Topical Review

Salivary carbonic anhydrase isoenzyme VI

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The carbonic anhydrases (CAs) participate in the maintenance of pH homeostasis in various tissues and biological fluids of the human body by catalysing the reversible reaction $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$ (Davenport & Fisher, 1938; Davenport, 1939; Maren, 1967). Carbonic anhydrase isoenzyme VI (CA VI) is the only secretory isoenzyme of the mammalian CA gene family. It is exclusively expressed in the serous acinar cells of the parotid and submandibular glands, from where it is secreted into the saliva. In this review, we will discuss recent advances in research focused on the physiological role of salivary CA VI in the oral cavity and upper alimentary canal.

General aspects of salivary secretion and buffer capacity

Whole saliva is a mixture of the secretions from the parotid, submandibular, sublingual and minor salivary glands and gingival crevicular fluid. Saliva contains inorganic compounds and multiple proteins that affect conditions in the oral cavity and locally on the tooth surfaces. It brings various defence mechanisms, including leukocytes, secretory IgA, agglutinating proteins and a number of enzymes, to the actual sites of microbial growth on the tooth and mucosal surfaces. Salivation is initiated by the salivary centres in the medulla oblongata, which receive afferent signals from the sensory terminals of the oral and nasal cavities and from the higher centres in the brain. The secretion of saliva is regulated by the autonomic nervous system (Asking & Gjörstrup, 1980; Helm *et al.* 1982; Olsen *et al.* 1988; Calvert *et al.* 1998), and its composition follows circadian rhythms (Dawes, 1972, 1975; Parkkila *et al.* 1995; Kivelä *et al.* 1997*b*). Water and electrolyte secretion are mainly controlled by parasympathetic activity, whereas protein synthesis and exocytosis are mainly controlled by sympathetic activity (Jensen *et al.* 1991; Nederfors & Dahlöf, 1992, 1996; Nederfors *et al.* 1994).

Salivary buffer capacity is a factor of primary importance in maintaining oral homeostasis. The main buffer systems known to contribute to the total buffer capacity of saliva are the bicarbonate and phosphate systems and those based on proteins (Leung, 1951, 1961; Lilienthal, 1955; Izutsu & Madden, 1978; Helm *et al.* 1982). These systems have different pH ranges of maximal buffer capacity, the phosphate and bicarbonate systems having pK (−log of

the dissociation constant) values of 6·8–7·0 and 6·1–6·3, respectively, whereas the proteins contribute to the salivary buffer capacity at very low pH values only. Most of the salivary buffer capacity operative during food intake and mastication is due to the bicarbonate system, which is based on the equilibrium $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$. The concentration of bicarbonate in the saliva is greatly increased at increased flow rates (Dawes, 1969, 1974). Another essential feature of this buffer system under the conditions prevailing in the oral cavity is the phase conversion of carbon dioxide from a dissolved state into a volatile gas. When acid is added, this phase conversion considerably increases the efficacy of the neutralization reaction, as there is no accumulation of the end products but complete removal of the acid, a phenomenon referred to as 'phase buffering'.

Phosphate makes a minor contribution to the total salivary buffer capacity relative to bicarbonate (Leung, 1951; Lilienthal, 1955). Its system is in principle analogous to that of bicarbonate but without the important phase-buffering effect. Within the pH range of the oral cavity, the phosphate buffer is based on the reversible reaction $H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$. The concentration of HPO_4^{2-} in saliva is relatively independent of the salivary secretion rate, and thus the capacity of the phosphate buffer system does not increase during food intake or mastication.

Evaluation of the salivary buffer effect based on proteins has produced controversial results. In general the effect has been regarded as insignificant, or at least of minor importance, although data suggesting an alternative conclusion have also been presented (Leung, 1961; Izutsu & Madden, 1978).

