The anion exchanger Ae2 is required for enamel maturation in mouse teeth


a Department Oral Cell Biology, Academic Center for Dentistry Amsterdam (ACTA), Universiteit van Amsterdam and Vrije Universiteit, The Netherlands
b Department Functional Anatomy, Academic Center for Dentistry Amsterdam (ACTA), Universiteit van Amsterdam and Vrije Universiteit, The Netherlands
c AMC Liver Center, Universiteit van Amsterdam, The Netherlands
d Division of Gene Therapy and Hepatology, University Hospital/School of Medicine/CIMA, University of Navarra, and Ciberehd, Pamplona, Spain
e Department Biochemistry, University of Oulu, Oulu, Finland

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Abstract

One of the mechanisms by which epithelial cells regulate intracellular pH is exchanging bicarbonate for Cl−. We tested the hypothesis that in ameloblasts the anion exchanger-2 (Ae2) is involved in pH regulation during maturation stage amelogenesis. Quantitative X-ray microprobe mineral content analysis, scanning electron microscopy, histology, micro-computed tomography and Ae2 immuno-localisation analyses were applied to Ae2-deficient and wild-type mouse mandibles. Immuno-localisation of Ae2 in wild-type mouse incisors showed a very strong expression of Ae2 in the basolateral membranes of the maturation stage ameloblasts. Strikingly, zones of contiguous ameloblasts were found within the maturation stage in which Ae2 expression was extremely low as opposed to neighbouring cells. Maturation stage ameloblasts of the Ae2−/− mice failed to stain for Ae2 and showed progressive disorganisation as enamel development advanced. Maturation stage enamel of the Ae2−/− mice contained substantially less mineral and more protein than wild-type enamel as determined by quantitative X-ray microanalysis. Incisor enamel was more severely affected than molar enamel. Scanning electron microscopy revealed that the rod-inter-rod structures of the Ae2−/− mice incisor enamel were absent. Mineral content of dentine and bone of Ae2−/− mice was not significantly different from wild-type mice. The enamel from knockout mouse teeth wore down much faster than that from wild-type litter mates. Basolateral bicarbonate secretion via the anionic exchanger Ae2 is essential for mineral growth in the maturation stage enamel. The observed zonal expression of Ae2 in the maturation stage ameloblasts is in line with a model for cyclic proton secretion during maturation stage amelogenesis.

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1. Introduction

Enamel is an epithelial product deposited by a unique, one cell-thick layer of tall cylindrical cells, the ameloblasts. These cells form the enamel in a two-step process. First, the ameloblasts secrete a provisional organic matrix, in which enamel crystals form. The enamel matrix consists mainly of amelogenins that initiate and stabilise the long thin enamel crystals. The initial enamel crystals are apatitic and grow preferentially in length within the organic matrix until almost the full enamel thickness is attained. This stage of enamel formation is called secretory amelogenesis. After completion of the secretory stage, the ameloblasts cease matrix deposition, shorten their cell bodies and after a short transitional stage, enter the maturation stage. Maturation stage triggers accelerated degradation of the extracellular matrix proteins mediated by serine proteases, such as kallikrein 4 (enamel matrix serine proteinase-1 or EMSP1) (Bartlett and Simmer, 1999) and is also accompanied by massive volumetric increase in enamel crystal size, mainly due to growth in width.

During formation of the hydroxyapatite crystals in the enamel compartment, protons are generated that need to be neutralised to maintain a physiological pH in order to drive the
mineralisation process (Smith et al., 2005). The number of protons generated is dependent upon the phosphate precursor used and can vary between four and 14 moles of hydrogen per mole of apatite formed (Simmer and Fincham, 1995). The initial mineral deposited during the secretory stage of amelogenesis is thought to be mainly octacalcium phosphate (OCP) (Brown and Wallace, 1965; Brown et al., 1987). The relatively low rate of mineralisation during the secretory stage and the presence of large amounts of amelogenins with high buffering capacity in the enamel extracellular space are thought to be the mechanism responsible for neutralising the protons formed during OCP formation (Ryu et al., 1998; Simmer and Fincham, 1995). During the maturation stage, however, the situation is different: the rate of mineralisation is very high and the generation of protons is expected to be high as well while at the same time, the amelogenins that were responsible for buffering the extracellular compartment during the earlier secretory stage are being removed from the enamel compartment (Smith et al., 2005). If maturation ameloblasts are actively involved in proton buffering, one would expect that they secrete bicarbonate into the forming enamel. Indeed, the presence of cytoplasmic carbonic anhydrase type 2 (CA-II, which generates HCO$_3^-$ and protons) in maturation stage ameloblasts supports this concept (Lin et al., 1994; Toyosawa et al., 1996). Assuming that bicarbonate is extruded into the forming enamel by the distal membranes, such concept requires that to maintain electro-neutrality protons are pumped away from the enamel compartment by a proton pump in the basolateral membranes (see for ion transporting epithelial cells: (Alper et al., 2002; van Adelsberg et al., 1993)). Remarkably however, maturation stage ameloblasts express a proton pump in the apical membrane (Lin et al., 1994), identical to the one found in the bone-resorbing osteoclasts.

