Jukka Leinonen

CARBONIC ANHYDRASE ISOENZYME VI: DISTRIBUTION, CATALYTIC PROPERTIES AND BIOLOGICAL SIGNIFICANCE
Secretory carbonic anhydrase isoenzyme VI (CA VI) catalyses the reversible hydration of carbon dioxide ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$). Low concentrations of salivary CA VI are associated with high decayed, missing or filled teeth (DMFT) index scores and a high incidence of acid injury in the upper gastrointestinal tract plus lowered taste and smell perception. Two mechanisms of action for CA VI have been proposed: acid neutralisation and growth factor function.

In the present study the distribution and catalytic properties of CA VI have been examined in order to further clarify its mechanisms of action and biological significance. CA VI was found to be present and secreted by the alveolar epithelium of the mammary gland, serous acinar cells of lingual von Ebner’s glands, serous demilune cells of posterior lingual mucous glands and serous cells of submucosal tracheobronchial glands. CA VI was also found in the serous cells in the tracheobronchial mucosal epithelium, taste pore, taste bud, base of the tracheobronchial cilia, bronchiolar Clara cells and enamel pellicle. An immunofluorometric assay showed that the mean concentration of CA VI in colostral milk was eight times higher than that in mature milk (35 mg/l vs. 4.5 mg/l). Stopped-flow spectroscopy measurements revealed that the dehydration activity of CA VI is moderate (maximum $k_{cat} = 3.0 \times 10^5 \cdot s^{-1}$).

The finding that CA VI is a potent catalyst of acid neutralisation emphasizes the possible role of the pellicle bound CA VI in local neutralisation of the acidic metabolic products of dental biofilm. The function of CA VI in von Ebner’s glands’ saliva is likely taste stimuli modification via CA activity although other functions may exist. Its role in milk or respiratory tract mucus remains open, however, as these secretions do not have significant acid predispositions that would need enzymatic catalysis for removal.

Keywords: carbonic anhydrase, catalysis, immunochemistry, milk, saliva, trachea, von Ebner’s glands
To my family
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSA-PBS</td>
<td>Bovine serum albumin in phosphate-buffered saline</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
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<tr>
<td>CA VI</td>
<td>Carbonic anhydrase isoenzyme VI</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMFT</td>
<td>Decayed, missing or filled teeth</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>Turnover number, the maximum number of enzymatic reactions catalysed per second</td>
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<tr>
<td>$K_m$</td>
<td>Michaelis constant, substrate concentration at which the rate of the enzyme reaction is half of the maximum</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton, unit of molecular mass</td>
</tr>
<tr>
<td>nonO</td>
<td>Non-POU (Pit-Oct-Unc) domain-containing octamer-binding protein</td>
</tr>
<tr>
<td>p54$^{nr}$</td>
<td>Nuclear RNA-binding protein, 54 kDa</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>pK_a</td>
<td>Acid dissociation constant</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


Additionally, unpublished original data on CA VI are included.
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1 Introduction

Carbonic anhydrases (CAs) catalyse the reversible hydration of carbon dioxide: $\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{H}^+ + \text{HCO}_3^-$. This reaction is the basis of the most important acid-base buffer system in mammals (Pocock & Richards 2001). The thirteen active CAs known today have overlapping functions, which, at least to some degree, explains the fact that only a few exact functions of single CA isoenzymes have been identified. Carbonic anhydrase isoenzyme VI (CA VI) is the only secretory CA (for review see e.g. Pastorekova et al. 2004). Its functions have been connected to dental caries, acid injury of the gastrointestinal mucosa, and taste and smell dysfunction in clinical studies (Parkkila et al. 1997, Henkin et al. 1999a, b, Kivelä et al. 1999). Patients with low salivary CA VI concentrations are susceptible to dental caries and acid injury of the gastroesophageal mucosa, as well as a decreased sense of taste and smell. The first two pathogenic consequences are hypothesised to result from decreased CA activity, whereas the defects in sensory functions are probably attributable to different mechanisms of action. An intracellular form of CA VI (CA VIB) has been found in the cytosol, but its enzymatic activity and function have not been studied (Sok et al. 1999).

Since CA VI is the only secreted CA, it is probably expressed in several glands and their secretory products. In previous studies, CA VI has been found in the saliva, salivary glands, lacrimal glands, pancreas, nasal glands, serum, bovine milk, oesophagus, hair follicle, forestomach, large intestine, enamel organ, mucosa of the upper alimentary tract and liver (Fernley et al. 1979, Parkkila et al. 1990, Ogawa et al. 1995, 2002, Kivelä et al. 1997b, Fujikawa-Adachi et al. 1999, Ichihara et al. 2003, Murakami et al. 2003, Ishimatsu-Tsuji et al. 2005, Kubota et al. 2005, Kaseda et al. 2006, Kasuya et al. 2007, Nishita et al. 2007). This thesis provides novel findings on the expression and enzyme kinetics of CA VI as well as functional linkages suggesting that CA VI is a widely expressed constituent of excretory fluids and a potent catalyst for pH regulation.
2 Review of the literature

2.1 Saliva

Human saliva is secreted into the oral cavity from the salivary glands, primarily by the paired large salivary glands (parotid, submandibular and sublingual glands). Numerous minor salivary glands are distributed all over the oral submucosa. Most of them are comprised of only one acinus but four larger groups can also be identified. In the posterior tongue beneath the taste buds lie the von Ebner’s glands and further back lie the posterior lingual mucous glands. Paired accessory parotid glands are occasionally situated in the cheeks along the parotid ducts. The salivary glands consist of tubuloacinar secretory endpieces and a branching ductal system. Acinar and tubular endpieces are comprised of serous and mucous cells, respectively. Serous cells produce protein-rich saliva while mucous cells produce carbohydrate-rich saliva. Mixed endpieces contain serous demilune cells among the mucous cells. The ductal cells modify the ion concentrations of saliva by absorbing water and ions and secreting ions (Burkitt et al. 1993). The secretion rate and composition of saliva follow circadian periodicity (Parkkila et al. 1995, Kivelä et al. 1997b) and are controlled by the autonomic nervous system. Taste stimuli affect the saliva composition and secretion rate (Froehlich et al. 1987). The total daily output of saliva is approximately 0.5–1.5 litres. Non-stimulated saliva has a mean flow rate of 0.3 ml/min whereas mastication or tastant stimulated saliva is secreted at the rate of about 2 ml/min (Kivelä et al. 1999, Humphrey & Williamson 2001). The flow rate and composition of saliva vary among people. Low flow rates are associated with dental caries and a sensation of oral dryness (Leone & Oppenheim 2001, Pedersen et al. 2005). Hyposalivation denotes a measurably lowered flow rate of saliva whereas xerostomia denotes a subjective feeling of oral dryness (Nederfors 2000).

Whole saliva is composed of secretory products of salivary glands, oral bacteria, their metabolic products, blood components diffused through crevices and oral epithelial cells. About 99% of whole saliva is water. Although saliva is initially isotonic to serum, the ductal cells absorb sodium and chloride. This renders whole saliva hypotonic compared to serum but rich in potassium, chloride, sodium, bicarbonate, phosphate and magnesium. The mean protein concentration of whole saliva is 1.15 g/l and the pH is 7.3 (Streckfus et al. 1994, Kivelä et al. 1999). Salivary α-amylase and proline-rich proteins constitute most of the protein in saliva.
whereas histatins, statherin, lysozyme, lactoferrin, peroxidases, secretory immunoglobulin A and CA VI are present in lower concentrations.

