike the other drugs, ethanol is a substantial source of energy, with 7.1 kcal (29.7 kJ) per gram, a value that exceeds the energy content of carbohydrates or proteins. On average, ethanol accounts for half an alcoholic's caloric intake, and therefore, displaces normal nutrients, causing malnutrition including deficiencies of folate, thiamine, and other vitamins. Secondary malnutrition also occurs through malabsorption due to gastrointestinal complications, such as pancreatic insufficiency and impaired hepatic metabolism of nutrients (Fig. 2). In addition, alcohol promotes the degradation of nutrients, as exemplified by its effects on vitamin A. Although ethanol is rich in energy, long-term consumption of up to 2000 calories per day in the form of alcohol does not produce the expected gain in body weight. The extent to which ethanol becomes the preferred fuel for the total body has been demonstrated in humans: it decreased total body fat oxidation by 79% and protein oxidation by 39%, and almost completely abolished the 249% rise in carbohydrate oxidation seen after glucose infusion. This deficit can be attributed, in part, to the poor yield of energy produced from fat oxidation in damaged

mitochondria and to microsomal pathways that oxidize ethanol without conserving chemical energy (Lieber 1995, Lieber 2005).

Oxidation of ethanol through the alcohol dehydrogenase pathway produces acetaldehyde, which is converted to acetate; both reactions reduce nicotinamide adenine dinucleotide (NAD) to NADH (Fig. 3). Excess NADH causes a number of metabolic disorders (Lieber 1992), including hyperlactacidemia, which contributes to acidosis and reduces urinary excretion of uric acid. This latter abnormality leads to secondary hyperuricemia, which is aggravated by alcohol-induced ketosis and the acetate-mediated breakdown of ATP and generation of purines. NADH also opposes gluconeogenesis (thereby favoring hypoglycemia), raises glycerophosphate levels, and inhibits the Krebs cycle and fatty-acid oxidation. The inhibition of fatty-acid oxidation favors steatosis and hyperlipidemia involving all lipid classes, including high-density lipoprotein (HDL). The acetaldehyde produced by the oxidation of ethanol also has toxic effects. It inhibits the repair of alkylated nucleoproteins (Espina et al. 1988), decreases the activity of key enzymes, and markedly reduces oxygen utilization in mitochondria damaged by long-term ethanol consumption (Lieber et al. 1989). Moreover, acetaldehyde promotes cell death by depleting the level of reduced glutathione, inducing lipid peroxidation, and increasing the toxic effect of free radicals. By binding to the tubulin of microtubules, acetaldehyde blocks the secretion of proteins. The increases in protein, lipid, water, and electrolyte contents cause hepatocytes to enlarge, which is a histopathological hallmark of alcoholic liver disease (Wondergem and Davis 1994). Acetaldehyde–protein adducts promote collagen production and may also act as neoantigens, which stimulate an immune response (Hoerner et al. 1986, Niemela et al. 1987). Acetaldehyde crosses the placenta, impairs fetal DNA methylation, and contributes to the fetal alcohol syndrome - the most preventable congenital abnormality (Abel and Sokol 1991).

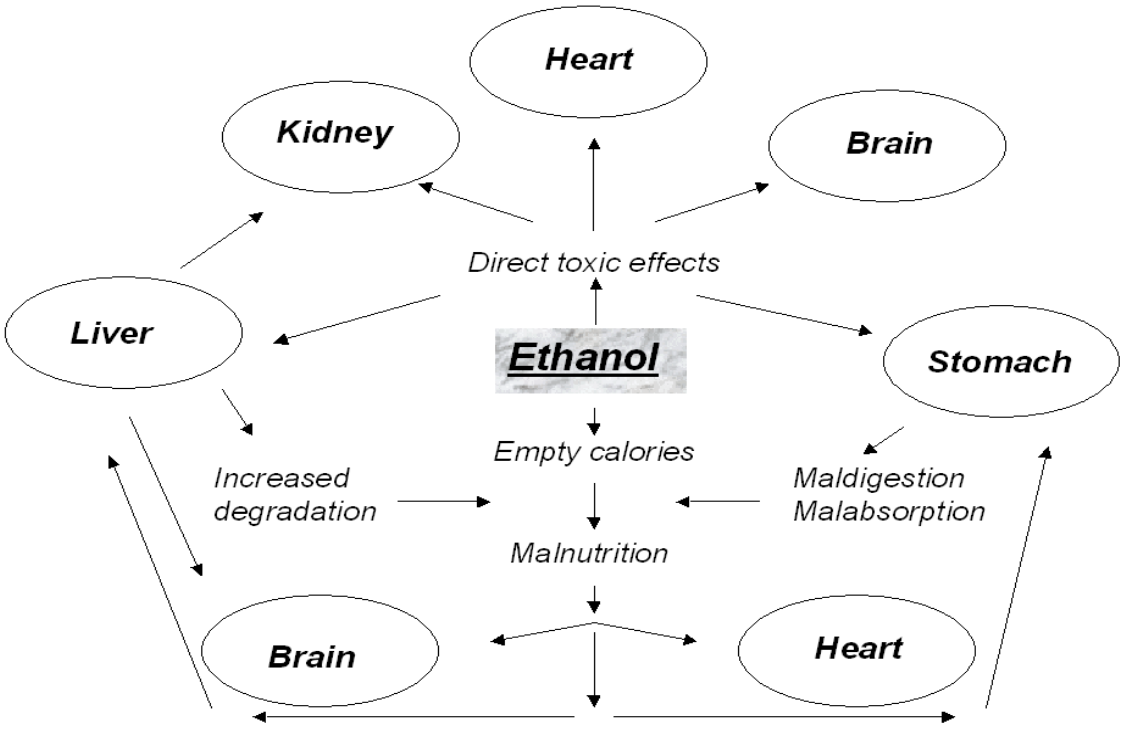

Fig. 2. The direct toxic effects of ethanol on various organs. Figure has been modified from (Lieber 1995).

