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Bicarbonate Transport During Enamel Maturation

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Abstract

Amelogenesis (tooth enamel formation) is a biomineralization process consisting primarily of two stages (secretory stage and maturation stage) with unique features. During the secretory stage, the inner epithelium of the enamel organ (i.e., the ameloblast cells) synthesizes and secretes enamel matrix proteins (EMPs) into the enamel space. The protein-rich enamel matrix forms a highly organized architecture in a pH-neutral microenvironment. As amelogenesis transitions to maturation stage, EMPs are degraded and internalized by ameloblasts through endosomal–lysosomal pathways. Enamel crystallite formation is initiated early in the secretory stage, however, during maturation stage the more rapid deposition of calcium and phosphate into the enamel space results in a rapid expansion of crystallite length and mineral volume. During maturation-stage amelogenesis, the pH value of enamel varies considerably from slightly above neutral to acidic. Extracellular acid–base balance during enamel maturation is tightly controlled by ameloblast-mediated regulatory networks, which include significant synthesis and movement of bicarbonate ions from both the enamel papillary layer cells and ameloblasts. In this review we summarize the carbonic anhydrases and the carbonate transporters/exchangers involved in pH regulation in maturation-stage amelogenesis. Proteins that have been shown to be instrumental in this process include CA2, CA6, CFTR, AE2, NBCe1, SLC26A1/SAT1, SLC26A3/DRA, SLC26A4/PDS, SLC26A6/PAT1, and SLC26A7/SUT2. In addition, we discuss the association of miRNA regulation with bicarbonate transport in tooth enamel formation.

Keywords

Amelogenesis; Bicarbonate; Carbonic anhydrase; Enamel; Solute carrier genes

Introduction

Amelogenesis is the process of tooth enamel formation. This developmental process features two major stages—secretory and maturation, which are clearly demarcated by a transition zone [59]. As the inner epithelium of the enamel organ, composed of ameloblast cells, transitions from secretory to maturation stage, the ameloblasts undergo both morphological

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and functional changes. During secretory-stage amelogenesis, ameloblasts are tall and columnar in shape and are highly polarized with Tomes' processes extending into the enamel matrix front [59]. Ameloblasts in this stage mainly function to synthesize and secrete a small number of structural enamel matrix proteins (EMPs), and also MMP20, such that the full thickness of ordered enamel volume is achieved concurrent with necessary proteolytic activity. In secretory-stage amelogenesis the protein-rich enamel matrix is maintained in a neutral pH condition [45, 74, 75].

Maturation-stage ameloblasts maintain their apical-basal polarity, and alternate between a 'ruffle-ended' ameloblast (RA) and 'smooth-ended' ameloblast (SA) phenotype [38, 74]. Cyclical changes between RA and SA morphology have been discussed in terms of dynamic changes in tight junctional seals at the apical region of the lateral membrane during the RA phase, and leaky junctions at the apical and distal region of the lateral membrane during the SA phase. Both tight and leaky junctional complexes have been observed at the proximal region of the lateral membranes in both RA and SA phenotypes [27, 31, 72, 74, 83]. The resulting dynamic intercellular permeability pattern may allow for intercellular movements of mineral ions and small molecules from the circulation, and into and out of the enamel matrix [39, 74]. In maturation-stage amelogenesis, the enamel structure experiences rapid mineral volumetric expansion associated with the ionic supersaturation of enamel fluid [74, 76]. Concurrently, extracellular EMPs are degraded by extracellular proteolytic enzymes, and the resulting protein debris is internalized by ameloblasts through endosome-lysosome pathways [42, 69, 77]. It is commonly accepted that the deposition of calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions during enamel maturation occurs in a mildly acidic extracellular environment [45, 68, 71, 74]. However, the extracellular pH varies considerably, ranging from slightly above neutral to acidic conditions, with a return to a physiologic pH level at the end of maturation stage [56, 74, 75]. Most previous studies suggest that the drop in pH during maturation stage results from proton release induced by enamel crystal nucleation and growth, and ameloblasts then react at the molecular level to buffer the acidic extracellular environment [45, 68, 71, 74]. Although there is still some discussion regarding the temporal and spatial working mode of enamel pH regulation, acid-base balance in the microenvironment of enamel maturation requires bicarbonate (HCO_3^-) synthesis and transport mediated by ameloblasts [45].

Carbonic Anhydrases (CAs)

In mammals, carbonic anhydrases (CAs) are a large family of enzymes with 16 homologs each generated from a distinct gene locus, of which 13 are enzymatically active [16, 81, 82]. CAs catalyze the reversible conversion of carbon dioxide (CO_2) and water (H_2O) to bicarbonate (HCO_3^-) and a hydrogen ion (H^+). CAs are clearly involved in ameloblast-mediated pH regulation of the developing enamel matrix, and the carbon provided may also contribute to the initial mineralization of tooth enamel [21, 40, 53, 78, 79, 85]. A systematic RT-PCR survey of CA expression conducted by Lacruz et al. revealed that all CA genes are expressed by mouse enamel organ epithelium (EOE) cells, with CA2, CA3, CA6, CA9, and CA13 being the most highly expressed catalytic isozymes [44, 47].