Saliva has several functions in the gastrointestinal tract. The importance of saliva to oral well-being is demonstrated in patients with hyposalivation who have rampant caries, a feeling of oral dryness, and difficulty in swallowing and speaking (Christensen et al. 2001, Pedersen et al. 2005, for review see Soto-Rojas & Kraus 2002). Saliva coats the mucosal surfaces, lubricating and protecting them (Bradway et al. 1989, 1992, Pedersen et al. 2002). It also contains growth factors that are essential for the oral mucosa and rinses food debris and bacteria from tooth and mucosal surfaces (Lipps 2000, Morris-Wiman et al. 2000, Markopoulos et al. 2001, Gröschl et al. 2005). Several factors of saliva (e.g. histatins and peroxidases) possess antimicrobial activity (De Smet & Conteras 2005, Ihalin et al. 2006). Salivary minerals are important in maintaining tooth integrity. Acidity derived from foods, drinks and bacterial metabolism is buffered by saliva. Saliva also prevents acid injury to the gastrointestinal tract mucosa (Helm et al. 1984). The most important buffering system in saliva is based on bicarbonate, although other significant systems are based on phosphate and protein (Helm et al. 1982, Bardow et al. 2000a, b). A low salivary buffering capacity is associated with high DMFT index score (Kivelä et al. 1999).

Specific salivary proteins attach to the dental enamel forming a 0.3–1.1 µm thick protein layer termed the acquired enamel pellicle (Kousvelari et al. 1980, Al-Hashimi & Levine 1989, Amaechi et al. 1999). In vitro studies have shown that the pellicle prevents demineralisation of the enamel caused by cariogenic bacteria and acidity (Zahradnik et al. 1976, 1977, 1978, Featherstone et al. 1993, Nekrashevych & Stösser 2003). The pellicle matures over time and 7-day-pellicle decreases demineralisation more effectively than 1-day-pellicle (Sönju & Rölla 1973, Zahradnik et al. 1979, Featherstone et al. 1993, Skjorland et al. 1995). However, incubation of teeth with saliva for only three minutes forms a pellicle that decreases demineralisation as effectively as a 2-hour pellicle (Hannig et al. 2004). The mechanism by which the pellicle protects teeth from demineralisation appears to result from the neutralisation of acid that restricts hydrogen diffusion to the enamel (Hannig & Balz 2001, Nekrashevych & Stösser 2003).

2.2 Dental caries

Dental caries is tooth decay caused by oral bacteria. Oral bacteria build up dental plaque on the pellicle that together comprise a dental biofilm. The biofilm matures
over time enabling e.g. wide bacterial diversity, pH gradient and formation of functional matrix (Garcia-Godoy & Hicks 2008). Some strains of bacteria in biofilm, especially *Streptococcus Mutans*, produce substantial amounts of acid as an end product of their sugar metabolism (Kleinberg 2002). The solubility constant of hydroxyapatite, the principal mineral of enamel, is increased by acidity (Larsen & Jensen 1989). Saliva, enamel and pellicle neutralise biofilm borne acids to some extent but considerable and long-lasting acid exposure causes demineralised caries lesions (Zahradnik *et al.* 1976, 1977, 1978, Nekrashevych & Stösser 2003). Accordingly, patients with high caries prevalence have lower initial dental biofilm pH and lower minimum dental plaque pH after sugar consumption plus slower recovery time to initial pH after sugar consumption than caries-free patients (Stephan 1944). However, the association of dental plaque pH with caries prevalence has not been found in some other studies (Fejerskov *et al.* 1992, Dong *et al.* 1999). Minor caries lesions, especially the ones not extending to the dentin, may remineralise if favourable surroundings are restored (Zahradnik 1979). On some instances even large lesions may remineralise. But if the lesion extends through the enamel and oral bacteria infect the dentin the patient’s need for restorative dental care is probable (Fejerskov & Kidd 2003).

Dental caries is a common global health problem (Petersen 2003). In Finland 37% of adolescents reported tooth ache during the preceding two years (Honkala *et al.* 2001). Most of the dental care is related to caries. In 2005 the dental care expenditure was 606 million euros comprising 5.1% of our total health-care expenditure and 0.4% of gross domestic product in Finland (Matveinen & Knape 2007). A subgroup of 20–25% of children has 50% of all caries (Winter 1990). The high caries prevalence of these particular individuals results from several factors e.g. diet, oral microbiota, personal oral hygiene plus saliva composition and flow rate.

### 2.3 Taste perception

Taste stimuli dissolved in saliva are converted into afferent neural signals by neuroepithelial taste receptor cells. The stimuli elevate intracellular calcium levels and trigger the release of neurotransmitters to the proximity of gustatory afferent nerve fibres. These transmit the signal to the central nervous system where the actual taste perception takes place (for review see Lindemann 2001, Gilbertson & Boughter 2003). Taste receptor cells are located in taste buds which are distributed all over the oral mucosa but most of them are found in the tongue (Avery 1987).
About one fourth of all taste buds are situated in the tops of fungiform papillae that are located all around the oral cavity. Nearly all of the remaining three quarters of taste buds are situated in the lateral walls of foliate and circumvallate papillae. In each taste bud there are 50 to 150 individual cells of which 10 to 14 are taste receptor cells. They don’t divide but new cells arise from the proliferative basal cell layer (Stone et al. 1995). The space where taste receptor cell microvilli protrude to the oral cavity is called a taste pore. The microvilli are rich in chemoreceptors and ion channels that react to taste stimuli. Taste sensation is concentration-dependent but also the size of the taste stimulus application area affects sensation (Smith 1971). Five basic tastes can be perceived: sour, salty, bitter, sweet and umami. Single taste receptor cells can respond to several taste stimuli (Gilbertson et al. 2001). Responses to sweet, bitter and umami tastes are mediated by receptor proteins (Bernhardt et al. 1996, Lin & Kinnamon 1999, Zhao et al. 2003, Pronin et al. 2004). Salty taste reception is mediated by direct movement of the cations sodium, potassium, ammonium and calcium to taste receptor cells (Kloub et al. 1997). Sour taste reception is a result of acidification of a subset of taste receptor cells, although acid-sensing ion channels have been proposed to take part in sour sensing as well (Miyamoto et al. 1998, 2000, Lyall et al. 2001, Stevens et al. 2001, for review see DeSimone et al. 2001). Weak organic acids (e.g. acetic acid and dissolved carbon dioxide) are believed to enter taste receptor cells more readily than protons, because they elicit stronger reception and perception of sourness plus lower intracellular pH in taste receptor cells than their virtual acidity would suggest (Lyall et al. 2001, Lugaz et al. 2005).

Systemic carbonic anhydrase inhibition causes alterations in basic taste sensations when carbon dioxide containing beverages are ingested (Graber & Kelleher 1988, Miller & Miller 1990). In rats the inhibition of carbonic anhydrase causes inhibition of carbon dioxide sensing (Komai & Bryant 1993, Simons et al. 1999, Lyall et al. 2001, 2002). Rat taste buds contain CA activity and CA II has been localised there (Brown et al. 1984, Dnaikoku et al. 1999). Interestingly, CA activity is also present in the lingual von Ebner’s glands that excrete saliva to the bottom of the trenches surrounding the taste receptor cell-rich circumvallate and foliate papillae (Brown et al. 1984). The function of von Ebner’s glands is closely associated with taste reception, above all in dissolving and diluting the stimuli and washing the clefts (Gurkan & Bradley 1988, Sbarbati et al. 1999). Removal of submandibular and sublingual salivary glands damages all taste receptor cells in the dorsal tongue but not those located in the circumvallate papillae suggesting that
the saliva of the von Ebner’s glands is an important source of growth factors for taste receptor cells (Morris-Wiman et al. 2000).