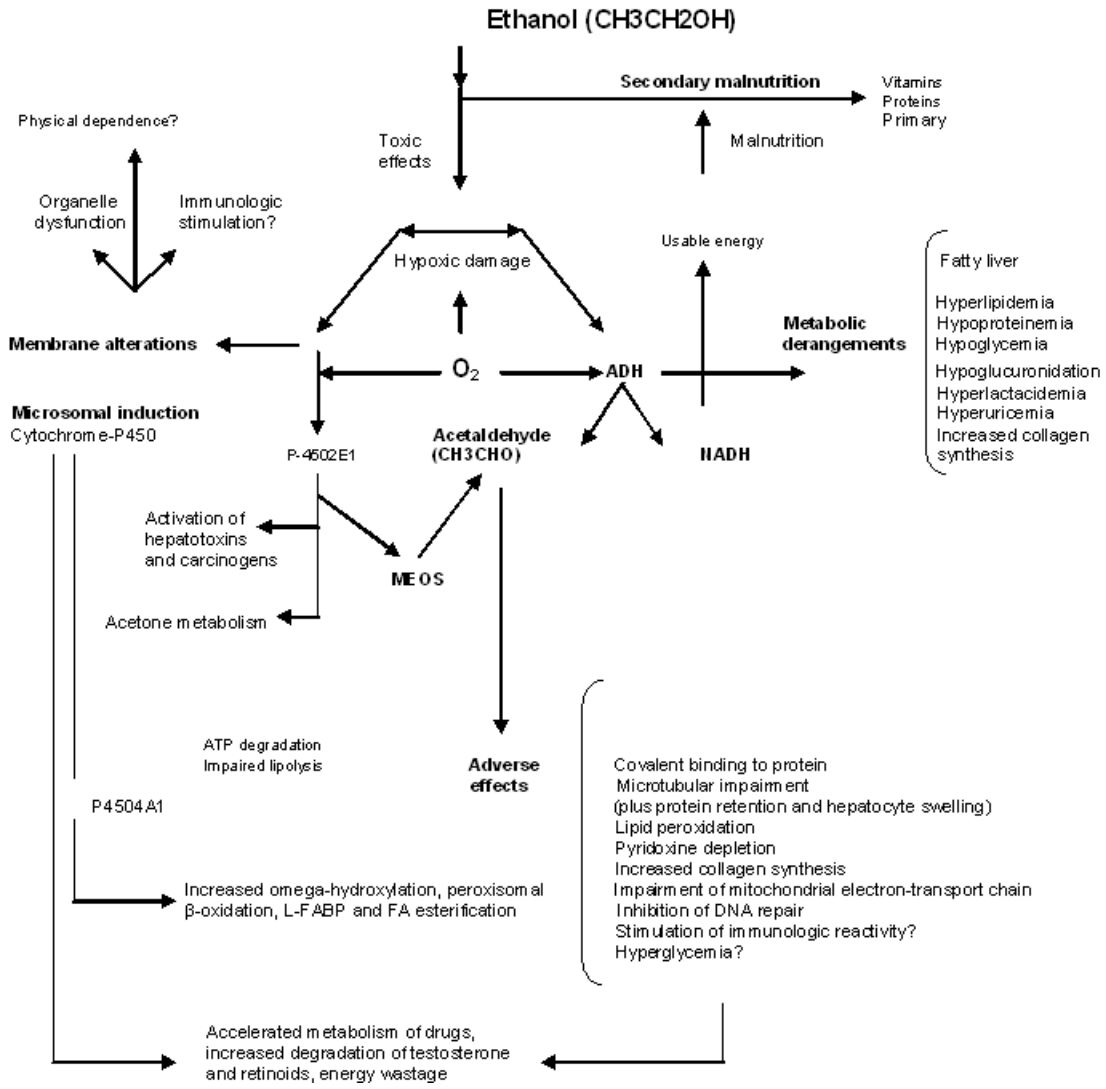

Fig. 3. Toxic and metabolic disorders caused by ethanol abuse. Most of the toxic and metabolic disorders that result from long-term ethanol abuse can be explained on the basis of the metabolism of ethanol by alcohol dehydrogenase (ADH) and the associated generation of reduced NADH or by the microsomal ethanol-oxidizing system (MEOS), with the induction of cytochrome P-4502E1 and other microsomal enzymes, as well as by the toxic effects of acetaldehyde produced by both pathways, primarily in the liver. FA denotes fatty acids and L-FABP liver fatty-acid–binding protein. Figure has been modified from (Lieber 1995).

2.3 Formation and role of acetaldehyde-protein adducts

Acetaldehyde is the first oxidation product of ethanol, which is responsible for a variety of adverse effects of ethanol (Freeman et al. 2005, Lieber 1995). The primary mechanisms of ethanol-induced tissue damage have been suggested to include aldehyde-derived protein

modifications resulting from ethanol metabolism and lipid peroxidation. Conjugation of reactive aldehydes to a variety of target proteins and cellular constituents, such as amino acid and nucleophilic biomolecules, can form both unstable and stable adducts (Niemela 1999, Niemela et al. 1994, Stevens et al. 1981, Tuma and Klassen 1992, Worrall et al. 1993). The presence of protein adducts in the centrilobular region of the liver in alcohol abusers with an early phase of histological liver damage indicates that adduct formation is one of the key events in the pathogenesis of alcoholic liver disease (Niemela 1999). Adduct formation leads to different changes and disturbances in the physiochemical properties of proteins, nucleic acid, and lipids. It can also trigger the formation of antibodies directed against the proteins modified with reactive metabolites of ethanol and products of lipid peroxidation (Mauch et al. 1987, Viitala et al. 2000). Aldehyde adducts make different type of protein modifications, which have been investigated *in vitro* and in alcoholic patient's *in vivo* (Fowles et al. 1996, Niemela 2001, Niemela and Parkkila 2004). The formation of both stable and unstable acetaldehyde-albumin adducts was shown to occur primarily via the reaction of acetaldehyde with lysine residues (Donohue et al. 1983). Reducing agents, $NaBH_3(CN)$ and ascorbic acid, both increased stable adduct formation via increased binding of acetaldehyde to lysine residues; however, a different elution profile of modified lysine residues was observed for these reducing agents. $NaBH_3(CN)$ increased the formation of N-ethyllysine residues exclusively, whereas ascorbate increased the formation of the acidic adduct of lysine and also caused the formation of an additional modified lysine residue which was present only in the ascorbate-treated polypeptides (Braun et al. 1995, Jennett et al. 1989a,b, Tuma et al. 1991, Xu et al. 1989).

Even though acetaldehyde reacts primarily with reactive lysine residues of preferred target proteins (Donohue et al. 1983, Jennett et al. 1987, Jennett et al. 1989a,b, San George and Hoberman 1986, Tuma et al. 1987a,b, Tuma et al. 1991, Xu et al. 1989), it also binds to other amino acids such as tyrosine and valine, and acetaldehyde residues may be exchanged among peptides (San George and Hoberman 1986). Previous studies on acetaldehyde-derived modifications investigated adduct formation with hemoglobin (Stevens et al. 1981), erythrocyte membrane proteins (Gaines et al. 1977), albumin, lipoproteins, transferrin, tubulin (Niemela and Parkkila 2004, Savolainen et al. 1987, Tuma et al. 1987a, Wickramasinghe et al. 1986), ketosteroid reductase (Zhu et al. 1996), and collagens (Jukkola and Niemela 1989). Even though acetaldehyde-derived modifications have been demonstrated in several proteins, the functional consequences of such binding have largely remained unclear.

3. AIMS OF THE STUDY

The aims of this study were:

1) To express, purify and characterize recombinant human CA VII long and short forms (II).

2) To study the expression of CA VII in normal tissues and malignant gliomas and investigate the role of this isozyme as a possible biomarker (II, III).

3) To investigate the generation and functional significance of acetaldehyde adducts on cytosolic CAs I, II, III, VII, and XIII (I, IV).

4. MATERIALS AND METHODS

4.1 Distribution of carbonic anhydrase VII (II, III)

4.1.1 RT-PCR (II, III)

RT-PCR was used to investigate the mRNA expression of full-length and shortened CA VII forms in the human brain. Cerebral cortex and hippocampus QUICK-Clone™ cDNAs were purchased from Clontech (Mountain View, CA). The specific sets of primers for the PCR reaction were designed using the published information on CA VII mRNAs in GenBank (accession numbers BC033865 and NM_001014435). The forward primers were matched with the 5´ untranslated region (UTR). The CA VII forward primers for long (F_1A and F_1B) and short forms (F_2A) and the reverse primer (R) are listed in Table 2. The primers were produced by Oligomer (Helsinki, Finland), and the reagents for the PCR reactions were obtained from KAPA Biosystems (Cape Town, South Africa). The reagents for the PCR reaction comprised of 25 µl RNase-free water, 5 µl of buffer B, 0.5 µl of dNTP, 1.25 µl of forward and reverse primers, 5 µl of Enhancer 1, 1 µl of Enhancer 2, 1 µl of each cDNA template from the human hippocampus and cerebrum, and 0.1 µl of robust polymerase. An amplification process was then performed as follows: initial denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. The results of the PCR reaction were analyzed on 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).

Table 2. Primers used for human CA VII RT-PCR

Primers	Sequence	Product (bp)
F_1A	5´- CGGTAGCGAGCGGGGCCGGAGCCGCAGCCC-3´	884
F_1B	5´- GGACCGAGCCGACCGGGCAGGTGCACGGCT-3´	842
F_2A	5´- AGAGCTCCTTCATCCTGCCTCAGTTTCCTT-3´	816
Reverse (R)	5´-CCGCTCGAGTCAGGAAAGGAAGGAGGCCTT-3´	

4.1.2 Western blotting (II)

Polyclonal anti-human CA VII serum was raised by Innovagen AB (Lund, Sweden) in a rabbit against the purified full-length recombinant human CA VII. Isozyme-specificity of the produced antibody against CA VII was confirmed by western blotting using purified human CA I, CA II, CA VI, CA VII, and CA XIII proteins (data not shown).
Recombinant CA proteins with or without glutathione S-transferase (GST) tag or total homogenates from different mouse tissues were subjected to SDS-PAGE under reducing

conditions (Laemmli 1970). The proteins were then transferred by electrophoresis from the gel to polyvinylidene fluoride (PVDF) membrane (Macherey-Nagel; Düren, Germany). The membrane was treated with the blocking solution (Santa Cruz, Heidelberg, Germany) for 30 min and then rinsed once with TBST buffer (20 mM Tris, 500 mM NaCl, 0.3 % Tween-20, pH 7.5). The membranes were incubated with primary antibody (anti-CA VII) diluted 1:1000 in TBST buffer at 4°C overnight. Alternatively, the recombinant proteins were also detected with goat anti-GST (Amersham Biosciences) diluted 1:1000. Membranes were washed 4 times for 5 min in TBST buffer followed by incubation with monoclonal horseradish peroxidase-labeled anti-rabbit IgG (Acris, Herford, Germany) (1:30000) for 1 h at room temperature. Binding of the goat antibody was detected using monoclonal peroxidase-conjugated anti-goat IgG (1:50000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Membranes were washed 4 times for 5 min in TBST buffer. Target proteins were detected by electrochemiluminescence (ECL) method (Thermo scientific, Rockford, IL) according to the manufacturer's instructions.