Carbonic anhydrase VI

The existence of CA activity in human saliva has been known for 60 years (Becks & Wainwright, 1939), but until recently only a few studies had been carried out on the physiological role of salivary CA (Rapp, 1946; Szabó, 1974; Parkkila *et al.* 1997; Kivelä *et al.* 1997*a*,1999; Leinonen *et al.* 1999). The ovine salivary CA isoenzyme expressed in the parotid gland was described in 1979 by Fernley *et al.* The enzyme was purified from human saliva by Murakami & Sly (1987), and designated CA VI. In 1991, Aldred *et al.* cloned and characterized the cDNA encoding human carbonic anhydrase (HCA) VI. The next major step in research into salivary CA was the development of specific immuno-fluorometric and radioimmunoassays for HCA VI (Parkkila *et al.* 1993; Fernley *et al.* 1995), which allowed accurate quantification of CA VI in biological matrices such as saliva and serum.

CA VI is the only known secreted isoenzyme of the mammalian CA gene family, and has several properties that distinguish it from the well-characterized cytoplasmic isoenzymes. Its reported subunit molecular weight is 42 kDa (Murakami & Sly, 1987). The enzyme molecule has two N-linked oligosaccharide chains, which can be cleaved by endo-β-*N*-acetylglucosaminidase F but not by endo-β-*N*-acetylglucosaminidase H, indicating that the oligo-saccharides are of a complex type (Murakami & Sly, 1987). Neuraminidase has no effect on the endo-β-*N*-acetyl-glucosaminidase F-digested protein, suggesting that HCA VI has no O-linked oligosaccharide, which contains neuraminidase-sensitive sialic acid residues. The HCA VI protein has a sequence identity of 35% to HCA II, while residues involved at the active site of the enzyme are conserved. HCA VI has three potential N-linked glycosylation sites and two cysteine residues (Cys25 and Cys207) (Aldred *et al.* 1991), the latter presumably forming a disulphide bond, as in the ovine enzyme (Fernley *et al.* 1988). The *HCA6* gene is located on chromosome 1 (Aldred *et al.* 1991).

In humans, immunohistochemical studies have demonstrated the location of CA VI exclusively in the secretory granules of the acinar cells of the parotid and submandibular glands (Parkkila *et al.* 1990), from where it is secreted into the saliva. Salivary concentrations of ovine and human CA VI have been investigated using radioimmunoassay (Fernley *et al.* 1991, 1995) and time-resolved immunofluorometric assay techniques (Parkkila *et al.* 1993, 1995; Kivelä *et al.* 1997*a*). Radioimmunoassay of ovine CA VI showed that its mean ± S.D. concentration in sheep parotid saliva is 5.61 ± 3.01 mg l^{-1} in the normal conscious animal, while feeding increased the concentration to 33.0 ± 19.0 mg l^{-1} (Fernley *et al.* 1991; Fernley, 1991). The mean ± S.D. concentration of CA VI in human parotid saliva was shown by the radioimmunoassay method to be 47.0 ± 39.2 mg l^{-1}, representing about 3% of total protein in the parotid saliva (Fernley *et al.* 1995). In young men, the mean ± S.E.M. concentration of CA VI in whole saliva, measured by time-

resolved immunofluorometric assay, is 5.0 ± 0.2 mg l^{-1}, and the concentration has a weak positive correlation with salivary secretion rate (Kivelä *et al.* 1997*a*).

Secretion of CA VI into the saliva has been observed to follow a circadian periodicity, its concentration being very low during sleep and rising rapidly to the daytime level after awakening and breakfast (Parkkila *et al.* 1995; Kivelä *et al.* 1997*b*). This circadian periodicity is very similar to that of salivary α-amylase, and a significant positive correlation is found between salivary amylase activity level and CA VI concentration (Parkkila *et al.* 1995; Kivelä *et al.* 1997*b*). These findings suggest that the two enzymes are secreted via similar mechanisms and may possibly be present in the same secretory granules. Since the role of the autonomic nervous system in amylase secretion is well established (Asking & Gjörstrup, 1980; Olsen *et al.* 1988; Nederfors & Dahlöf, 1992), it is conceivable that the autonomic pathways also control CA VI secretion. This concept was supported by Fernley *et al.* (1991) who demonstrated, using nerve stimulation and cholinergic drug administration, that both parasympathetic and sympathetic pathways can regulate the CA VI concentration in sheep saliva.