2.4 Milk

The mammary glands are paired modified sweat glands located within the subcutaneous tissue. The development of the mammary glands is completed during pregnancy as the glandular tissue increases and the amount of fat and connective tissue decreases. After parturition the alveoli become dilated with milk and have only a thin epithelial lining. The secretory process for milk proteins is merocrine and for lipids apocrine. The constituents vary during pregnancy and subsequent nursing. At the end of the pregnancy and for 1–5 days postgestationally the mammary glands secrete protein- and lactose-rich colostrum. Thereafter the protein content of the milk decreases, and it is called mature milk that contains 88% water, 7% carbohydrate, 3.5% lipids, 1.5% protein and ions (particularly sodium, potassium, chloride, calcium and phosphate). Milk proteins are classified into three major groups: caseins, whey proteins and mucins (fat globule membrane proteins). The mucin concentration of milk remains the same during lactation but the whey-to-casein ratio varies from 9:1 in early lactation to 1:1 in late lactation. The total protein concentration of milk gradually decreases from 15 g/l in early lactation to 8 g/l at 6 months and onwards (Lönnerdal 1976, Kunz & Lönnerdal 1992). Colostrum has especially high levels of immunological components. Milk not only provides nutrients to the infant but also possesses growth factors and components that protect the infant from viral, yeast and bacterial infections (Dewey et al. 1995, Shi & Kong 2000, Lee et al. 2004). β-lactoglobulin, α-lactalbumin and caseins α1-2, β and κ constitute most of the milk protein. The caseins take part in curd formation which lengthens the time the milk is in the stomach. β-lactalbumin induces lactose synthesis but the function of β-lactoglobulin is uncertain (for review see Threadgill & Womack 1990, Kontopidis et al. 2004). Several other proteins are found in minor amounts in milk. Some of them are growth promoting like epidermal growth factor that stimulates the proliferation of intestinal cells (Chang & Chao 2002, Takeda et al. 2004). Others, for example lactoferrin, have an anti-microbial effect (van der Strate et al. 2001).

Some intact milk proteins can be found in the stools of breastfed infants suggesting that they have enzymatic, immunological or other functional significance throughout the gastrointestinal tract of the infant (Davidson & Lönnerdal 1987).
Interestingly CA activity has been found in histochemical studies of the goat mammary gland and the amount of activity correlates with the volume of secreted milk (Cvek et al. 1998).

2.5 Structure, function and development of the respiratory tract

The lower airways consist of the trachea, the primary bronchi and the lungs. The luminal surfaces of airways are covered by mucosa and surrounded by the submucosa. The mucosal epithelium consists of ciliated cells, mucous goblet cells, brush cells, small granule cells and basal cells. In rats there are also serous cells in the epithelium. In humans, serous cells are found in the tracheal epithelium only in the foetus. The submucosa is composed of a relatively loose connective tissue that also contains seromucous glands. Their ducts penetrate the mucosa. The surface liquid on the airway mucosa is produced predominantly by the submucosal glands, but also by the epithelial secretory cells. The airway surface liquid can be divided into the periciliary liquid layer and the mucus covering it. Inhaled small particles, e.g. bacteria, attach to the mucus and are deployed to the larynx by a coordinated sweeping motion of the cilia. Cilia are flexible membrane extensions of the cell that are found in vast numbers on the respiratory tract luminal surfaces (more than 10 million per square millimetre). The low viscosity of the periciliary liquid is essential for the function of cilia (for review see Boucher 2004). The airway surface liquid also possesses antibacterial proteins and properties (Nakayama et al. 2002, for review see Verkman et al. 2003).

The trachea divides gradually into several smaller airways (bronchi, bronchioles, respiratory bronchioles, alveolar ducts and alveoli). Simultaneously the mucosal epithelium becomes cuboidal and may be ciliated. The height of the epithelial cells and the number of seromucous glands decreases as the lumen becomes smaller in diameter. There are also some goblet cells, brush cells, dense-core granule cells and protein-secreting Clara cells. The airways end in gas exchanging units called alveoli. They are composed of type I and II pneumocytes with some macrophages and brush cells. The type II pneumocytes are secreting cells producing the surfactants that e.g. prevent the adhesion of alveoli walls during expiration. The walls of the alveoli are very thin in parts. Between the inhaled air and the blood there are only three layers including an extremely thin type I pneumocyte, the basal lamina of the alveolar epithelium and the capillary endothelium (Burkitt et al. 1993).
2.6 Carbonic anhydrases

2.6.1 General properties

Carbonic anhydrases are proteins that catalyse the reversible hydration of carbon dioxide: $\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{H}^+ + \text{HCO}_3^-$ (for review see Lindskog 1997, Pastorekova et al. 2004). CAs have various functions e.g. in pH homeostasis, CO$_2$ transfer and ion exchange (for review see Chegwidden et al. 2000). Three gene families named α, β and γ have been recognized. In vertebrates the α-CAs are dominant (Hewett-Emmett 2000). So far thirteen active α-CAs and three genetically related, but acatalytic CAs, have been identified in vertebrates. Isoenzymes CA I-III, CA VIB, CA VII and XIII are cytosolic, CA V A and B are mitochondrial, CA VIA is secreted and CA IV, CA IX, CA XII, CA XIV and CA XV are membrane-bound (Fig. 1) (Pastorekova et al. 2004, Hilvo et al. 2005). The nuclear protein nonO/p54$^\text{nh}$ has CA activity but only little sequence homology to CAs and therefore nonO/p54$^\text{nh}$ is considered a non-classical CA (Karhumaa et al. 2000).

![Fig. 1. Location of the active $\alpha$-CA isoenzymes in a vertebrate cell.](image)

The genes of the membrane-bound and secreted $\alpha$-CAs form a group that has evolved separately from the cytosolic and mitochondrial $\alpha$-CAs (Fig 2) (Jiang & Gupta 1999, Hewett-Emmett 2000).
Fig. 2. Phylogenic table of α-CAs.
The catalytic site is conserved in the active CAs (Hewett-Emmett & Tashian 1996). Three conserved histidine residues (His93, 96, 119) bind a zinc ion (Berg et al. 2002). The zinc ion binds water lowering the ionisation constant pK\textsubscript{a} of water from 15.7 to 7. In the first reaction step of CA catalysed carbon dioxide hydration, the zinc-bound water ionises by releasing one of its protons to form a CA bound OH\textsuperscript{−}-group as in [1]: (Chegwidden & Carter 2000)

\[ [1]: \text{CA-Zn}^{2+}\text{-H}_2\text{O} \Leftrightarrow \text{CA-Zn}^{2+}\text{-OH}^- + \text{H}^+ \]

In the second reaction step [2] the CA bound OH\textsuperscript{−}-group attacks CO\textsubscript{2} to produce HCO\textsubscript{3}\textsuperscript{−}, which is then replaced by water as in [3]:

\[ [2]: \text{CA-Zn}^{2+}\text{-OH}^- + \text{CO}_2 \Leftrightarrow \text{CA-Zn}^{2+}\text{-HCO}_3^- \]
\[ [3]: \text{CA-Zn}^{2+}\text{-HCO}_3^- + \text{H}_2\text{O} \Leftrightarrow \text{CA-Zn}^{2+}\text{-H}_2\text{O} + \text{HCO}_3^- \]