4.1.3 Immunohistochemistry (II, III)

Paraffin-embedded tissues obtained from several NMRI mice (from Animal Care Center of Tampere University, Finland) were cut into 5 µm-thick sections and dried onto Superfrost™ microscope slides. The tissues included the kidney, liver, stomach, small intestine, colon, spleen, pancreas, heart, skeletal muscle, and lung. The collection of mouse tissues was conducted according to the provisions of the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, France). According to the national guidelines, no permission was required by authorities to collect tissue specimens from sacrificed mice.

The immunostaining method included the following steps: (1) deparaffinization of the sections using xylene and ethanol series; (2) treatment with 3 % H_2O_2 in methanol for 5 min; (3) rinsing in TBS; (4) blocking with Rodent Block M™ (Biocare Medical, Concord, CA) for 30 min; (5) washing 3 times for 5 min with TBS; (6) incubation with polyclonal anti-CA VII serum or preimmune serum (both diluted 1:100) for 1 h; (7) washing 3 times for 5 min with TBS; (8) incubation with a mixture of Rabbit on Rodent HRP-Polymer™ and blocking reagent XM Factor™ (20 µl XM Factor™ in 1 ml HRP-Polymer) (Biocare Medical, Concord, CA) for 30 min; (9) washing 3 times for 5 min with TBS; (10) incubation in DAB (3,3'-diaminobenzidine tetrahydrochloride) solution (Zymed, Carlsbad, CA) for 5 min; (11) rinsing in ddH_2O; (12) counterstaining with hematoxylin (13); and rinsing again with ddH_2O. The sections were mounted in Entellan Neu™ (Merck; Darmstadt, Germany) and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss; Göttingen, Germany).

4.2 Production and purification of recombinant human cytosolic CAs (I, II, IV)

4.2.1 Construction and cloning of CA I, III, and VII on bacterial expression system (II, IV)

The complete coding sequences for human *CA1* and *CA3* genes were obtained from GenBank (for *CA1* accession number BC027890 and for *CA3* BC004897). The *CA1* and *CA3* cDNAs were amplified by PCR using designed primers based on published information on *CA1* and *CA3* mRNAs in GenBank. In order to generate the GST-CA I and GST-CA III constructs the following primers were designed which are listed in Table 3. The reagents for the PCR reactions were purchased from Fermentas GmbH (St.Leon-Rot, Germany), and the PCR reactions were performed on a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA). The protocol consisted of a 98°C denaturation step for 3 min followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 7 min. The PCR product was analyzed using 1.5% agarose gel containing 0.1 mg/ml ethidium bromide. DNA standards (100 bp and 1000 bp DNA Ladders) were obtained from New England Biolabs. The amplified cDNAs for both CA I and CA III were digested with BamHI and XhoI (New England Biolabs) and ligated into the BamHI-XhoI site of the digested pGEX-4T-1 vector (Invitrogen, Carlsbad, CA) that would express GST fusion protein in bacteria and would contain the cleavage site of thrombin protease between the GST and CA domains. The pGEX-4T-1/GST-CA I and pGEX-4T-1/GST-CA III constructs were transformed into *E.coli* BL21 (DE3) (Invitrogen) for subsequent expression.

The complete coding sequence of human *CA7* gene was obtained from GenBank (accession number NM_005182). The *CA7* cDNAs were amplified by PCR using designed primers based on published information on the corresponding mRNA in GenBank (NM_005182 for CA VII). In order to generate the GST-CA VII constructs, the primers were designed (Table. 3). The reagents for the PCR reactions were purchased from Fermentas GmbH (St. Leon-Rot, Germany), and the PCR reaction was performed on a GeneAmp PCR System 9700 (Applied Biosystems). The protocol consisted of a 98°C denaturation step for 3 min followed by 33 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 7 min. The PCR products were analyzed using 1.5% agarose gel containing 0.1 mg/ml ethidium bromide. DNA standards (100 bp and 1000 bp DNA Ladders) were obtained from New England Biolabs. The amplified cDNA was digested with EcoRI and XhoI (NewEngland Biolabs) and ligated into the EcoRIeXhoI site of the digested pGEX- 4T-1 vector (Invitrogen) that would express a GST fusion protein in bacteria and would contain the cleavage site of thrombin protease between the GST and CA domains. The pGEX-4T-1/GST-CAVII constructs were transformed into *E.coli* BL21 (DE3) (Invitrogen) for subsequent expression.

Table 3. Primers used for the PCR of human *CA1*, *CA2*, and *CA7* constructs

Restriction site		Sequence	Product (bp)
CA I	BamHI-F	5´-CGCGGATCCATGGCAAGTCCAGACTGGGGA-3´	804
	HxoI-R	5´-CCGCTCGAGTCAAAATGAAGCTCTCACTGT-3´	
CA III	BamHI-F	5´-CGCGGATCCATGGCCAAGGAGTGGGGCTAC-3´	807
	HxoI-R	5´-CCGCTCGAGTTACTATCATTTGAAGGAAGC-3´	
CA VII	EcoRI-F1	5´-CGCGAATTCATGACCGGCCACCACGGCTGG-3´	819
	HxoI-R	5´- CCGCTCGAGTTACTATCAGGAAAGGAAGGA-3´	

4.2.2 Production and purification of human recombinant CA I, II, III, VII, and XIII proteins (I, II, IV)

The recombinant human CA II enzyme production in *E.coli* has been described earlier (Hu et al. 1995). Similarly, the expression of human CA XIII has been previously described (Hilvo et al. 2008). During the present investigations human CA I, III, VII cDNAs were cloned into the expression vector, pGEX-4T-1, and expressed as fusion proteins with GST.

To produce GST fusion proteins with CA I, CA III or CA VII single colonies of *E.coli* BL21 (DE3) with the pGEX-4T-1/GST-CA I, III or VII construct transformants were grown overnight in 10 ml culture, then mixed and grown in 1 l of LB Broth Base containing 50 mg/ml ampicillin with shaking (250 rpm) at 37°C until the optical density (OD) at 600 nm reached 0.6. The expressions of the recombinant CA enzymes were optimized by induction using isopropyl-b-D-thiogalactopyranoside (IPTG) (Fermentas) with a final concentration of 1 mM at 37°C for 3 h. The cells were harvested by centrifugation (Sorvall RC 5C) at 5000 × g for 7 min at 4°C.

Human CA I, III and XIII proteins were purified under native conditions using a Glutathione Sepharose^{TM} column (GE Healthcare, Buckinghamshire, UK). Restriction-grade, site-specific thrombin (GE Healthcare) (15 μl) was used for the specific cleavage of the GST under shaking at RT overnight. After cleavage the target protein was eluted out with PBS as suggested by the manufacturer.

The CA VII cell pellet was suspended in 25 ml Tris-buffer containing 0.1 M Tris-HCl, pH 7, 0.1% Triton X-100, 0.45 g lysozyme, 1 tablet of protease inhibitors and 20 ml DNase (Roche,

Basel, Switzerland). The *E.coli* cell suspension was kept on ice while lysed and sonicated for 3 min. The suspension was clarified by centrifugation at 12 900 × g for 15 min at 4°C, and the clear supernatant was used for affinity purification in a Glutathione Sepharose™ column (GE Healthcare, Buckinghamshire, UK). CA VII recombinant protein was isolated under native conditions as described by the manufacturer. A restriction-grade, site-specific thrombin (GE Healthcare) (15 ml) was used for specific cleavage of the GST under shaking at RT overnight. After cleavage the target protein was eluted out using the glutathione elution method (GE Healthcare) according to the manufacturer's instructions. The size of the expressed CA VII protein was determined under reducing conditions by SDS-PAGE analysis.