![Fig. 1](image_url)

Fig. 1. A–H. A is a macro-photograph of an erupted Ae2a,b$^{+/-}$ mandibular incisor and B the $\mu$CT image of the same tooth. In A, the enamel can be clearly seen. C is a macro-photograph of an Ae2a,b$^{-/-}$ incisor. Note that there is virtually no enamel present, only some remnants (white arrows). The corresponding $\mu$CT image of the same tooth is shown in D. The black arrow shows the eroded enamel just before it enters the oral cavity. The morphology of the Ae2a,b$^{+/-}$ molars is shown in E (macro-photograph) and F ($\mu$CT), respectively. The cusps of all molars (M1=first, M2=second and M3=third maxillary molars) are sharp (arrows) and are not eroded. The molars from the Ae2a,b$^{-/-}$ specimen (G, macro-photograph and H, the corresponding $\mu$CT image of the same tooth) are severely eroded (asterisks).
(vacuolar H\(^+\)-ATPase, (Mulari et al., 2003)). The location of this vacuolar H\(^+\)-ATPase suggests paradoxically that the ameloblasts are secreting protons into the forming enamel instead of bicarbonate.

So far there is hardly any data pertaining to how ameloblasts regulate intracellular and extracellular pH during enamel formation. Smith et al. (2006) reported the presence of carbonic anhydrase (CA-VI) in the enamel compartment that may function to buffer protons. These data suggest that bicarbonate exchange in ameloblasts may be crucial for the development of fully functional enamel. In addition, mice in which cystic fibrosis transmembrane conductance regulator (CFTR) responsible for chloride transport across epithelial membranes is mutated have severely softened enamel, suggesting that apical chloride secretion is also essential for amelogenesis (Sui et al., 2003).

Na\(^+\)-independent anion exchanger (Ae) is a ubiquitous gene family that mediates electro-neutral exchange of Cl\(^-\) for HCO\(_3\)^- ions across cell membranes. Four different genes have been identified in mammals so far: Ae1, Ae2, Ae3 and Ae4. Of these four genes, Ae2 is most ubiquitously expressed (Alper, 2002). The primary function of Ae2 is thought to be intracellular pH regulation, cell volume regulation and involvement in trans-epithelial hydroionic fluxes. In the mouse, five N-terminal isoforms of Ae2 have been recognised: Ae2a, Ae2b1 and Ae2b2, Ae2c1, and Ae2c2. Ae2a is expressed in all tissues examined so far, while Ae2b1 and Ae2b2 are restricted to epithelial tissues and Ae2c is expressed mainly in the stomach (Alper et al., 2002).

Based on these reports we hypothesised that Ae2, localised in the membranes of maturation ameloblasts, (co)regulates the intracellular pH by the exchange of chloride for bicarbonate generated by carbonic anhydrase II. In the present study, we investigated the localisation of the Ae2 protein in ameloblasts and examined its function by analysis of the enamel of Ae2\(^{-/-}\) knockout mice in which the Ae2a, Ae2b1 and Ae2b2 isoforms were disrupted (Medina et al., 2003).

2. Results

The heterozygous animals were phenotypically indistinguishable from their wild-type litter mates. In addition, preliminary studies could not demonstrate any differences between the heterozygous and wild-type animals with respect to all the parameters reported in this study. Therefore, only the results pertaining to the knockout and wild-type animals will be reported further in this study.