The properties of the buffer solution affect the catalytic activity of CAs by accelerating the diffusion or release of protons (Berg et al. 2002). Some buffers are too large to reach the active site of CAs. CAs possess proton shuttle structures that enhance the movement of protons between the active site of the enzyme and the outer surface (Tu et al. 1989, Duda et al. 2001, Fisher et al. 2005). The buffer and the proton shuttle enable CA II to catalyse the hydration of carbon dioxide a hundred times faster than in their absence (Berg et al. 2002). The hydration of carbon dioxide occurs spontaneously at a rate of k\text{cat}=0.15 \text{ s}^{-1} (k\text{cat}/K\text{m}=0.0027 \text{ M}^{-1} \text{ s}^{-1}), but CAs accelerate the reaction to over a million molecules per second (Khalifah 1971, Berg et al. 2002). The uncatalysed rate of bicarbonate dehydration k\text{cat} is 50 \text{ s}^{-1} but CAs are physiologically significant because they catalyse the dehydration rate to an even faster velocity (Tsuruoka & Schwartz 1998, Berg et al. 2002). The enzyme activity profile for the hydration of carbon dioxide as a function of pH has been characterised for CA isoenzymes I–VA, VII, IX and XII (Khalifah 1971, Heck et al. 1994, Baird et al. 1997, Hurt et al. 1997, Earnhardt et al. 1998, Ulmasov et al. 2000, Wingo et al. 2001, Nishimori et al. 2005). The pH profile is a saturation curve that maintains near-maximal values in the pH range 7.5–9 whereas the values decrease considerably as a function of acidity below pH 7.5. CA II has very high activity (k\text{cat}=1.4 \times 10^6 \text{ s}^{-1} and k\text{cat}/K\text{m}=1.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}) whereas CA III has low activity. The other enzymes have 20–70% of the activity of CA II (Wingo et al. 2001, Nishimori et al. 2005, Hilvo et al. 2008). In pH 5.5–
7.5 the kinetic constants $k_{cat}/K_m$ and $k_{cat}$ for the CA catalysed dehydration of bicarbonate increase as a function of acidity. The maximal value for $k_{cat}/K_m$ of CA IV for dehydration is $2.8 \times 10^7$ M$^{-1}$ s$^{-1}$ in pH 5.5, being 2–3 times higher than the value for CA II and CA VII (Baird et al. 1997, Earnhardt et al. 1998).

2.6.2 CA VI

Two subtypes of CA VI are known. The A-type is thus far the only known secreted member of the α-CA family and the B-type a stress-induced cytoplasmic form (Sok et al. 1999, Kubota et al. 2005). They have separate promoters but the polypeptides differ only in that the B-type lacks the signal peptide (Sok et al. 1999). The B-type has been found from mouse embryonic fibroblasts, salivary glands and ameloblast-derived cell lines (Sok et al. 1999, Kubota et al. 2005). So far there is no information about CA activity in the B-type, and therefore in this thesis, the A-type is called simply CA VI. CA VI has also been called gustin, although Thatcher et al. (1998) reported that gustin and CA VI are in fact the same protein.

The gene encoding CA VI is located in the tip of the short arm of chromosome 1 (Sutherland et al. 1989). Human CA VI has 68%, 71.5% and 74% amino acid sequence identity with sheep, ovine and canine CA VI, respectively (Aldred et al. 1991, Jiang et al. 1996). In the C-terminus of CA VI there is a 10 amino acid extension compared to cytosolic CAs (Aldred et al. 1991). Human CA VI has 308 amino acids that include a 17 amino acid leader sequence in the N-terminus (Aldred et al. 1991). Bovine, sheep and canine CA VI have about 10 amino acids more in the C-terminus than does the human CA VI (Fernley et al. 1988b, Aldred et al. 1991, Jiang et al. 1996). According to the cDNA sequence of human CA VI the molecular size of the mature polypeptide backbone is 33.6 kDa (Aldred et al. 1991). Using the predicted polypeptide sequence, the mature bovine CA VI polypeptide has been estimated to be 35.5 kDa (Fernley et al. 1988b, Jiang et al. 1996). Using SDS-PAGE the size of the mature CA VI has been estimated to be 37–46 kDa (Feldstein & Silverman 1984, Kadoya et al. 1987, Murakami & Sly 1987, Fernley et al. 1988a, Parkkila et al. 1990, Ogawa et al. 1992, 2002, Kivelä et al. 1997b, Thatcher et al. 1998, Kimoto et al. 2004). CA VI has two potential N-glycosylation sites and oligosaccharide side chains that total 4–9 kDa (Murakami & Sly 1987, Fernley et al. 1988a, Thatcher et al. 1998). Different glycosyltransferases present in the parotid and submandibular glands produce different glycoforms of CA VI (Hooper et al. 1995a, b). CA VI appears also as a 7–12 fold larger mole-
cule, suggesting that the enzyme forms homologic complexes (Fernley et al. 1979, 1988a). There is a disulphide bond between cysteine residues Cys^{25} and Cys^{207} of ovine CA VI (Fernley et al. 1988b).

The catalytic activity for hydration by CA VI has been reported to be moderate with \( k_{\text{cat}}/K_{m} = 4.9 \times 10^{7} \text{ M}^{-1} \text{ s}^{-1} \) (Feldstein & Silverman 1984, Fernley et al. 1988a, Nishimori et al. 2007a, b). CA VI has been found from saliva, salivary glands, lacrimal glands, pancreas, nasal glands, serum, bovine milk, oesophagus, hair follicles, forestomach, large intestine, enamel organ the mucosa of upper alimentary tract and liver (Table 1) (Fernley et al. 1979, Parkkila et al. 1990, Ogawa et al. 1995, 2002, Kivelä et al. 1997b, Fujikawa-Adachi et al. 1999, Ichihara et al. 2003, Murakami et al. 2003, Ishimatsu-Tsuji et al. 2005, Kimoto et al. 2004, Kubota et al. 2005, Kaseda et al. 2006, Kasuya et al. 2007, Nishita et al. 2007). CA VI is primarily expressed in the serous acinar cells but is also expressed in the ductal cells (Parkkila et al. 1990, Ogawa et al. 1993, Ichihara et al. 2007).
Table 1. Expression of CA VI in mammalian tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Species</th>
<th>Immunochemistry</th>
<th>Western blot</th>
<th>mRNA</th>
<th>Immunoassay</th>
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<tr>
<td>Parotid gland</td>
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<td>*</td>
<td>*</td>
<td>RT-PCR</td>
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<tr>
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<td>*</td>
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<td>1.8 mg/g</td>
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<td></td>
<td></td>
<td>0.29 mg/g</td>
</tr>
<tr>
<td>Minor salivary glands</td>
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<td></td>
<td></td>
<td>RT-PCR</td>
<td></td>
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<tr>
<td>Saliva</td>
<td>Human, sheep, rat, bovine</td>
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<td></td>
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<td>7.8 mg/L</td>
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<tr>
<td>Mammary gland</td>
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<td>7.9 mg/L</td>
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<tr>
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<td>Large intestine</td>
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<td></td>
<td>(+)</td>
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<td>Liver</td>
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<tr>
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<tr>
<td>Sublingual gland</td>
<td>canine</td>
<td>+</td>
<td></td>
<td>RT-PCR</td>
<td></td>
</tr>
</tbody>
</table>

The concentration of CA VI in saliva varies diurnally and has also a high individual variation (Kivelä et al. 1997a, b). During sleep the CA VI concentration is very low in saliva and serum, while it increases after awakening followed by breakfast (Kivelä et al. 1997b). The mean CA VI concentration in whole saliva is 1.8 mg/L (0.1–10.0 mg/L) for non-stimulated and 5.0 mg/L (0.6–16 mg/L) for stimulated whole saliva. Most of the CA VI in saliva is secreted by the parotid gland (Fernley et al. 1991). Interestingly, low salivary CA VI concentrations have
been shown to be associated with an increased risk of acid injury to the gastroesophageal mucosa and high DMFT index values (Parkkila et al. 1997, Kivelä et al. 1999), although low CA VI concentrations are not associated with low saliva pH (Kivelä et al. 1999). Low salivary CA VI concentrations are also associated with a decrease in taste perception and an increase in apoptosis of taste receptor cells in the circumvallate papilla taste buds (Henkin et al. 1999a, b). Therefore CA VI has also been suggested to act as a growth factor. However, no direct evidence is available to support this suggestion.
3 **Aims of the present study**