4.3 Biochemical characterization of human recombinant CAs (I, II, IV)

4.3.1 Structural modeling of CA VII (II)

CA VII (UniProt sequence P43166) was modeled in Dr. Vesa Hytönen's laboratory (Institute of Biomedical Technology, University of Tampere) with Modeller 9v7 (Sali and Blundell 1993), using the crystallographic structure of CA II, 2ILI (Fisher et al. 2007) as template. PyMOL v0.99 (DeLano Scientific, Palo Alto, CA) was used for visualization of the structures. For comparison we also used the published crystal structures of CA I, 2NMX (Srivastava et al. 2007), CA III, 1FLJ (Mallis et al. 2000), and CA XIII, 3D0N (Di Fiore et al. 2009).

4.3.2 Mass spectrometry (I, II, IV)

Mass spectrometry experiments were performed in Dr. Janne Jänis's laboratory (Department of Chemistry, University of Eastern Finland) using a 4.7-T hybrid quadrupole–Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (BioAPEX-Qe™; Bruker Daltonics, Billerica, MA), equipped with Apollo-II™ electrospray ionization (ESI) source. Samples were directly infused at a flow rate of 1.5 μl min^{-1} and ESI-generated ions were externally accumulated in a hexapole ion trap for 1.0 s. Ions were then transmitted through a high-voltage optics region to an Infinity ICR cell for "sidekick" trapping, conventional "RF-chirp" excitation and broadband detection. For each spectrum, a total of 1000 co-added (512-kWord) time-domain transients were zero-filled once prior to fast Fourier transform and magnitude calculation. For electron capture dissociation (ECD), ions of interest were mass-selectively accumulated in the hexapole (quadrupole isolation window of 15 *m*/*z* units), transferred into the ICR cell and subsequently irradiated for 220 ms with low-energy electrons (heating current 1.4 A, bias 0.5 V, lens 12 V) provided by the indirectly-heated dispenser cathode. Mass calibration was performed externally with respect to the ions of an ES Tuning Mix (Agilent Technologies, Santa Clara, CA) calibration mixture. All data was acquired and processed with the use of Bruker XMASS 7.0.8 software. Mass spectra were charge-deconvoluted (to zero charge state) with a dedicated Tcl-script. Peptide maps resulting from digestion experiments were analyzed with the use of GPMAW 8.11 software (Lighthouse Data, Odense, Denmark).

4.4 Modifications of human cytosolic CA I, II, III, VII, and XIII with acetaldehyde (I, IV)

4.4.1 Labeling and acetaldehyde treatment of CA I, II, III, VII, and XIII (I, IV).

All cytosolic human recombinant CA enzymes (CA I, II, III, VII, and XIII) were treated with various concentrations of acetaldehyde either in the presence or absence of a reducing agent, $NaBH_3(CN)$. All reagents were maintained and pipetting was performed at 4°C to minimize acetaldehyde evaporation. The sample tubes containing CA enzymes with or without acetaldehyde were tightly sealed and incubated at 37°C for 2 h. Then 10 mM $NaBH_3(CN)$ or an equal volume of H_2O was added to each sample tube, and the incubation at 37°C was continued for 22 h followed by a quick cooling down to 4°C.

4.4.2 Activity measurements of CA I, II, III, VII, and XIII (I, IV)

CA catalytic activity was first (I) determined using a slightly modified end-point titration method (Maren 1960). In short, the steps included: 150 ng of human CA II or 1 µl of 1/10 diluted blood sample was added to 500 µl of ice-cold assay buffer (20 mM imidazole, 5 mM Tris, and 0.2 mM p-nitrophenol). The cuvette containing the sample and assay buffer was placed in a Lambda 35 UV/VIS spectrophotometer (Perkin Elmer Instruments, Waltham, MA) and 500 µl of ice-cold CO_2-saturated H_2O was added to the cuvette. The exact time taken for the yellow colour to disappear was counted. In the control experiments without the CA II enzyme, the colour disappeared in 50 sec. Statistical significance of the enzyme activity results was assessed using Student's t-test and was denoted as p-values.

The enzymatic activities of native recombinant human CA II and the enzyme treated with 100 mM acetaldehyde under reducing conditions were also assayed using an Applied Photophysics (Leatherhead, UK) stopped-flow instrument (I). Only the stopped-flow instrument was used for the kinetic studies on CA I, III, VII, and XIII enzyme samples (IV). This method was available at the laboratory of Dr. Claudiu Supuran (Laboratory of Bioinorganic Chemistry, University of Florence, Italy). The reaction was measured using 0.2 mM phenol red as an indicator, in 10 mM HEPES, 0.1 M Na_2SO_4, pH 7.5, for a period of 10–100 s. To determine the kinetic parameters, the CO_2 concentration ranged from 1.7 to 17 mM. For the inhibitor assay, at least six traces of the initial reaction were used for determining the initial velocity, which were substracted from the uncatalyzed reaction. A stock solution of 1 mM acetazolamide in 10-20% (v/v) DMSO was used to prepare dilutions up to 0.01 nM. To form enzyme-inhibitor complexs, inhibitor and enzyme solutions were preincubated for 15 min, at 25°C, prior to inhibition measurements. Kinetic parameters were obtained from Lineweaver- Burk plots, as reported earlier, and represent the mean from at least three different determinations.

4.5 Expression of carbonic anhydrase VII in gliomas (III)

4.5.1 Tumor materials and immunohistochemistry (III)

To investigate the expression of human CA VII in gliomas our material consisted of diffusely infiltrating astrocytoma and oligodendroglioma samples, which were obtained from patients surgically operated on in Tampere University Hospital, Tampere, Finland, during 1983-2001. First, the tumor specimens were fixed in 4% phosphate-buffered formaldehyde and processed into paraffin blocks. On the basis of hematoxylin and eosin staining a neuropathologist (Dr. Hannu Haapasalo, Deparment of Pathology, Laboratory Centre of Tampere University Hospital) performed an evaluation of the tumors according to the WHO 2007 criteria (Kleihues et al. 2002, Louis et al. 2007). Diffusely infiltrating astrocytomas were grouped into three grades (2-4) according to the WHO criteria. The evaluated parameters included the presence of atypia, mitotic activity, necrosis, and endothelial proliferation. The samples selected for investigation included 107 astrocytic tumors (grade 2: 14; grade 3: 11; grade 4: 82) and consisted of 90 primary tumors and 17 recurrences. The mean follow-up time for 14 survivors was 67 months (range 31 – 165), and 76 patients died during the five-year follow-up The neuropathologist selected one histologically representative section in each specimen for the CA VII immunohistochemistry. The WHO criteria grouped oligodendroglial tumors into two main categories: pure oligodendrogliomas and mixed oligoastrocytomas, which were further divided into two grades (2 and 3) according to the presence of atypia, increased cellularity, and mitotic activity. From 47 cases of oligodendroglial tumor samples which were included in our analyses, there were both pure oligodendrogliomas (18 of grade 2 and 12 of grade 3) and oligoastrocytomas (11 of grade 2 and 6 of grade 3). These tumors included 35 primary tumors and 12 recurrences.