Compared to the undifferentiated expression patterns of all other CA gene family members during amelogenesis, both CA2 (which is cytosolic) and CA6 (which is secreted) exhibit significantly upregulated expression during maturation-stage amelogenesis when compared to secretory stage [47, 90]. In maturation stage, CA2 mainly localizes to the ruffled border of the RA [7, 53]. The specific localization of CA2 led to the hypothesis that the function of CA2 may be coupled with a series of proton pumps, such as the V-type ATPase proton pump, which is also highly expressed in the ruffled border of RA [45, 68]. CA6 has been identified as the only secreted member of the CA family [61, 63, 73]. The expression of CA6 in maturation-stage enamel organ cells, including ameloblasts, has been confirmed by RT-PCR, northern blot analysis, and genomewide mRNA profiling [45, 47, 48, 78, 90]. Nevertheless, there is still a lack of information regarding the exact localization of CA6 in the enamel space once secreted, and as such, the likely function of CA6 in enamel space is yet to be clarified. For example, if CA6 is a secretory product of the RA rather than SA, then it could be speculated that the V-type ATPase proton pump and CA6 work to finely control extracellular pH conditions during hydroxyapatite formation [51]. It has been postulated that CA6 may function to generate HCO_3^- in enamel matrix and facilitate the recycling of proton ions [45, 78]. It should be noted that to date no enamel pathologies have been reported as associated with genetic defects in any CA gene family members.

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

The Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-regulated chloride (Cl^-) channel localized to the apical membrane of epithelium in many organs. CFTR also contributes to the trans-epithelial transport of HCO_3^- by direct permeation through the channel or by regulating $\text{Cl}^-/\text{HCO}_3^-$ exchangers [62, 84]. Mutations in *CFTR* are responsible for cystic fibrosis (CF), which is the most common and most lethal genetic disorder in the population of European origin [17]. In CF, disruption of Cl^- and sodium (Na^+) transport induces viscous mucosal secretion in the lungs, liver, pancreas, and intestine, which leads to failure or insufficiency of the target organs [17].

CFTR maintains a high level of expression across the different stages of amelogenesis. During enamel maturation, the expression of *CFTR* is significantly upregulated compared to secretory stage [42, 47, 90]. In the rodent enamel organ, CFTR is localized to the apical membrane of maturation-stage ameloblasts [8, 91]. Additionally, co-localization of CFTR and multiple solute carrier 26A (SLC26A) gene family members on the apical end of maturation-stage ameloblasts has been reported, suggesting the potential functional linkage between CFTR and other SLC26A anion exchanger proteins [91]. CFTR is critical in maintaining the pH homeostasis required for mineral deposition in the enamel space. It has been proposed that CFTR mainly serves to transport intracellular Cl^- to the enamel matrix, and the released Cl^- is recycled in exchange for HCO_3^- through the activities of transmembrane anion exchangers, including members of the SLC26A gene family [9, 45]. *Cftr*-deficient mice have decreased Cl^- levels in the enamel [11, 80]. Enamel cells treated with a CFTR inhibitor and targeted siRNA show increased intracellular pH and endocytotic activities [23].

The phenotypes of *Cfir* knockout animal models (mice and pigs) have been thoroughly documented. *Cfir*-null mice exhibit a significantly reduced composition of enamel minerals, while the enamel crystallites appear normal in their architecture and thickness under a scanning electron microscope [86]. The enamel in these mutant mice is characterized by a chalky-white appearance and degenerates prematurely upon completion of secretory stage [4, 87]. *CFTR*-F508 and *CFTR*-null pigs show hypomineralized enamel and abnormal crystal growth [15]. In human CF patients there have been a great variety of enamel abnormalities observed, which might be attributed to disrupted ameloblasts or secondary effects from therapeutic medications and other systematic problems of CF [3, 5, 18, 33, 45, 93].

Anion Exchanger 2 (SLC4A2/AE2)

Anion exchangers (AEs) or solute carrier 4 proteins (SLC4s) are a group of $\text{Cl}^-/\text{HCO}_3^-$ exchangers. Mutations in *AE1* and *AE3* cause severe human hereditary disorders, including spherocytosis, distal renal tubular acidosis, episodic movement disorder, and seizure [1, 13, 24, 36]. Human hereditary diseases associated with AE2 mutations have never been reported; however, reduced expression of AE2 may be related to primary biliary cirrhosis in human patients [57].