Carbonic anhydrase isoenzyme VI is the only known secreted CA isoenzyme. Earlier findings have demonstrated that the enzyme is present in saliva and tear fluid where it is proposed to participate in maintaining the acid-base homeostasis of the gastrointestinal tract lumen and surface of the eye, respectively. The hypotheses of this thesis are that CA VI is a general constituent of exocrine gland secretions and a factor protecting teeth and the mucosae of the alimentary and respiratory tracts. The specific aims were:

1. To determine the distribution of CA VI in rat and human exocrine glands.
2. To study whether CA VI is bound to the human enamel pellicle and to assess its possible physiological role there.
3. To determine the bicarbonate dehydration activity of human CA VI.
4 Materials and methods

4.1 Saliva, milk and tissue samples (I–IV)

Collection of the saliva and tissue samples was carried out according to the provisions of the Declaration of Helsinki and informed consent was obtained from each volunteer. Human whole saliva was collected from healthy volunteers into test tubes. Saliva from human von Ebner’s glands was collected from two healthy volunteers’ circumvallate papillae with a micropipette and from foliate papillae with nitrocellulose sheets (Spielman et al. 1993). Frozen human milk was obtained from the Oulu University Hospital Milk Centre. Colostrum samples were obtained from 9 mothers on the 2–4th day postpartum. Samples from saliva drops were obtained at the same time period from two 3-day-old infants prior to their breast feeding. Mature milk samples were also obtained from four mothers on the 90th day postpartum. Human tongue and parotid gland specimens were obtained from the Department of Forensic Medicine, University of Oulu and lingual carcinoma samples of patients undergoing surgery at the Clinic of Otorhinolaryngology, Oulu University Hospital.

All animal experiments described below had the approval of the Animal Care and Use Committee of the University of Oulu. Rat saliva was collected under anaesthesia and pilocarpine-stimulation. Rat mammary gland specimens were taken from sexually mature non-pregnant female rats and from rats two days before or after parturition. Rat trachea and lung samples were collected from foetuses and from 1-, 2-, 5-, 10-, 20-, 30-day-old and adult male rats. Tongue and salivary gland samples were collected from adult male rats.

The tissue samples used for protein purifications and immunoblotting were homogenised in ice-cold 0.1 M Tris-SO₄ buffer pH 8.7, containing either 1 mM phenylmethanesulphonylfluoride, 1 mM benzamidine and 1 mM o-phenanthroline or protease inhibitors from Complete™ (Roche Applied Science) which were applied according to the manufacturer’s instructions. The samples were then centrifuged and the supernatants were used for the experiments. Samples for immunohistochemistry were fixed in Carnoy’s fluid or 4% paraformaldehyde in PBS for 18 h at 4 °C, and embedded in paraffin, and sections of 5 µm were subjected to immunohistohemical stainings.

Permanent non-caries human teeth extracted in routine dental care were obtained from the Department of Oral and Maxillofacial Surgery, University of Oulu and from the Parolannummi Garrison Hospital. Three methods were used to
produce enamel pellicle. 1) Prior to extraction the teeth were polished with pumice and pellicle was allowed to form in situ for 2 h. 2) Crown halves were polished with pumice and incubated in vitro in saliva or in PBS in a rotary shaker for 2 h at room temperature. 3) Crown quarters were polished with pumice and incubated in a rotary shaker for 2 h at room temperature with purified human CA VI (3 or 9 mg/mL in PBS) or with purified salivary α-amylase (230kU/L in PBS) or in PBS alone. Enamel pellicle proteins were collected for immunoblotting experiments from two volunteers using a curette.

4.2 Purification of carbonic anhydrase isoenzyme VI (II)

An affinity gel matrix was prepared by coupling the CA inhibitor p-aminomethyl benzenesulphonamide to CM Bio-Gel A (Bio-Rad) according to Khalifah et al. (1977). Samples were diluted with buffer containing 0.1 M Tris-SO₄ and 0.2 M sodium sulphate at pH 8.7 and incubated with inhibitor affinity gel matrix in constant rotation. After the incubation the gel was washed first with buffer solution containing 0.1 M Tris-SO₄, 0.2 M sodium sulphate, 1 mM benzamidine, and 20% (v/v) glycerol, pH 8.7 followed by the same solution at pH 7.0. CA VI was eluted from the inhibitor affinity gel matrix with a specific elution buffer containing 0.4 M NaN₃, 0.1 M Tris-SO₄, 1 mM benzamidine, and 20% (v/v) glycerol, pH 7.0. The eluted proteins were dissolved in SDS loading buffer and then subjected to SDS-PAGE followed by Coomassie Blue staining.

4.3 Production and characterisation of rabbit anti-rat CA VI serum (II)

The purified rat salivary CA VI was dissolved in sodium dodecyl sulphate sample buffer and the proteins were separated using SDS-PAGE (Laemmli 1970). The major 41 kDa band representing the mature glycolysed CA VI was used for the immunisation of rabbits. Anti-rat CA VI serum was tested using immunoblots and immunoprecipitation. The antiserum identified two major polypeptides of 41 and 37 kDa in rat saliva and purified rat salivary CA VI preparations (II). The molecular masses of these bands correspond to the masses of the glycosylated and partially glycosylated forms of CA VI reported earlier for human CA VI (Murakami & Sly 1987). A minor 56 kDa polypeptide was found in rat saliva (II). Preimmune serum did not recognize any polypeptide bands in the control immunoblots. To further analyse the antiserum, the antibodies were coupled to
Protein A Sepharose CL-4B (Pharmacia Biotech), which was subsequently incubated with a saliva sample. The bound proteins were eluted using SDS loading buffer and then subjected to SDS-PAGE. All three abovementioned bands were found in the eluate obtained from the Protein A Sepharose CL-4B containing CA VI antibodies (data not shown). To obtain CA VI antiserum free from antibodies to the 56 kDa proteins, 500 µg of rat saliva was transferred onto a PVDF sheet and the 56 kDa band detected by immunoblotting was cut out. Then 2.5 µl rabbit anti-rat CA VI serum diluted in 500 µl 1% BSA-PBS was incubated with the PVDF band stripe bearing the 56 kDa proteins for 24 h in constant shaking at room temperature. The resulting supernatant (later 56 kDa absorbed antiserum to rat CA VI) identified only the 41 and 37 kDa protein bands in rat saliva (data not shown). The anti-human CA VI serum and anti-rat CA II serum have been characterised earlier (Parkkila et al. 1990, Kaunisto et al. 1995). Antibody to human salivary α-amylase was purchased from Sigma Chemicals.

4.4 Immunoblotting (I–IV)

The proteins were separated on SDS-PAGE and thereafter electroblotted onto nitrocellulose or PVDF sheets according to a standard method (Towbin et al. 1979). The sheets were washed first with Tween-20 containing buffer, incubated then with blocking solution (TWEEN-20 containing buffer with BSA or colostrum) followed by incubating with primary antiserum. The sheets were subsequently washed with Tween-20 containing buffer, treated with blocking solution, washed briefly with Tween-20 containing buffer and incubated with peroxidase-conjugated anti-rabbit immunoglobulin G. After extensive washing with Tween-20 containing buffer the bands were visualised with enhanced chemiluminescence (ECL) protein visualising method according to the manufacturer’s instructions.