Five µm sections were processed for immunoperoxidase staining, which was performed using an automated Lab Vision Autostainer 480 (LabVision Corporation, Fremont, CA, USA). Automated immunostaining was performed using the Power Vision+ Poly-HRP Immunohistochemistry kit (ImmunoVision Technologies Co) reagents. The immunostaining procedure included the following steps: (1) rinsing in wash buffer; (2) treatment in 3% H_2O_2 in ddH_2O for five min and rinsing with wash buffer; (3) blocking with cow colostrum diluted 1:2 in Tris-buffered saline (TBS) containing 0.05% Tween-20 for 30 min and rinsing in wash buffer; (4) incubation with primary antibody (rabbit anti-human CA VII for 30 minutes; (5) rinsing in wash buffer three times for five min; (6) incubation in poly-HRP-conjugated anti-rabbit IgG for 30 min and rinsing in wash buffer three times for five min; (7) incubation in DAB (3,3`-diaminobenzidine tetrahydrochloride) solution (one drop of DAB solution A and one drop of DAB solution B in 1 ml of ddH_2O) for six min; (8) $CuSO_4$ treatment for five min to enhance the signal; and (9) rinsing with ddH_2O. All procedures were performed at room temperature. The intensity (INT) of the staining was scored on a scale of 0 to 3 by three of the investigators (H. Haapasalo, F. Bootorabi and S. Parkkila) as follows: 0, no reaction; 1, weak reaction; 2, moderate reaction; and 3, strong reaction. The extent (EXT) of staining was also scored as 1 when 1-10% of the cells stained, 2 when 11-50% of the cells stained and 3 when 51-100% of the cells stained. A negative score (0) was given to tissue sections that had no evidence of specific immunostaining. The relative staining indices (SI) were calculated for each tumor sample using the formula SQRT (EXT x INT) as described earlier (Leppilampi et al. 2003). The tumors were then divided into three groups based on the staining index: -, $SI \leq 1$ (immunonegative); +, $SI > 1$ and ≤ 2 (immunopositive); ++, $SI > 2$ (strongly immunopositive).

Expression of CA VII was correlated to the extent of several other molecular markers, such as Ki-67 (MIB-1, marker of cell proliferation) and p53 (Haapasalo et al. 1999). EGFR amplification was detected in astrocytic tumors using chromogenic *in situ* hybridization (CISH) (Jarvela et al. 2006). The immunostaining results for CA IX and CA XII were described earlier (Haapasalo et al. 2008, Haapasalo et al. 2006).

4.5.2 Statistical analyses (III)

All statistical analyses were performed using SPSS for Windows (SPSS Inc. Chicago, IL). The significance of associations was defined using chi-square test, Mann-Whitney test, variance analysis and Kruskal-Wallis test. The log rank test, Kaplan-Meier curves, and Cox multivariate regression analysis were used in the survival analysis. P-values < 0.05 were considered statistically significant.

4.6 Carbonic anhydrase autoantibodies (IV)

4.6.1 Serum samples (IV)

Serum samples for autoantibody assays were obtained from abstainers (n = 16, 10 men, 6 women, mean age 44±9 years) and alcoholic subjects (n = 32, 28 men, 4 women, mean age 46±10 years). The alcoholic patients were all subjects who had been consecutively admitted for detoxification with a history of continuous ethanol consumption or binge drinking, which had consisted of over 80 g/day during the period of 4 weeks prior to sampling. All participants gave their informed consent and the study was carried out according to the provisions of the Declaration of Helsinki.

4.6.2 Detection of autoantibodies against CA I, II, and VII enzymes (IV)

Autoantibody levels were determined using an enzyme-linked immunosorbent assay (ELISA) developed in our laboratory. The protocol for the immunoassay included the following steps: purified CA I, II, and VII proteins (50 ng/well) in sodium carbonate buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6) were incubated on 96-well plates (Thermo Scientific Pierce, Waltham USA) overnight at 4^{o}C. The wells were washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBST) and blocked with 2% bovine serum albumin/PBST for 2 h at room temperature. After washing three times with PBST, serum samples were diluted (1:5000) in 2% bovine serum albumin/PBST and incubated (50 µl/well) overnight at 4^{o}C. Each sample was analyzed as triplicate. After washing three times with PBST, diluted (1:25000) peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was added (50 µl/well) and incubated for 1 h at room temperature. The plates were washed three times with PBST and incubated with TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate (Thermo Scientific Pierce, Waltham USA) for 15 min. Reaction was stopped with 2M H_2SO_4 and absorbance measured at 450 nm.

5. RESULTS

5.1 Sequence and RT-PCR analyses of CA VII short and long form (II)

Sequence information obtained from GenBank data suggested that human CA VII exists as two forms, a full-length CA VII (266 residues) and shorter form (210 residues). Protein alignment of these forms showed a deletion of 56-residues in the N-terminal sequence of CA VII-s. Computer modeling of CA VII structures showed that the deletion of N-terminal residues (1-56) and loss of a potential intramolecular disulfide bridge (from Cys-56 to Cys-180) in CA VII-s may cause structural instability of the protein. The central part of the sequence, where all catalytic residues exist, is conserved in CA VII-s, and therefore it could possess some CA activity if expressed.
The mRNA sequences for the full-length CA VII and CA VII-s are similar between the nucleotides 169-781, but the 5´-end and untranslated regions (UTRs) are different. This was an advantage, which allowed us to design forward primers specific to each form in order to detect the different mRNAs separately in the human brain cDNA samples. The results from RT-PCR experiments demonstrated that mRNAs for both CA VII forms were expressed in both the human cerebrum and hippocampus.

5.2 Distribution of human CA VII (II)

Recombinant human CA VII protein was used to raise anti-CA VII serum. This antibody recognized both forms of CA VII in western blotting. In principle, the 6 kDa difference between the full-length and shortened CA VII allowed us to use western blotting to screen and see which form was expressed in which tissue. Only a single ~30 kDa polypeptide corresponding to the full-length CA VII was detected in the mouse colon, liver, and muscle, indicating that it represents the major form expressed at the protein level in tissues. Western blotting showed no signal for CA VII in the mouse cerebrum and cerebellum, probably due to low and restricted expression of CA VII protein in the brain. In our study, we also performed immunohistochemical staining of CA VII protein in selected mouse tissues. In the stomach, weak staining was observed in a limited number of cells locating in the upper segment of the gastric glands, CA VII was also detectable in the Brunner glands of the duodenum. In the colon, CA VII immunostaining was restricted to the lamina propria, and even though several other CA isozymes (CA I, II, IV, V, IX, XII, and XIII) are expressed in the colonic epithelium (Fleming et al. 1995, Kivela et al. 2000, J Lehtonen et al. 2004, Parkkila et al. 1994, Pastorekova et al. 1997), we observed no specific signal for CA VII in the epithelial cells. The strongest positive expression for CA VII was detected in the liver. The staining was localized in the hepatocytes and showed no significant zonal variation within the hepatic lobule. No immunoreaction for CA VII was found in the pancreas and lung. Expression of CA VII was also demonstrated in the skeletal muscle where the signal appeared slightly stronger in near proximity to the sarcolemma, although it was also present in the sarcoplasm of the myocytes. No staining for CA VII was found in the heart, muscle, or spleen.
The control immunostaining using preimmune serum instead of the anti-CA VII serum showed no staining in the CA VII-positive tissues, including the liver, stomach duodenum, colon, and skeletal muscle. The immunohistochemical results of CA VII clearly showed a unique distribution for this enzyme in comparison to the other CA isozymes.

5.3 Production and purification of recombinant human cytosolic CA I, III, and VII (I, II, IV)

The human CA I, III, and VII cDNAs were cloned into the expression vector pGEX-4T-1 and expressed as fusion proteins with GST. The recombinant proteins were first characterized for the apparent molecular mass using SDS-PAGE. Upon digestion with thrombin and purification by affinity chromatography CA I, III, and VII appeared as 28-30 kDa polypeptides in SDS-PAGE. The bands were isolated from the gel and confirmed to represent the correct proteins by mass spectrometry at the Meilahti Mass Spectrometry Center (University of Helsinki).
Recombinant human CA II enzyme was produced in *E.coli* and purified to homogeneity using CA inhibitor affinity chromatography (Hu et al. 1995). Production and purification of human CA XIII has also been described earlier (Hilvo et al. 2008).