2.1. Gross morphology of erupted teeth

All molar and incisor teeth erupted into the oral cavity indicating that tooth eruption was not affected (Fig. 1A–H). The enamel of the incisors in the Ae2\(^{-/-}\) mice (Fig. 1C and D) lacked the typical yellow–orange pigmentation normally seen in the wild-type mice (Fig. 1A and B). At 40× magnification, the most profound effect of the Ae2\(^{-/-}\) gene knockout was severe abrasion of the erupted teeth (Fig. 1C and D, and G and H). Very little or no enamel at all could be observed in the Ae2\(^{-/-}\) incisors after eruption (cf. Fig. 1A–B and C–D). The μCT image (Fig. 1D) clearly showed that the incisor enamel was lost immediately after the tooth entered the oral cavity. The characteristic sharp cusp pattern seen in the Ae2\(^{+/+}\) specimens (Fig. 1E and F) was lost in the Ae2\(^{-/-}\) specimens (Fig. 1G and F), i.e., the molars were flattened due to severe abrasion (cf. Fig. 1E–F and G–H). Although clearly abraded, an enamel layer was present in the erupted molars.

2.2. Light microscopy

Maturation stage ameloblasts in the Ae2\(^{-/-}\) teeth were severely affected as observed by the cellular disorganisation and retention of organic matrix and cell fragments in the underlying enamel layer (cf. Fig. 2A and B). The stratum intermediate and papillary cell layers adjacent to the ameloblast layer also appeared disorganised with extensive intercellular spaces (Fig. 2B). The structure of presecretory and secretory ameloblasts on the other hand, was not affected. Such lack of phenotypic changes was also true for the alveolar bone cells as well as the cells in the pulp chamber.

2.3. Mineral content (EPMA)

The positions of the analysed areas are shown in Fig. 3A–D. Using back-scattered detector (BSD) electron optics, the enamel
of $Ae_{2a,b}^{-/-}$ incisors appeared darker than the enamel of $Ae_{2a,b}^{+/+}$ specimens (cf. Fig. 3A and B) suggesting a lower mineral content in the $Ae_{2a,b}^{-/-}$ enamel. The mineral elements calcium, phosphorus and magnesium account for most of the back-scattered electrons responsible for the BSD image acquisition. It should be noted here that the images were acquired under exactly the same microscope parameters, a prerequisite for direct comparison of BSD images.

2.3.1. Incisor and molar enamel

The cumulative mineral content data for the incisors and molars are shown in Fig. 4A and B, respectively. Both calcium and phosphorus contents in the $Ae_{2a,b}^{-/-}$ enamel specimens were significantly decreased in the incisor and molar when compared to the $Ae_{2a,b}^{+/+}$ specimens. This decrease in mineral content was however more pronounced in the $Ae_{2a,b}^{-/-}$ incisors than in the $Ae_{2a,b}^{-/-}$ molars (cf. Fig. 4A and B). On the other hand, sulphur, a measure for amelogenin protein content, as well as magnesium were significantly higher in both incisor and molar enamel of the $Ae_{2a,b}^{-/-}$ mice.

2.3.2. Crown (enamel-related) and root (cementum-related) dentine in incisors

No significant differences in mineral content could be demonstrated between the $Ae_{2a,b}^{+/+}$ and $Ae_{2a,b}^{-/-}$ crown or root dentine (Fig. 4C and D). It should be noted here that although no significant differences were found between the magnesium content in the root dentine of $Ae_{2a,b}^{+/+}$ and $Ae_{2a,b}^{-/-}$ animals, the magnesium content in root dentine of both the wild-type and knockout mice was always significantly higher than in the crown dentine.

2.3.3. Molar crown dentine

The only mineral parameter measured in the molars of the wild-type mice that significantly differed from the $Ae_{2a,b}^{-/-}$ mice was magnesium; i.e., in the $Ae_{2a,b}^{-/-}$ mice magnesium content in molar crown dentine was significantly higher than in the $Ae_{2a,b}^{+/+}$ molars (Fig. 4E).

2.3.4. Bone

Although magnesium content in the $Ae_{2a,b}^{-/-}$ alveolar bone specimens tended to be lower than in the $Ae_{2a,b}^{+/+}$ specimens, this difference was not significant either for magnesium or for all other parameters measured (Fig. 4F).

2.4. Scanning electron microscopy

The characteristic rod-inter-rod structure seen in the $Ae_{2a,b}^{+/+}$ specimens (cf. Fig. 5A and B) was completely absent in the $Ae_{2a,b}^{-/-}$ specimens after acid etching (cf. Fig. 5C and D). The surface of the $Ae_{2a,b}^{-/-}$ enamel appears amorphous with no evidence of enamel rod structure. No obvious differences in surface texture between the $Ae_{2a,b}^{+/+}$ and $Ae_{2a,b}^{-/-}$ specimens could be demonstrated in either bone or dentine.