4.5 Immunohistochemical staining (I–IV)

The 5µm-thick sections for immunoperoxidase staining were deparaffinised and pre-treated with 3% (v/v) H₂O₂. After incubation with blocking solution (1% (w/v) BSA-PBS or cow colostrum whey or 1% (v/v) goat colostrum) the sections were rinsed with PBS, incubated with antiserum diluted in 1% (w/v) BSA-PBS, rinsed again with PBS and re-incubated with biotin-conjugated swine anti-rabbit immunoglobulin G diluted in 1% (w/v) BSA-PBS. Subsequent to rinses with PBS the sections were incubated with streptavidin-conjugated peroxidase diluted 1:600.
in PBS and rinsed with PBS. Finally, the enzyme localisation was visualised by incubating the sections with a solution containing 9 mg of 3,3’diaminobenzidine tetrahydrochloride in 15 ml of PBS + 5 µl of 30 % (v/v) H$_2$O$_2$ and rinsing with PBS. The sections for immunofluorescence staining were deparaffinised and pretreated with 1% (w/v) BSA-PBS. After rinsing with PBS, the sections were incubated with antiserum diluted in 1% (w/v) BSA-PBS, rinsed with PBS, reincubated thereafter with rhodamine-conjugated swine anti-rabbit immunoglobulin G diluted in PBS and rinsed with PBS. All sections were examined with a Nikon Eclipse E600 microscope and photographed using a Nikon Coolpix 950 digital Camera. The images were further processed with Corel Draw and Adobe Photoshop computer software.

4.6 Histochemical staining of CA activity (I)

A pellicle was formed on tooth samples by incubating them in saliva or with purified salivary CA VI. Control samples were incubated in PBS alone. The samples were thereafter stained for CA activity using the Hansson’s method (Hansson 1967) briefly as follows:

1. Incubation of the samples for 1 min with washing buffer solution containing 9 mL 1/15 M KH$_2$PO$_4$ and 1 mL 1/15 M Na$_2$HPO$_4$ in 1 L of saline.

2. Rinsing of the samples for 8 min with fresh Hansson’s medium (solution containing 1 mL 0.2 M CoSO$_4$, 6 mL 0.5 M H$_2$SO$_4$ and 10 mL 1/15 M KH$_2$PO$_4$ added to a freshly prepared solution of 0.75 mg NaHCO$_3$ in 40 mL distilled water). The medium was changed continuously to enable air contact of the samples. Carbon dioxide was blown over the surface of the CoSO$_4$-containing solution for 10 min prior to addition of the NaHCO$_3$-containing solution and over the samples during the rinsing.

3. Washing the samples for 1 min with the washing buffer containing 9 mL 1/15 M KH$_2$PO$_4$ and 1 mL 1/15 M Na$_2$HPO$_4$ in 1 L of saline.

4. Incubation of the samples for 5 s with a freshly prepared solution containing 1% (NH$_4$)$_2$S in distilled water.

5. Incubation for 1 min with the washing buffer containing 9 mL 1/15 M KH$_2$PO$_4$ and 1 mL 1/15 M Na$_2$HPO$_4$ in 1 L of saline.
37

Another set of control samples were incubated in Hansson’s medium containing the carbonic anhydrase inhibitor acetazolamide at a final concentration of 50 mM.

4.7 Polypeptide sequencing and molecular mass analysis (III)

The peptide sequence analyses were performed using matrix-assisted laser desorption ionisation mass spectrometry followed by analysis with the ProFound and PeptideSearch programs. The molecular mass of purified CA VI from human milk was analysed using Voyager DE RP matrix-assisted laser desorption ionisation time-of-flight mass spectrometer analysis (PerSeptive Biosystems).

4.8 Deglycosylation of purified CA VI (III)

CA VI samples purified from human milk and saliva were deglycosylated with N-glycosidase F (Roche diagnostics) as described earlier (Petäjä-Repo et al. 1991) and analysed on SDS-PAGE.

4.9 Immunofluorometric assay (III)

Concentrations of CA VI in colostrum (n=9), mature milk (n=4) and 3-day-old infants’ saliva samples (n=2) were measured using competitive time-resolved immunofluorometric assay as described earlier (Parkkila et al. 1993). The mean intra-assay coefficient of variation of the present series was 9.4% and the interassay coefficient of variation determined in three assays was 9.7%.

4.10 Characterisation of CA VI dehydration activity

The initial velocities of bicarbonate dehydration were determined by stopped-flow spectrophotometry measuring the change in absorbance of a pH indicator at 25 °C (Khalifah 1971). The final concentrations of bicarbonate varied from 1.3 to 50 mM. The buffer-indicator pairs used were p-nitrophenol (pKₐ 7.10) / 3-(N-morpholino) propanesulphonic acid (pKₐ 7.02) for pH 7 and chlorophenol red (pKₐ 6.03) / 2-(N-morpholino) ethanesulphonic acid (pKₐ 6.05) for pH 6.5, 6 and 5.5. During the measurements the ionic strength was maintained at 0.2 M by the addition of Na₂SO₄ to the solution. The mean initial rate was determined from 8–10 reaction traces comprising the initial 10% of the reaction. The measurements in pH 6.5 were repeated twice using two different concentrations of CA VI. The
uncatalysed rates were subtracted, and the kinetic constants $k_{\text{cat}}$, $K_m$ and $k_{\text{cat}}/K_m$ were determined by a nonlinear least-squares method (Enzfitter, Biosoft).
5 Results

5.1 Binding of CA VI to enamel pellicle

In immunohistochemical staining, CA VI specific immunoreactivity was found in both the in vivo and in vitro formed enamel pellicle (I). The supragingival enamel surface showed a uniform peroxidase reaction (I). The staining intensity of in vitro formed enamel pellicle was dependent on CA VI concentration, suggesting a concentration-dependent enamel binding profile for CA VI (I). Interestingly, the CA VI bound to the enamel was found to display carbonic anhydrase activity in histochemical staining (I). Human enamel pellicle proteins subjected to immunoblotting with CA VI antiserum revealed a 38.5 kDa polypeptide band, while normal rabbit serum revealed no positive staining (I).

5.2 Dehydration kinetics of CA VI

The pH dependence of the kinetic constants $k_{cat}$, $k_{cat}/K_m$ and $K_m$ are shown in figures 3–5. The maximal values for dehydration by CA VI were $3.0 \times 10^5 \text{ s}^{-1}$ for $k_{cat}$ and $2.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for $k_{cat}/K_m$ (Figs 3 and 4). The $K_m$ for dehydration ranged from 5.2–16 mM (Fig. 5) (unpublished results). The dehydration kinetics of CA VI are similar to those of its closest genetic relatives, the membrane-bound CA isoenzymes IV, XII and XIV (Baird et al. 1997, Earnhardt et al. 1998).

Fig. 3. pH dependence of $k_{cat}$ for dehydration of bicarbonate.
Fig. 4. pH dependence of $k_{cat}/K_m$ for dehydration of bicarbonate.

Fig. 5. pH dependence of $K_m$ for dehydration of bicarbonate.