5.4 Characterization of human CA VII (II)

5.4.1 Mass spectrometry

High-resolution ESI FT-ICR mass spectrometry was used to confirm the amino acid sequence and disulfide arrangements in CA VII. A mass spectrum of CA VII, measured in acetonitrile/water/acetic acid (49.5:49.5:1.0, v/v) solvent, demonstrated a typical charge state distribution (from 17þ to 27þ) of a single protein form in solution, with the most abundant isotopic mass determined as 30 172.79 ± 0.07 Da. No higher protein oligomers (e.g. covalent protein dimers) or other impurities were observed.
There are four cysteine residues available (Cys-56, Cys-180, Cys-185, and Cys-219) in CA VII according to the sequence to form disulfide bridges; (note that the amino acid numbering is based on the cDNA of the full-length CA VII). Homology modeling showed that Cys-56 is likely to form a disulfide with Cys-180 and that the other two, Cys-185 and Cys-219, could also possibly form a disulfide, although Cys-219 is only conserved among primates and not present, for instance, in the CA VII sequences of rodents, sheeps or dogs.
A CA VII sample was treated with DTT to reduce possible disulfides in CA VII. The charge-deconvoluted mass spectra for native (disulfide-intact) and disulfide-reduced enzyme clearly indicated a mass difference of 2 Da. Following the DTT treatment, we also treated the native protein with NEM which specifically reacts with free thiol groups in polypeptides, resulting in a 125 Da mass increase per free cysteine residue. In fact, CA VII reacted with NEM consistently showed a 250 Da mass increase upon NEM-treatment, confirming that two cysteine residues were effectively conjugated with NEM. No unconjugated protein was present after 30 min, indicating a high reactivity of NEM towards free thiols. To further verify cysteine pairing in CA VII, we performed on-line pepsin digestion with the native protein. Immobilized pepsin has a high preference towards C-terminal cleavages of Phe, Leu and Ala residues, but other aliphatic and aromatic residues may also be cleaved at variable rates (Jänis, unpublished results). The used digestion protocol also efficiently works with disulfide-intact proteins. An on-line digestion of CA VII resulted in 13 specific peptic peptides with a sequence coverage of 74% (200 out of 268 residues found), most appearing after C-terminal cleavages of Phe, Leu and Ala residues. Peptide identification was assured by the high mass accuracy (0.8 ppm on average) and some of the peptic peptides were further identified by ECD tandem mass spectrometric measurements (data

not shown). One of the observed peptides corresponded to the sequence, IIe-Val-Leu-Arg-Glu-Pro-IIe-Cys-IIe-Ser-Glu-Arg-Gln-Met-Gly-Lys-Phe (residues 212-228), had a free Cys-221 residue. Since this sample did not react with DTT, it indirectly confirmed the presence of a disulfide bridge between Cys-56 and Cys-180 in CA VII. In CA VII-s, Cys-56 is missing (sequence starts from the neighbouring Met-57). This means that there is either one disulfide bridge (Cys-185 to Cys-219) and one free cysteine (Cys-180) or all the three cysteines are free in CA VII-s.

5.5 Modifications of human cytosolic CAs I, II, III, VII, and XIII with acetaldehyde (I, IV)

5.5.1 Mass spectrometry (I, IV)

High-resolution mass spectrometry was used to detect acetaldehyde-derived covalent modifications in the produced recombinant CA isozymes. The protein samples were treated with acetaldehyde either in the presence or absence of a reducing agent, $NaBH_3(CN)$, to induce chemical modifications and analyzed directly with ESI FT-ICR mass spectrometry. The reactions were carried out at the constant CA concentration of 100 μM and increasing acetaldehyde concentrations up to 200 mM (CA-to-acetaldehyde molar ratio of 1:10 to 1:2000) after which the reaction mixtures were diluted with acetonitrile/water/acetic acid (49.5:49.5:1.0, v/v) to CA concentration of 5-10 μM prior to the measurement. High resolution afforded by the technique allowed unambiguous detection and identification of different unstable and stable modifications. Molecular masses were first determined for native proteins (not treated with acetaldehyde) and they were in good agreement with the theoretical masses calculated from the protein sequences, except for CA III.

A Schiff base (imine) formation between the ε-NH_2 (side chain amino group) of lysine and acetaldehyde is highly pH-dependent and reversible (a rehydration of imine gives free amine and aldehyde) making these adducts unstable and difficult to detect. In contrast, under reducing conditions imine is rapidly and irreversibly converted to corresponding amine (N-ethyllysine; +28 Da), which is a stable covalent adduct.

Under nonreducing conditions, only a very modest adduct formation with human CA II was observed at the highest acetaldehyde concentrations (100-200 mM). Interestingly, two different adducts were formed, one consistent with the Schiff base formation (+26 Da) and the other displaying +44 Da mass increment, presumably representing unstable carbinolamine intermediate (direct amine-aldehyde addition product from which imine is formed by dehydration). In contrast, under reducing conditions a number of stable N-ethyllysine residues (+28 Da) were formed already at 1 mM acetaldehyde concentration (3-4 on average) and considerably increasing amount at 10 mM (13-14 on average).

Similar results were obtained for the other CAs, with some minor differences. CA VII did not form any detectable adducts in nonreducing conditions, even at the highest aldehyde concentrations. In reducing conditions, a number of stable adducts (n × +28 Da) were formed, similar to what was observed with CA I and II. A modest adduct formation (+26 Da and +44 Da) was observed also for CA XIII in nonreducing conditions, but only at the highest acetaldehyde concentration. Similar to other isozymes, many more adducts were formed in reducing conditions, but decreasing spectral resolution with the highest aldehyde concentrations did not

allow us to quantify the amount of adducts formed. The least amount of adducts were formed with CA III. Only one unstable (+26 Da) adduct was detected at low level with 100 mM acetaldehyde in nonreducing conditions but again considerably more (roughly 5-7 stable adducts) in reducing conditions. Again, spectral resolution was decreased and did not permit to accurately quantify the amount of adducts formed with this protein sample.

5.5.2 Activity measurement (I, IV)

Cytosolic recombinant human CA I, II, III, VII, and XIII isozymes were treated with acetaldehyde in the presence or absence of a reducing agent, $NaBH_3(CN)$, in order to induce chemical modifications in these proteins. The kinetic effects of the formed acetaldehyde-derived modifications were analyzed by a stopped-flow CA activity assay. The kinetic data indicated that the activity of CA II was significantly reduced in the presence of 100 mM acetaldehyde. Under reducing conditions, the k_{cat}/K_M-values for native and acetaldehyde-treated CA II enzyme were 1.5×10^8 and 0.5×10^8 $M^{-1}s^{-1}$, respectively. The results on CA VII, an important candidate enzyme for acetaldehyde-derived modifications with the highest expression in hepatocytes, showed that low, physiological acetaldehyde concentrations (up to 1 mM) had virtually no effect on CA VII activity nor did they change the K_I value for acetazolamide. The studies on the other cytosolic enzymes (CA I, CA III, and CA XIII) showed that even the supraphysiological acetaldehyde concentrations ranging from 9 to 500 mM showed only modest changes in the activity levels. CA XIII showed the highest changes, although they were still smaller than in CA II.

5.6 Expression of CA VII in gliomas (III)

5.6.1 Oligodendroglial and diffuse astrocytic tumors

Our tumor specimens included 47 oligodendrogliomas of which 30 cases represented pure and 17 represented mixed oligoastrocytomas. We observed that 85% of oligodendrogliomas showed positive immunostaining for CA VII. There was no significant difference in CA VII expression levels between astrocytomas and oligodendrogliomas (p = n.s., chi-square test). Recurrent oligodendrogliomas were more immunopositive for CA VII than the primary tumors, and the difference was found to be statistically significant (p = 0.011, chi-square test). There was no statistically significant correlation between the patient age and CA VII status nor did CA VII correlate with the tumor grade, cell proliferation (assessed by MIB-1), p53 immunostaining, or EGFR amplification (p = n.s., chi-square test). A near significant correlation (p = 0.117, chi-square test) was observed between CA VII and CA IX staining results.
In diffuse astrocytomas, strongly positive areas were often located in close proximity to necrotic regions. The positive staining was usually unevenly distributed within the tumor. It also appeared that the cell cytoplasm was more intensively stained in the tumors with anaplastic features. In addition to the positive staining of tumor cells, CA VII was often located to the endothelial cells of the neovessels. Within the group of WHO grade 2 astrocytomas, 86% of the tumors were CA VII-positive and 82% of the grade 3 astrocytomas were CA VII-positive. Importantly, almost all (98%) cases of the grade 4 tumors were immunopositive for CA VII. The statistical comparison of cytoplasmic CA VII staining index and tumor grade revealed significantly higher CA VII

staining in tumors with higher malignancy grade ($p = 0.02$, chi-square test). All recurrent tumors were immunopositive ($p = 0.015$, chi-square test). The variance analysis showed that there was no significant correlation between the CA VII staining index and patient age.
The possible correlation between the patient survival and CA VII staining index in diffuse astrocytomas was tested by log-rank test. CA VII staining results divided the tumors into three significantly different prognostic subsets ($p = 0.017$, log-rank test). When different factors, including CA VII, CA IX and CA XII (Haapasalo et al. 2008, Haapasalo et al. 2006), (all divided into three categories by staining indices -, + and ++) as well as histological grade (2, 3, and 4) and patient age (<50, 50 – 65 and >65 years) were tested in the Cox multivariate analysis, CA VII, CA IX, histological grade, and patient age were found to be of independent prognostic value.