CA VI has been found to be transferred into the blood circulation in humans (Kivelä *et al.* 1997*b*). The mean serum concentrations of CA VI, measured by time-resolved immunofluorometric assay, are only about 1/22 of those of simultaneous saliva samples. The enzyme levels in serum show much intra-individual variation ($0.15–0.33$ mg l^{-1}), although the circadian rhythm is less evident than in saliva. Western blotting of CA VI indicated that it is associated with IgG in serum, whereas it mainly occurs as a monomeric enzyme in saliva. Association with IgG may protect the enzyme from proteolytic degradation or target it to cells which do not express CA VI.

According to recent investigations, CA VI seems to be one of the key enzymes in oral physiology. It was originally predicted to regulate salivary pH or buffer capacity (Feldstein & Silverman, 1984; Kadoya *et al.* 1987). Interestingly, results from our laboratory indicate that these variables are not directly associated with CA VI concentration in saliva (Parkkila *et al.* 1993; Kivelä *et al.* 1997*a*), suggesting that the enzyme may have a different role or that it may participate in these processes together with other CAs. Another novel aspect to the function of CA VI was provided by Thatcher *et al.* (1998), who identified gustin, a salivary protein that has been reported to be associated with the function of taste buds, as human CA VI by protein sequencing, activity profiles and other physical data.

Role of saliva and salivary CA VI in protecting the dental hard tissues

Dental enamel is the hardest tissue in the human body, and the main challenge to it comes from acidic conditions in the oral cavity, which can cause dissolving of the mineral,

i.e. dental caries or erosion. The metabolism of the microbial flora on the dental surfaces produces considerable amounts of acid, mainly in the form of lactic, acetic, formic and propionic acids (Clarke, 1924; Muntz, 1943; Stephan, 1944; Geddes, 1975, 1981). Moreover, various foods and drinks add to the acid charge on these surfaces. Saliva can be considered the oral tissue fluid of the enamel, and the maintenance of homeostasis on the dental surfaces is totally dependent on salivary factors, including inorganic compounds and multiple proteins. Saliva is involved in the clearance of food debris, detached epithelial cells and microbes. It provides inorganic ions for the neutralization of the acid and alkaline metabolic products of oral bacteria and for the remineralization of the enamel. It also brings various defence mechanisms, including leukocytes, secretory IgA, agglutinating proteins and a number of enzymes, to the actual sites of microbial adherence and growth on the tooth surfaces. The importance of saliva for dental health is demonstrated by the rampant caries seen in patients with grave salivary hypofunction (Rudney, 1995; Peeters *et al.* 1998).

Recent research has indicated that salivary CA VI has a remarkable role in protecting teeth from caries (Kivelä *et al.* 1999). It is the first salivary protein reported to be associated with the occurrence of caries in individuals as measured using the DMFT index (number of decayed, missing, or filled teeth). The negative correlation between salivary CA VI concentration and DMFT index was most distinct in the group of subjects with neglected oral hygiene. This finding, together with the observation that salivary CA VI concentration does not correlate with salivary pH or buffer capacity, has led to a search for the site of salivary CA VI function.

The enamel pellicle is a thin layer of proteins covering the enamel. Its formation is initiated by the adsorption of specific salivary proteins to the hydroxyapatite surface (Kousvelari

et al. 1980; Al-Hashimi & Levine, 1989; Lamkin *et al.* 1996). This adsorption is assumed to be dependent on the chemical characteristics of the surface as well as the properties of the particular proteins. The enamel pellicle evidently prevents demineralization of the surface hydroxyapatite, increasing its acid resistance, although the mechanisms responsible for this effect remain elusive (Zahradnik *et al.* 1976, 1977, 1978; Kousvelari *et al.* 1980; Meurman & Frank, 1991; Featherstone *et al.* 1993). It is also thought to prevent calculus formation on the dental surfaces by controlling the precipitation of calcium phosphate from supersaturated saliva (Hay & Moreno, 1989).

Recent research has indicated that CA VI binds to the enamel pellicle and retains its enzyme activity on dental surfaces (Leinonen *et al.* 1999). In the enamel pellicle, CA VI is located at the optimal site to catalyse the conversion of salivary bicarbonate and microbe-delivered hydrogen ions to carbon dioxide and water (Fig. 1). These findings suggest that CA VI may protect teeth by catalysing the most important buffer system in the oral cavity, thus accelerating the removal of acid from the local microenvironment of the tooth surface.