5.3 Expression and secretion of CA VI by human and rat von Ebner’s glands

Immunochemical staining demonstrated that the serous acinar cells of both the human and rat von Ebner’s salivary glands in the posterior tongue expressed CA VI. In rat, the serous demilune cells of the posterior lingual mucous gland and ductal cells of both glands also expressed CA VI. The ductal content of both rat
glands stained strongly for CA VI, suggesting that both glands also secrete the enzyme into saliva (II). Immunostaining for CA VI was also seen in the rat taste pores and in unidentified cells within the taste buds (II). The staining pattern in rat lingual salivary glands was identical when the 56 kDa absorbed antiserum to rat CA VI was used (results not shown). Immunoblotting of the von Ebner’s gland extract and saliva confirmed the presence of CA VI in the von Ebner’s glands and the secretion into saliva. The antiserum to human CA VI recognized a 40 kDa polypeptide in immunoblots of von Ebner’s gland saliva collected from the orifices of circumvallate and foliate papillae (II). An additional weaker 56 kDa polypeptide band was recognized in saliva collected from the foliate papilla orifice (II). The antiserum to rat CA VI recognized 41 kDa and 37 kDa polypeptide bands in immunoblotting of the CA preparation purified from rat von Ebner's glands (II). Sections and immunoblots stained with normal rabbit serum or preimmune serum showed no staining, confirming the specificity of CA VI immunostaining (II).

5.4 Secretion of CA VI into milk

The alveolar epithelium of rat mammary glands were found to express CA VI, and the enzyme was also present in the postpartum alveolar milk (III). CAs purified from human milk showed one major 42 kDa polypeptide band in SDS-PAGE that was recognized by antiserum to human CA VI (III). The molecular mass of the major band corresponds to the glycosylated form of salivary CA VI. In mass spectrometry, the accurate size of the glycopeptide was 37.8 kDa. Occasionally a minor band of 29 kDa was purified from milk corresponding to CA II which likely originates from red blood cells contaminating the milk. The molecular weights of glycosylated and deglycosylated CA VI purified from human milk and saliva were identical (III). Rat mammary glands showed specific bands at 56 kDa, 42 kDa and 36 kDa in immunoblots (III). The most intense bands were found in the lactating gland, a moderate band in glandular tissue prior to parturition, and a faint band in the resting gland (III). The analysis of the protein sequence data of CA VI purified from human milk revealed 100% identity with human salivary CA VI (40% coverage) (III). The mean concentration of CA VI in colostrum was 34.7 mg/l (range 10.0–78.4 mg/l, n=9) and in mature milk 4.5 mg/l (2.6–6.9 mg/l, n=4) (III). The CA VI levels in the saliva of two infants were 1.9 and 3.6 mg/l (III).
5.5 Expression and secretion of CA VI in the lower airways

The serous cells of submucosal tracheobronchial glands, the ductal cells of the glands, the serous cells of tracheobronchial mucosal epithelium and bronchiolar Clara cells were found to express CA VI (IV). CA VI was also present in the ductal content of the glands. Interestingly, CA VI was localised in the base of the cilia in the bronchiolar ciliated cells (IV). The expression of CA VI in the glandular cells appeared as soon as the glandular structures were detected, in the 20th postnatal day. In contrast, the Clara cells expressed CA VI from the 1st postnatal day onwards. A similar staining pattern was obtained when the 56 kDa absorbed antiserum to rat CA VI was used (results not shown). Sections stained with anti-rat CA VI serum absorbed with purified rat CA VI or preimmune serum showed no positive staining, confirming the specificity of the immunostaining (IV).
6 Discussion

6.1 Delivery of CA VI into the gastrointestinal tract and its physiological role there

CA activity in human saliva was reported for the first time over 60 years ago by Rapp (1946). This activity was demonstrated to result from CA VI secreted by the large salivary glands (Fernley et al. 1979). The results here show that CA VI is delivered into the gastrointestinal tract not only in saliva secreted by the large salivary glands but also in von Ebner’s glands’ saliva and in milk. Saliva and milk contain inorganic compounds and several proteins that affect overall homeostasis in the oral cavity and locally on tooth surfaces. Patients with a low salivary flow rate, like patients suffering from hyposalivation, have usually rampant caries which points to the fact that saliva protects teeth from caries. The importance of salivary CA activity for dental health was first suggested by Szabo (1974) who reported that low CA activity in saliva is associated with high caries prevalence and later by Kivelä et al. (1999) who reported that low concentrations of CA VI in saliva are associated with high DMFT index scores. Salivary buffering capacity and pH are two long-known caries-associated factors. Interestingly, Kivelä et al. (1999) found, however, no correlation between salivary CA VI concentrations and salivary buffering capacity or pH. The finding here showing that CA VI attaches to the enamel in a concentration-dependent manner and as an active form suggests that CA VI functions rather as a local caries protective factor in the enamel pellicle by neutralising the acidity produced by the bacteria in the dental biofilm. In fact, Kimoto et al. (2006) recently showed that inhibiting CA activity in a mixture of saliva and oral bacteria yields lower pH after sugar exposure than in non-CA-inhibited control mixtures. The solubility constant of enamel hydroxyapatite increases as pH decreases and hence long-lasting acidity on enamel dissolves it resulting in caries lesions. The acidity on enamel results principally from bacterial sugar metabolism in the dental biofilm. Thus, CA VI located in the enamel pellicle is in an optimal position for neutralisation of the acidity produced by the dental biofilm. More importantly, the present results show that the catalytic activity of CA VI has a high maximal $k_{\text{cat}}$ ($3.0 \times 10^5 \text{ s}^{-1}$) for dehydration of bicarbonate. Also, CA VI has a low dehydration $K_m$ that is similar to the saliva bicarbonate concentration (Bardow et al. 2000a, b). To conclude, the results here suggest that CA VI bound to enamel facilitates locally the removal of the dental biofilm.
produced acidity as carbon dioxide by catalysing the reaction: $\text{H}^+ + \text{HCO}_3^- \rightarrow \text{H}_2\text{O} + \text{CO}_2$.