5.7 Carbonic anhydrase autoantibodies

Recently developed immunoassay methods were used to analyze the possible presence of anti-CA I, -CA II, and -CA VII autoantibody responses in alcoholic patients. The results were compared to the values obtained from abstainers. The mean absorbance levels in the immunoassays did not differ significantly between the abstainer and alcoholic groups, suggesting that alcohol abusers show no detectable IgG-class autoantibody response against these CA isozymes.

6. DISCUSSION

6.1 Carbonic anhydrase VII long and short forms

To date, 13 active α-carbonic anhydrase isozymes have been identified in mammals. Five of them (CA I, CA II, CA III, CA VII, and CA XIII) have been described as cytoplasmic enzymes (J Lehtonen et al. 2004, Pastorekova et al. 2004). Even though CA VII was first reported 20 years ago (Montgomery et al. 1991), it was not thoroughly characterized before the present studies.

The studies on CA VII were initiated after we discovered two forms of human CA VII mRNA in the GenBank. Based on that finding we aimed to investigate and characterize these two forms. The corresponding proteins were named CA VII (the full-length enzyme, 266 residues) and the shortened CA VII (CA VII-s, 210 residues). Prior to the present study there was no data available on the shorter form of CA VII. Also the data on human full-length CA VII protein was restricted to a few publications mainly on CA inhibitor and activator design and analysis (Vullo et al. 2007, Vullo et al. 2006, Vullo et al. 2005). During the first investigation we decided to determine whether both alternative forms are expressed in the brain. As they were encoded for by the same gene, and the coding sequence was identical in both from nucleotide 169 onwards, we performed RT-PCR utilizing the 5´ UTR region which was unique in both cases. The RT-PCR result confirmed that the corresponding mRNAs of full-length and short form CA VII were indeed detectable in the human cerebrum and hippocampus. Information on the expression of CA VII in the mouse hippocampus, cerebral cortex and cerebellum was already available (Lakkis et al. 1996, Lakkis et al. 1997). Data from mass spectrometry and comparative modeling showed that the CA VII protein contains a single intramolecular disulfide bridge (Cys-56 to Cys-180), which is lacking in the short form. Parallel to this result the CA VII short form showed fragmentation and degradation during different purification methods, which was later attributed to the lack of stability in the structure of this small protein. We originally predicted that CA VII short form could still conserve some activity because it contains the essential histidine residues in the central part of the sequence which is involved in Zn ion binding. The lack of 56 residues from the N-terminus made the modeled conformation of s-CA VII very different compared to the full-length form. The deletion of 56 residues from the N-terminus and missing Cys-56 left only three cysteines (Cys-180, Cys-185 and Cys-219) free, and the mass spectrometry later confirmed that they do not form any disulfide bridges. This was considered an important reason for the degradation, and thus, instability of the CA VII-s form. The SDS-PAGE demonstrated the size of 23.8 kDa for CA VII-s, indicating that it would have represented the smallest α-CA isozyme if it was expressed *in vivo*. A small amount of CAVII-s, which was isolated from the SDS-PAGE gel, was confirmed to represent the correct protein by peptide sequencing. Although it was technically challenging, we found that the CA VII-s protein exhibited about 20 % of the CA VII enzyme activity.

The full-length CA VII protein appeared as a 30 kDa polypeptide in SDS-PAGE. Although there were four cysteines (Cys-56, Cys-180, Cys-185, and Cys-219) available to form disulfide bridges, the mass spectrometry analysis, on-line peptide digestion and treatment of the protein sample with DTT (to reduce possible disulfides) confirmed that only Cys-56 and Cys 180 make a disulfide bridge in CA VII under these experimental conditions. Recently published crystal structures of human CA VII also suggested the presence of the disulfide bond in CA VII (Di Fiore et al. 2010). In eukaryotic cells, disulfide bonds are generally formed in the lumen of the

rough endoplasmic reticulum but not in the cytosol. This is due to the oxidative environment of the endoplasmic reticulum and the reducing environment of the cytosol (Hwang et al. 1992). Therefore, disulfide bridges are mostly found in secretory proteins, lysosomal proteins, and the exoplasmic domains of membrane proteins. This makes it questionable whether disulfide bridges can exist in native CA VII enzymes located within the cytosolic microenvironment. There are, however, some exceptions to the rule described above. Some cytosolic proteins have cysteine residues in proximity to each other and are regulated by reversible disulfide bond formation (Jakob et al. 1999, Kuge et al. 2001). It remains to be studied further whether a similar phenomenon can occur in CA VII.

Recombinant full-length CA VII enzyme was used to raise the anti-human CA VII serum for immunohistochemistry and western blotting. The predicted mass difference of 6 kDa between the full-length and shortened CA VII allowed us to determine which CA VII forms are expressed in native murine tissues. A single 30 kDa polypeptide band was observed in CA VII-positive tissues, indicating that only the full-length form is expressed at detectable levels. Positive signals for CA VII were detected in the mouse liver, muscle, and colon. Western blotting showed no signal for CA VII in the mouse cerebrum and cerebellum. The lack of signal was probably due to the low and restricted expression of the CA VII protein in the brain. This also agrees with *in situ* hybridization results, showing a very limited signal for CA VII in brain tissues (Lakkis et al. 1997).

In addition to the western blotting, we also used immunohistochemical techniques to investigate the CA VII distribution in selected mouse tissues. Our investigations revealed some weak signal for CA VII in a limited number of gastric cells, which were located in the upper segment of the gastric glands. In the duodenum, CA VII was observed in the Brunner's glands. In the colon, CA VII was restricted to the lamina propria. The highest signal was observed in the hepatocytes of the liver, which was a totally novel and important finding of our study. It was also in line with the information from GeneSapiens database (http://ist.genesapiens.org), which suggested higher CA VII mRNA levels in the human liver than in any other organ. The immunohistochemical signal for CA VII showed no singnificant zonal variation within the hepatic lobule. The high expression of CA VII in the liver placed this protein into a strategic position to regulate acid-base homeostasis and other bicarbonate ion-dependent processes together with the other CAs present in the hepatocytes, including mitochondrial CA VA and membrane-associated CA XIV (Parkkila et al. 2002, Shah et al. 2000).

6.2 CA VII expression in malignant tissues

The succesful production of a novel polyclonal antibody against human CA VII prompted us to test CA VII immunohistochemical staining not only in normal tissues but also in cancer. Malignant gliomas were selected, because the information from the GeneSapiens database (http://www.genesapiens.org) suggested that CA VII mRNA is highly expressed in these tumors. Prior to our present study some CA isozymes, namely CA II, IX, and XII, were shown to represent promising biomarkers for certain brain tumors. They do not only act as a marker for specific tumors but have also shown correlation with prognosis in some tumors (Haapasalo et al. 2007a,b, Haapasalo et al. 2006, Nordfors et al. 2010).