2.5. Immunohistochemistry

The highest staining intensity for the Ae2 protein was found in the maturation stage ameloblasts of the $Ae_{2a,b}^{+/+}$ specimens and the staining was associated with the basolateral plasma membrane of the cells (Fig. 6A and B).
However, not all the maturation stage ameloblasts were equally intensely stained; each specimen contained some few contiguous cell populations with strikingly low staining intensity (Fig. 6C). The staining in the secretory ameloblasts was very low or absent and when present, it was mainly associated with the Golgi region. Erythrocytes were always...
intensely stained with this antibody due to the Ae1 that is highly expressed in these cells. The staining pattern described above for the Ae2a,b+/− specimens was completely absent in the Ae2a,b−/− specimens (Fig. 6D). The only cells in Ae2a,b−/− tissues that stained with the antibody to Ae2 were the erythrocytes in the blood vessel lumina (Fig. 6D, arrows).

Control sections of Ae2a,b+/+ tissue stained with non-immune antibodies were completely negative, including erythrocytes.

3. Discussion

The data presented in this study demonstrate that a functional Ae2 gene is necessary for proper enamel maturation in mouse teeth. The histological data suggest that the transitional-and-maturation stage ameloblasts were the cells most sensitive to loss of function of Ae2.

The Ae2 exchanger in the wild-type mice was localised in the basolateral membranes of the maturation ameloblasts similar to parietal cells in the gastric gland (Recalde et al., 2006) and in osteoclasts (Jansen et al., 2007, personal communication). Both cell types secrete large amounts of protons, express cytoplasmic CA-II and have a proton pump in their apical membrane. Thus, our localisation data are consistent with the concept that the apical membranes of maturation ameloblasts may secrete protons and that basolateral membranes exchange bicarbonate for chloride to compensate for this proton flux. In line with this contention, Lin et al. (1994) demonstrated the apical localisation of v-type H+-ATPase in maturation stage ameloblasts. The lack of staining of maturation stage ameloblasts in Ae2a,b−/− mice confirmed that these cells do not express Ae2 and suggest that pH regulation in these cells is perturbed. Although weak immuno-staining for Ae2 was observed in the Golgi area of secretory ameloblasts in wild-type mice, no visible changes were noted in secretory stage amelogenesis in Ae2a,b−/− mice. In contrast to enamel, the other mineralising tissues, i.e., dentine and alveolar bone did not appear to have been markedly affected by Ae2 gene disruption, either morphologically or with respect to the degree of mineral deposition. So, defective mineralisation following a deletion of Ae2 gene appears to be unique for the tooth enamel rather than for the other mineralising tissues.

The antibodies used in this study recognise Ae1 as well as all Ae2 isoforms. The positive staining of erythrocytes in Ae2a,b−/− mice is attributed to Ae1 abundantly present in these cells. Since in Ae2−/− mice, the ameloblasts were completely negative for Ae2 immuno-staining, the data prove that Ae2a,b−/− mice ameloblasts do not express Ae1 nor the Ae2 isoforms that were not knocked out (Ae2c1, c2). From this it may be inferred that normal ameloblasts only express Ae2a, and/or Ae2b1 and Ae2b2.

Enamel maturation is characterised by a dramatic decrease of organic matrix in the developing enamel accompanied by a
massive increase in mineral content. During enamel maturation, the ameloblasts undergo a cyclic phenomenon, i.e., they are either smooth- or ruffle-ended at any given time. The pH of the enamel space under the smooth-ended ameloblasts is neutral (pH 7.0–7.2) while that under the ruffle-ended ameloblasts is acidic (pH 5–6) (Sasaki et al., 1991), a condition that may drive proteolysis of the matrix. Ruffle-ended ameloblasts comprise about 80% of the maturation stage ameloblast population in the rat incisor. These ameloblasts are thought to be responsible for controlling the movement of calcium and other ions into the enamel space and thus control enamel crystal growth as well as enamel matrix resorption (Smith et al., 2005). It is interesting to note here that within the population of wild-type maturation ameloblasts some groups of cells stained weakly for Ae2 and we assume that these cells were less active and may correspond to the smooth-ended ameloblasts. The low mineral content and the presence of high organic matrix content (high sulphur content) in the maturation stage enamel reported here for the Ae2a,b/mice suggest that in the absence of Ae2, mineral ion transport into the enamel space as well as protein resorptive capacity in maturation stage ameloblasts is seriously impaired. The SEM data indicate that after acid etching, the characteristic enamel prism structure was lost in the Ae2a,b/mice suggesting that disruption of the gene results in enamel crystal disorganisation and/or change in enamel mineral composition.