At the onset of maturation-stage amelogenesis, the expression of ameloblast-specific AE2 is significantly upregulated [47, 90]. AE2 shows a basolateral membrane distribution pattern in maturation-stage ameloblasts [39, 55, 91]. The major function of AE2 during enamel maturation is to transport HCO_3^- into the enamel space in exchange for extracellular Cl^- . Mutant mice deficient in AE2 expression show a disorganized maturation ameloblast morphology and substantially less mineralized enamel [55]. In these *Slc4a2*/AE2 mutant mice, there is a lack of normal rod-interrod enamel structure, and the enamel wears faster than in wild-type teeth [55]. In an *Slc4a2*-null mouse model, teeth fail to erupt from the poorly formed alveolar bone, which may be attributed to a coincident dysfunction of osteoclasts [12].

Electrogenic Sodium Bicarbonate Cotransporter 1 (SLC4A4/NBCE1)

The electrogenic sodium bicarbonate cotransporter 1 (NBCE1) is another member of the SLC4A family that has been identified as being expressed in ameloblasts [45, 46, 60]. NBCE1 is encoded by *SLC4A4* and has three major spliced variants: NBCE1-A, NBCE1-B, and NBCE1-C [67]. NBCE1-B is the predominant isoform detected in RNA samples isolated from ameloblasts or ameloblast-like cells [34, 46, 60]. Unlike the anion exchanger activity of AE2, NBCE1 mediates the transport of Na^+ and HCO_3^- across the plasma membrane. In human patients, mutations in *SLC4A4* have been reported to cause proximal renal tubular acidosis, glaucoma, cataracts, and enamel defects [20, 32].

NBCE1 protein immunostains positively to the basal membrane of both secretory- and maturation-stage ameloblasts, although it is during maturation-stage amelogenesis that NBCE1 is most highly expressed [34, 42, 46, 47, 50, 60, 90]. The enamel development of *Slc4a4*-null mice is severely disrupted, featuring disorganized ameloblasts, extremely

hypoplastic and thin enamel without typical prismatic structure [28, 34, 46]. The gross enamel defects seen in these mutant mice, which have been described as raised, white, chalk-like spots, are similar to those observed in human patients lacking proper NBCe1 function [32].

Solute Carrier 26A Family

The solute carrier 26A (SLC26A) gene family consists of 11 members/homologs in mammals, SLC26A1- SLC26A11 [2]. Most SLC26A family proteins are multifunctional anion exchangers and/or anion channels with high affinity to a broad range of substrates, including chloride, sulfate, bicarbonate, oxalate, iodine, and formate [2]. Human genetic deficiencies in SLC26As are associated with three recessive congenital diseases: diastrophic dysplasia (SLC26A2), chloride diarrhea (SLC26A3), and deafness with enlargement of the vestibular aqueduct and thyroid disorder (SLC26A4) [29, 30, 70].

Rodent models have established that *Slc26a1*, *Slc26a3*, *Slc26a4*, *Slc26a6*, and *Slc26a7* are all expressed in ameloblasts during amelogenesis [10, 35, 91]. Among the SLC26A genes in rodents, *Slc26a1*, *Slc26a6*, and *Slc26a7* are the only members that show significant upregulation at both the mRNA and protein levels during enamel maturation [90, 91]. The protein products of these three genes all exhibit functional activity involving $\text{Cl}^-/\text{HCO}_3^-$ exchange activities [64–66]. Using immunofluorescence (IF) staining and co-immunoprecipitation (CoIP) methodologies, Slc26a1, Slc26a6, and Slc26a7 were found to colocalize with Cftr on the apical membrane of maturation-stage ameloblasts [91]. Intracellular membranous vesicles near the apical end of maturation-stage ameloblasts also stain positively for Slc26a7 [91]. Deletion of *Slc26a1* or *Slc26a7* in mice disrupts ion homeostasis, leading to urolithiasis, hepatotoxicity, distal renal tubular acidosis, and impaired gastric secretion [19, 26, 37, 88]. However, the enamel phenotypes of these transgenic animal lines are not impacted when compared with wild-type littermates, except for significant accumulation of Cl^- in *Slc26a1*^{-/-} and *Slc26a7*^{-/-} enamel [35, 91]. Strong compensatory expression of other pH regulators, such as Cftr, Ae2, NBCe1, Ca2, and non-mutated family members, in the maturation-stage enamel organ seem to be responsible for the absence of any major enamel pathologies in these mouse models [35, 91]. Most recently, our group generated a double-null mutant mouse line with the absence of both *Slc26a1*^{-/-} and *Slc26a7*^{-/-} [89]. The enamel density in the double-mutant mice was significantly lower in the regions that represent late maturation-, maturation-, and secretory-stage enamel development in wild-type mandibular incisors. Elemental composition analysis revealed a lack of mineral deposition and an accumulation of carbon and chloride in the double-mutant enamel. Compensatory expression of pH regulator genes and ion transporters was also detected in maturation-stage enamel organs of doublemutant animals when compared to wild-type. Taken together, these data provide more evidence to support the hypothesis that multiple SLC26A gene family members participate in pH regulation during enamel maturation.