We investigated the expression of CA VII in three different categories of malignant gliomas, including the astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas. Our result indicated that CA VII expression is higher among the high grade infiltrating tumors. The positive staining was often very strong in a hypoxic region close to the necrotic area. Even though CA

VII has not been considered a HIF-regulated isozyme, in contrast to CA IX and CA XII, its immunostaining pattern showed that hypoxia may regulate CA VII expression by an unknown mechanism.
Our present study showed that CA VII immunoreactivity did not correlate with cell proliferation which was examined by MIB-1 immunostaining, nor did we detect any association with EGFR-amplification or p53 protein expression in malignant gliomas. Interestingly, our results showed that high expression of CA VII is associated with poor prognosis in malignant astrocytomas. Therefore, CA VII represents the fourth CA isozyme which seems to correlate with survival in astrocytoma patients. Prior to our study, CA II, CA IX and CA XII have been identified as promising biomarkers with prognostic value in diffuse astrocytomas (Haapasalo et al. 2008, Haapasalo et al. 2006, Korkolopoulou et al. 2007). The expression of several CAs in malignant gliomas probably reflects the requirement for a rapid turnover of acid metabolic products in tumor cells. The cytosolic isozymes, such as CA II and CA VII are probably important in rapid neutralization of the cell interior. Membrane-bound CA XII and CA IX conceivably contribute to extrusion of protons in a metabolon system, which contains ion transport proteins associated with CAs (Pastorekova et al. 2004, Pastorekova et al. 2006). The expression of several CAs in tumors also suggests promising opportunities to refine cancer therapies. Drug development research has been already focused on different compounds as potential CA inhibitors (Supuran 2008), which may act in several manners. Some of the CA inhibitors may inhibit the neoangiogenesis and reduce the tumor growth (Xiang et al. 2004), while others can play different roles involving e.g. decreased invasion of tumor cells (Supuran 2003, Supuran 2008, Supuran et al. 2001). Regardless the mechanism, the ultimate goal is to design novel drugs which can decrease the mortality rate in these devastating cancers.

6.3 Acetaldehyde-derived modifications in cytosolic CAs

Acetaldehyde, the first product of ethanol metabolism, has been established as a toxic molecule to form both stable and unstable adducts with different proteins and cellular constitutes (Lieber 1995, Niemela et al. 1994). Such adducts formation on different proteins, lipids and nucleic acid can cause disturbances in the physicochemical structures and functions of target molecules (Niemela and Parkkila 2004, Sorrell and Tuma 1987). In our present study, we investigated and detected such adduct formation on five cytosolic CAs I, II, III, VII, and XIII. Based on previous research, CAs are known to be important enzymes with remarkable biological functions, and thus disturbances in their structure and activity could potentially lead to severe pathophysiological consequences.
CA II, as the most active CA isozyme, was the first target for acetaldehyde-derived modifications in our analyses. Later, we also investigated all other cytosolic isozymes when suitable recombinant proteins were produced and became available for biochemical characterization. The structural and functional changes in CAs were monitored with mass spectrometry and kinetic assays, respectively. Mass spectrometric analysis confirmed that one of the primary NH_2 groups (e.g., in lysine side chain) in CA II had reacted with acetaldehyde under non-reducing condition, consistent with a "Schiff base" formation. In reducing conditions, each CA II molecule, which contains a high number of lysine residues (24 Lys), had reacted with 9-19 (14 on average) acetaldehyde molecules to form stable covalent adducts. The result suggested that most lysine residues present on the surface of CA II molecule might react with acetaldehyde under reducing conditions, which can occur e.g. in hepatocytes after chronic alcohol ingestion.

CA I, CA III, CA VII, and CA XIII contain a lower number of lysine residues in the sequence, and furthermore, the lysine residues may not be as easily accessible on the molecular surface when compared to CA II. We also believe that the adduct formation showed only minor effects on the kinetic properties of all these enzymes, because none of the lysine residues are located in the near proximity to the active site of the enzyme molecule.

Previous studies have indicated that acetaldehyde can bind to human proteins and generate antigenic determinants which trigger a corresponding immune response against such epitopes (Worrall et al. 1993). This humoral immune response may be implicated in autoantibody formation and liver damage associated with excessive alcohol consumption (Niemela et al. 1987). It has been also shown that acetaldehyde modified splenic cells can generate cytotoxic T lymphocytes (CTL), and the ability of the CTL to lyse specific targets is dependent on the formation of stable acetaldehyde adducts (Terabayashi and Kolber 1990). Although acetaldehyde has several direct disadvantageous effects on normal cell function, such as stimulation of carcinogenic processes (Yu et al. 2010), it is conceivable that induction of autoimmunity against the target antigens can be another harmful effect with very serious pathophysiological consequences. Although the structural modifications due to acetaldehyde treatment only mildly affected the kinetic properties of cytosolic CAs, it is conceivable that autoantibodies, whenever present, could have more deleterious effects on the catalytic function of CAs *in vivo*. We examined the presence of IgG-class autoantibodies against the CA isozymes in alcoholic patients. The results indicated no difference between the values obtained from alcoholic or normal subjects. Since previous studies have indicated significant inductions in IgA autoantibody responses (Viitala et al. 1997), suitable immunoassays to detect IgA-class antibodies will be developed in our future studies.

7. SUMMARY AND CONCLUSIONS

During the present study, human recombinant CA I, III, and VII enzymes were successfully cloned, and expressed in *E.coli* and then purified to homogeneity. Human CA VII was characterized with biochemical methods. CA VII expression was observed in some normal human tissues as well as in malignant gliomas.
The present results also provided the first *in vitro* evidence on acetaldehyde-adduct formation in cytosolic CA I, II, III, VII, and XIII enzymes. The following three conclusions are proposed based on the observations made during the present investigations:

1. Two forms of human CA VII mRNA exist in GenBank. Only the full-length CA VII is detectable at protein level in tissues. It is most highly expressed in the liver, colon, and skeletal muscle.

2. CA VII isozyme is expressed highly in malignant gliomas, including oligodendrogliomas, oligoastrocytomas and diffusively infiltrating astrocytomas. The positive immunostaining correlates with poor prognosis in patients with malignant astrocytomas.

3. Acetaldehyde, the first metabolite of ethanol, can bind to human cytosolic CA enzymes *in vitro* and form stable and unstable adducts. The acetaldehyde binding shows only minor effects on the CA catalytic activity. The highest inhibition by acetaldehyde treatment is observed for CA II, which can contain up to 19 acetaldehyde molecules / one enzyme molecule.

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هواخواه توام جانا و میدانم که می دانی که هم نادیده می بینی و هم ننوشته می خوانی

خدایا، تو را شکر و سپاس که روزهای جوانی مرا صرف آموختن علم کردی، پدرم را بیامرز که او نام نیکو بر من نهاد که هرچه در دنیا یافتم از برکت نام فاطمه بود، پس مرا در آخرت همنشین بانوی دو عالم قرار ده. به مادرم سلامت و عمر طولانی عطا کن که از او استقامت و سختکوشی آموختم و او مرام مولا و عشق اهل بیت به من آموخت. مادرم، بانوی زیبای من، بر دست ها و پاهای خسته ات بوسه می زنم و این رساله دکتری را با تمام عشقم تقدیم و پیشکش می کنم به تو و پدرم که دعای خیرتان همیشه بدرقه راه من بوده و هست. عزیزانم، امیر، علی، الیزابت، سارا و دانیال شما را بسیار دوست می دارم و برایتان آرزوی خوشبختی و سلامتی می کنم، متشکرم که همیشه مرا همراهی و تشویق کرده اید. عزیزانم، نازی، شیما، وحید، مینا، فریناز و عاطفه شما که برای من بهترین دوستان بوده و هستید، خاطرات زیبای با شما بودن را همیشه در یاد خواهم داشت، ممنون که در تمام این سال ها همیشه در کنار من بودید، برایتان بهترین آرزوها را دارم. از همه کسانی که مرا با دستی، قلمی، کلامی و نفسی همراهی کردند متشکر و سپاسگزارم. امیدوارم این رساله شاهدی باشد بر بندگی و خضوع من در پیشگاه آفریدگاریکتا که من در برابر پیچیدگی آفرینش بیش از پیش سر بر سجده فرود می آورم.

ایران، سرزمین زیبای من، افتخار من، غرور من که من دختری از ایران زمینم. این رساله دکتری با تمام سختی ها و مشقات به پایان نمی رسید مگر با یاری و اراده خداوند و عشق اهل بیت.

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بدین شعر تر شیرین ز شاهنشه عجب دارم

که سر تا پای حافظ را چرا در زر نمی گیرد

یاحق

فاطمه بوترابی فنلاند ۱۳۹۰(۲۰۱۱)

Fatemeh
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