Role of salivary CA VI on epithelial surfaces of the upper alimentary canal

The epithelia of the human oral cavity and oesophagus are exposed to widely varying conditions due to the physical and chemical properties of the ingested food, in addition to which the epithelium of the oesophagus is challenged by acid reflux from the stomach. The saliva is responsible for the luminal defence of the epithelia of the upper alimentary canal. A number of salivary proteins are known to bind to the epithelial surfaces of the oral cavity, including salivary mucins, amylase, salivary cystatins and acidic proline-rich proteins (Bradway *et al.* 1989, 1992). This epithelial pellicle
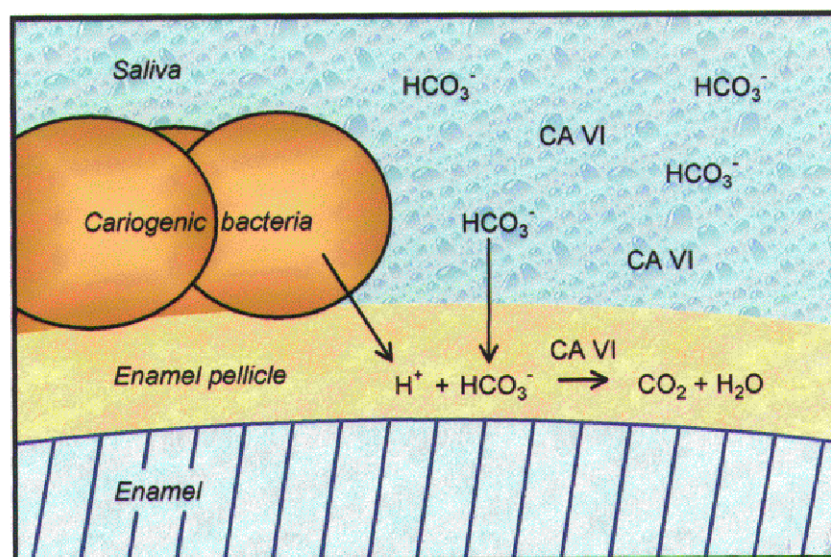

Figure 1. Model illustrating the suggested function of CA VI on dental surfaces

Modified from Leinonen *et al.* (1999).

provides a lubricatory film and an effective barrier against desiccation and environmental factors, and it is also thought to protect the epithelial cells from proteases emanating from bacteria attached to the mucosal surfaces and from degenerating polymorphonuclear leukocytes (Mandel, 1987; Vaahtoniemi *et al.* 1992).

Salivary bicarbonate secretion is known to be of vital importance for the maintenance of oesophageal pH homeostasis (Helm *et al.* 1982, 1984; Sarosiek & McCallum, 1995; Sarosiek *et al.* 1996), and recent observations have suggested that CA VI may also be involved in this process (Parkkila *et al.* 1997). CA VI has also been detected in the gastric mucus where it may contribute to the maintenance of the pH gradient on the surface epithelial cells. This view is supported by the observation that CA VI probably maintains its activity in the harsh environment of the gastric lumen and that patients with verified oesophagitis or oesophageal, gastric or duodenal ulcers have a reduced salivary CA VI concentration relative to patients with a non-acid peptic disease (Parkkila *et al.* 1997). The finding that CA VI is not expressed in the gastric epithelial cells (Parkkila *et al.* 1994) implies that gastric CA VI is of salivary origin. It has been proposed that CA VI and CA II may form a mutually complementary system for the regulation of pH homeostasis on the epithelial surfaces of the upper alimentary canal (Parkkila *et al.* 1990, 1994, 1997). Salivary CA VI probably catalyses the neutralization of excess acid in the mucous layer covering the oesophageal and gastric epithelial cells using endogenous and salivary bicarbonate produced by cytosolic CA II (Parkkila & Parkkila, 1996; Parkkila *et al.* 1997).

Conclusions

The recent advances in CA VI research suggest that salivary CA VI is one of the key enzymes maintaining homeostasis on the surfaces of the oral cavity and upper alimentary canal. Low salivary concentrations of CA VI appear to be associated with an increased prevalence of caries and acid peptic diseases. Since CA VI is present in the enamel pellicle and gastric mucus, it may function locally in the micro-environment of dental and epithelial surfaces and accelerate the neutralization of excess acid.

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