MicroRNA and Bicarbonate Transporters

MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate target gene expression by directly binding to the seeding sequence in the 3' UTR of their target mRNAs. In human patients with primary biliary cirrhosis (PBC), decreased expression of AE2 is associated with upregulation of miR-506 [6]. Luciferase assays and site-specific mutagenesis hosted by human cholangiocytes demonstrated that miR-506 targets AE2 mRNA and prevents protein translation. This suggests that dysregulation of AE2 expression by miR-506 may contribute to the pathogenesis of PBC.

The involvement of miRNA regulation in tooth development has been indicated in multiple previous studies [14, 52, 54, 58]. Genome-wide miRNA and mRNA expression profiling conducted by our group identified differentially expressed miRNAs during amelogenesis in rodent incisors [90]. Subsequent bioinformatic analyses established the linkage between miRNA regulators and the predicted key genes in HCO_3^- transport during enamel maturation [90]. The predicted regulatory role of miRs in pH regulation during enamel formation is further corroborated by several functional studies. MiR-224 has been reported to modulate the expression of Cftr and Slc4a4/NBCE1 in cultured human epithelial cells [25]. Microinjection of miR-224 agomir/mimics in mouse neonatal incisors resulted in disturbed enamel mineralization [25]. In another functional study, miR-153 was found to regulate ameloblast-mediated endocytotic and endosomal/lysosomal pathways by targeting *Cltc*, *Lamp1*, *Cln4*, and *Slc4a4* gene transcripts [92]. Finally, enamel hypomineralization was observed in mouse mandibular first molars after miR-153 overloading was introduced during the maturation stage [92].

Summary

Acid–base homeostasis mediated by ameloblasts is one of the key processes during maturation-stage amelogenesis. The involvement of CAs, CFTR, and SLC4A and SLC26A gene family members in transporting HCO_3^- and other related ions between intracellular lumens and the extracellular enamel space has been widely accepted [7, 9, 22, 39, 41, 43, 49, 74]. Additionally, miRNA-centered epigenetic regulation of pH regulatory pathways involved in amelogenesis may provide novel insights into the genesis of enamel pathologies and potential preventative or therapeutic interventions. A summary of the localization of these ion transport proteins in the enamel organ, including ameloblasts, and relevant references are provided in Table 1 and Fig. 1.

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Fig. 1.
Schematic diagram of carbonic anhydrases, bicarbonate transporters/exchangers, and miR regulators involved in pH regulation during enamel maturation

Table 1

Localization of CA2, CA6, CFTR, SLC4A2, SLC4A4, SLC26A1, SLC26A3, SLC26A4, SLC26A6, and SLC26A7 in the enamel organ

Gene symbol	Name	Function	Localization in maturation-stage enamel organ	References
CA2	Carbonic anhydrase 2	Bicarbonate generation through H^+ , CO_2	Intracellular	[39, 53, 85]
CA6	Carbonic anhydrase 6	Bicarbonate generation through H^+ , CO_2	Extracellular (enamel space)	[78]
CFTR	Cystic fibrosis transmembrane conductance regulator	Cl^- and HCO_3^- transport	Apical membrane of ameloblast	[8, 80]
SLC4A2	Anion exchanger 2 (AE2)	1 $Cl^-/1 HCO_3^-$ exchange	Lateral membrane of ameloblast	[55, 60]
SLC4A4	Electrogenic sodium bicarbonate exchanger 1 (NBCe1)	3 $Na^+/1 HCO_3^-$ exchange	Basolateral membrane of ameloblast and papillary layer	[39, 46, 60]
SLC26A1	Sulfate anion transporter 1 (SAT1)	Cl^-/HCO_3^- exchange	Apical membrane of ameloblast and papillary layer	[91]
SLC26A3	Downregulated in adenoma (DRA)	Cl^-/HCO_3^- exchange	Apical membrane of ameloblast and papillary layer	[35]
SLC26A4	Pendrin	Cl^-/HCO_3^- exchange	Apical membrane of ameloblast	[10, 35]
SLC26A6	Putative anion transporter 1 (PAT1)	Cl^-/HCO_3^- exchange	Apical membrane of ameloblast	[35, 91]
SLC26A7	Sulfate transporter 2 (SUT2)	Cl^-/HCO_3^- exchange	Apical membrane of ameloblast and intracellular	[91]