Significantly more mineral was found in the mature molar enamel than in the maturation stage enamel of the incisor of the Ae2a,b/mice. It should be noted here that the incisor enamel of the knockout mice was lost just before or immediately after eruption into the oral cavity. This indicates that there are probably tissue differences with respect to the function and/or expression of Ae2 in cells. Whether ameloblasts in the molar regulate pH differently or express different isoforms of Ae2 than in the incisor remains to be investigated.

Our working model for the possible function of Ae2 in enamel maturation is shown in Fig. 7. We assume that the ruffle-ended maturation stage ameloblasts maintain a low pH in the enamel layer to catalyse protein hydrolysis. To maintain this low pH, ameloblasts pump protons, generated by carbonic anhydrase II that is abundantly present in these cells, into the enamel space by the vacuolar H⁺-ATPase associated with the ruffled border (Lin et al., 1994; Toyosawa et al., 1996). The Ae2 associated with the basolateral membranes of the ameloblasts exchanges extracellular Cl⁻ for intracellular HCO₃⁻. The accumulated intracellular Cl⁻ is probably transported into the enamel compartment by the cystic fibrosis trans-membrane conductance regulator (CFTR, (Sui et al., 2003)). Conversely, in the smooth-ended ameloblasts Ae2 expression is severely down-regulated, suggesting that these cells secrete much less or no bicarbonate at the basolateral aspect and thus little or no protons into the enamel space. Indeed, more data is required to validate our model, in particular, regarding the pH gradient in the Ae2-deficient mice.

In conclusion, our data demonstrate that Ae2 is highly expressed in the basolateral membranes of maturation stage ameloblasts and is essential for the completion of mineral crystal growth in the maturation stage enamel in order to achieve its final hardness.

4. Experimental procedures

4.1. Animals and tissue processing

Mice carrying a targeted disruption of Ae2 that prevents the expression of three Ae2 isoforms (Ae2a, Ae2b1, and Ae2b2) were generated as reported (Medina et al., 2003).

Mice, aged between six and twelve weeks were euthanised and the intact lower jaws dissected free of the adhering soft tissue and fixed in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4 overnight in the cold. After fixation, one hemimandible from each mouse was demineralised in 10% EDTA buffer, pH 7.4 overnight in the cold. After fixation, one hemimandible from each mouse was demineralised in 10% EDTA containing 0.8% formaldehyde, pH 7.4 for six weeks at room temperature on a shaker and then embedded in Technovit 8100 (Kulzer AG, Germany) according to manufacturers’ instructions. The polymerised blocks were sectioned at 2 μm thickness and routinely stained with either toluidine blue or haematoxylin–eosin (HE). The other (non-decalcified) hemimandible was dehydrated in ascending alcohol series, embedded in methyl methacrylate (MMA) and further analysed for mineral content.

In order to visualise the three-dimensional architecture of the mineralised tissues in the jaw, one representative MMA-embedded Ae2a,b/- and one Ae2a,b/- hemi-mandible were scanned at a resolution of 6 μm voxels using μCT-40 high resolution scanner (Scanco Medical, AG, Bassersdorf, Switzerland) before preparation for the analysis of mineral content.

4.2. Immunohistochemistry

For the detection of Ae2 isoforms we used polyclonal rabbit antibodies raised against a synthetic peptide, identical to the 12
of 20 μm. Analysis times were 25 s for calcium and phosphorus, 36 s for magnesium, and 50 s for sulphur.

4.5. Scanning microscopy

After microanalysis, the graphite coating was removed from the surface of the blocks by gentle polishing and then the surface etched with 40% phosphoric acid solution for 30 s, thoroughly washed with distilled water, dried, gold-palladium-coated and then observed in Jeol Super Probe scanning electron microscope.

4.6. Statistics

Values are presented as means and standard deviation (±SD). Significant differences were calculated using Student’s t-test, and values were judged significant at \( p \leq 0.05 \) (two-sided).

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