Henkin et al. (1999a, b) have reported that CA VI has an important role in taste perception. They found that low concentrations of CA VI in saliva are associated with lowered taste perception and morphological deterioration of the taste buds of the circumvallate papillae and that oral zinc administration elevated salivary CA VI concentration and restored the normal morphology (Henkin et al. 1999a, b). A zinc deficient diet is also associated with lowered trigeminal nerve responses to carbonated water (Komai et al. 2000). The connection of CAs to taste perception is also demonstrated in studies by Miller and Miller (1990), McMurdoo et al. (1990), and Graber and Kelleher (1988) who showed that oral use of CA inhibitors causes a multitude of side-effects on taste perception e.g. decrease in sensing carbonated water. Carbon dioxide sensing recovers fast after topical application of the CA inhibitor dorzolamide on the dorsum of the tongue suggesting that salivary CA VI plays a major role in carbon dioxide sensing but is not the only CA involved as the taste buds also express other CAs e.g. CA II (Daikoku et al. 1999, Simons et al. 1999). The taste buds are abundantly located in the walls of the trenches surrounding lingual circumvallate and foliate papillae. Since whole saliva can not readily enter the trenches the source of CA VI in the trenches is principally, as the present study suggests, the von Ebner’s glands, which are located beneath the papillae and open directly into the bottom of the trenches. The importance of the von Ebner’s glands to the functions of taste buds of circumvallate papillae is also supported by a report that shows that the removal of the rat submandibular and sublingual glands results in damage to the taste buds located in the dorsum of the tongue but not in those located in the circumvallate papillae (Morris-Wiman et al. 2000). In the trenches, CA VI may remove excess protons or carbon dioxide by catalysing the dehydration/hydration reaction and CA VI may thereby affect taste perception by modifying the actions of the proton-gated sodium and potassium channels or decreasing the concentration of carbon dioxide that readily penetrates the plasma membrane of the taste receptor cells. Acid neutralisation may also protect taste receptor cells from apoptosis or alternatively, CA VI may be a trophic factor for the taste bud cells as has been proposed by Henkin et al. (1999a, b). The trophic effects of CA VI may be mediated via the widely expressed family of calmodulin dependent cyclic nucleotide phosphodiesterases, some of which have been shown to be activated by CA VI (Law et al. 1987, Omori & Kotera 2007).
Low salivary CA VI concentrations are also associated with acid injury of the gastrointestinal mucosa suggesting that CA VI is involved in pH balancing on the mucosa (Parkkila et al. 1997). CA VI retains its activity in the low pH present in gastric juice and hence the acid neutralisation effect of CA VI likely extends from the stomach to the intestinal tract as well (Parkkila et al. 1997). For maximal acid neutralisation it would be beneficial if CA VI would be immobilised on the luminal surface of the mucosal epithelia e.g. by receptor proteins as proposed by Hooper et al. (1995a, b). The present study shows that CA VI is delivered into the gastrointestinal tract not only in saliva but also in milk and particularly in high concentrations in colostrum. In the gastrointestinal tract, CA VI possibly has several physiological effects alone or together with other milk proteins. Milk bicarbonate concentration (3.5–8.3 mM) (Anderson 1992) is near the dehydration K_m of CA VI (5.2–16 mM) enabling efficient catalysis by CA VI. The finding that CA VI appears in particularly high concentrations in colostrum suggests that CA VI originating from milk possibly has other mechanisms of action in addition to the neutralisation effect in the newborn gastrointestinal tract. Concentrations of many whey proteins, especially of immunoglobulins, are also high in colostrum (Korhonen et al. 2000, Ballabio et al. 2007). Since CA VI is known to form a complex with immunoglobulin G in serum (Kivelä et al. 1997b) it may also form complexes with immunoglobulins in milk and thereby participate in the antimicrobial functions of milk in the newborn’s gastrointestinal tract. Milk CA VI may also have similar trophic effects in the gastrointestinal tract as has been proposed for salivary CA VI on the taste receptor cells (Henkin et al. 1999). Thus it is apparent that CA VI can act as a multifunctional protein like e.g. lactoferrin in the gastrointestinal tract (Ward et al. 2005) and have a specific role not only in tooth and mucosal protection and taste perception but also in the development of the newborn gastrointestinal tract, and that the role of milk CA VI is to compensate for the low CA VI levels in the newborn’s own saliva.

6.2 Delivery of CA VI into the respiratory tract and its physiological role there

In the respiratory tract, efficient defence mechanisms of the mucosa and strictly regulated cell renewal systems are needed to surmount various chemical, physical and microbial stresses. The present results show that CA VI secreted by the serous cells of the tracheobronchial glands and bronchiolar Clara cells can participate in these defence and renewal processes of the mucosa. Secreted CA VI is likely
linked to acid removal from mucosal surfaces as carbon dioxide by catalysing the reaction: $\text{HCO}_3^- + \text{H}^+ \rightarrow \text{CO}_2 + \text{H}_2\text{O}$. A neutral pH on the mucosa is important for the mucosal defence against microbes in at least two ways. First, acidity reduces bactericidal activity in the surface liquid of human airway epithelia and hence increases their susceptibility to bacterial infection (Nakayama et al. 2002). Second, acidity lowers the beat frequency of bronchial cilia (Clary-Meinesz et al. 1998), which also increases the susceptibility of the airway epithelia to bacterial infection (Afzelius 1976, Whitelaw et al. 1981). Accordingly, CA VI could be implicated in protection of the mucosa by increasing the bactericidal activity of the surface liquid and/or increasing cilia motility via pH neutralisation of the surface liquid. Interestingly, CA VI is localised in the base of the cilia suggesting that its function is locally linked with cilia motility. However, the lack of significant acid predispositions in the respiratory tract also suggests alternative roles and mechanisms of action for CA VI there. For instance, CA VI could have trophic functions as proposed for CA VI in the gastrointestinal tract. The fact that CA VI is present in airway surface liquid and in milk points to the fact that CA VI is not only a constituent of saliva and tear fluid but also a more general constituent of glandular secretions.
7 Conclusions

1. CA VI attaches to the acquired enamel pellicle in a concentration-dependent manner and in an enzymatically active form. In the enamel pellicle, CA VI is in an optimal location to neutralise the acidity produced by the dental biofilm and thus to protect the teeth from caries.

2. CA VI displays a marked bicarbonate dehydration activity and a low $K_m$. The dehydration activity constant $k_{cat}/K_m$ of CA VI is the highest of all CAs. The $K_m$ values are close to the bicarbonate levels found in milk and saliva suggesting that CA VI is well able to accelerate the pH neutralisation in milk and saliva plus on tooth and mucosal surfaces.

3. CA VI is secreted into the mammalian oral cavity not only by the large salivary glands but also by the serous cells of lingual von Ebner’s salivary glands and posterior mucous glands. The saliva of the von Ebner’s glands is delivered into the trenches surrounding the taste bud-rich circumvallate and foliate papillae suggesting that CA VI is implicated in the taste stimuli modifying effect of the von Ebner’s saliva and might also protect the taste receptor cells from apoptosis.

4. CA VI is secreted into mammalian milk in high concentrations. The particularly high concentrations of CA VI in colostrum suggest that milk CA VI has a specific significance in the physiology and development of the newborn gastrointestinal tract.

5. CA VI is also secreted into the lower airways by the serous cells of the submucous gland. CA VI is also present in tracheobronchial epithelia, bronchiolar Clara cells and in the bases of the cilia of the ciliated epithelial cells of the lower airways. In the lower airways, CA VI may have other functions, in addition to its classical role in the regulation of pH homeostasis, such as trophic or anti-microbial effects or effects on ciliary motility.
8 General comments and future considerations

The results presented here provide supporting data on the hypothesis that CA VI is a potent acid neutraliser in the enamel pellicle. The data explains, at least in part, why a group of patients with high salivary CA VI concentration has a high resistivity to caries. Accordingly, oral hygiene products e.g. artificial salivas could be supplemented with CA VI. Especially patients who suffer from hyposalivation derived rampant caries could benefit from these products. However, additional direct studies are necessary to confirm the cariesprotective effect and the exact mechanism of action of CA VI. CA VI deficient mice, follow up patient studies and in vitro demineralisation experiments combined with specific CA VI inhibitors that do not hinder biofilm metabolism are essential to address the unresolved questions.

Also the second hypothesis that CA VI is a general constituent of exocrine gland secretions was proved valid. It was suggested that CA VI functions on gastrointestinal and respiratory tract mucosa principally as an acid neutraliser but also other mechanisms of action should be considered. As has been suggested earlier CA VI may be a multifunctional protein with e.g. growth factor properties. The high concentration of CA VI in mammalian milk, especially in colostrum, suggests further that CA VI has a significant role in the development of the newborn gastrointestinal tract. The CA VI deficient mice are expected to provide comprehensive answers to these questions, although double CA deficient mice may be needed since other CAs may compensate for CA VI deficiency.
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Original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


Additionally, unpublished original data on CA VI are included.


Original publications are not included in the electronic version of the dissertation.


992. Kunnari, Anne (2008) Genetic, epidemiological and cell culture studies on human resistin


994. Tuomisto, Anne (2008) The role of collagen XIII in B-cell lymphoma development, and characterization of its biosynthesis and tissue distribution


996. Erkko, Hannele (2008) TOPBP1, CLSPN and PALB2 genes in familial breast cancer susceptibility

Jukka Leinonen

CARBONIC ANHYDRASE ISOENZYME VI:
DISTRIBUTION, CATALYTIC PROPERTIES AND
BIOLOGICAL SIGNIFICANCE