Secretory carbonic anhydrase II – Finding the evolutionary key to the symbiosis of animal hosts and their cellulose-fermenting bacteria

Marcus Mau¹*, Karl-Heinz Südekum²

Grasses, leaves and wood contain large amounts of structural carbohydrates such as cellulose, which in animals are digested with the help of symbiotic, cellulolytic microorganisms living in fermentative chambers in the stomach or gut. Although we find the identical relationship in grass eaters (ruminants), in leave-eaters (Colobus monkeys) and in wood-eaters (termites), still little is known about the evolutionary pre-requisites that enabled the development of this very complex symbiosis. Two carbonic anhydrases (CA; CA-VI and the recently described CA-II) are prominently expressed in the saliva of ruminating animals, suggesting that the enzymatic production of bicarbonate is basic for the regulation of oral pH and for maintaining a consistent milieu in the alimentary tract allowing microbial digestion of cellulose. Interestingly, the fermentative chambers of termites (Arthropoda), Colobus monkeys and ruminants show a similar slightly acidic to basic pH. Therefore, the expression of high amounts of CA-II in saliva or mucus of ruminants, camelids, Colobus monkeys and termites is considered to be an essential basis for the development of cellulose fermentation with the help of symbiotic bacteria.

Introduction

The parotid gland has the highest carbonic anhydrase (CA) activity of all ruminant salivary glands (1). Since the saliva is well buffered at a constant pH of 8 and flows continuously into the rumen, salivary CA might play an important role in maintaining a constant oral and ruminal milieu (2; 3). Especially, CA-II was shown to be essential for the production of regular amounts of saliva (4). In fact, almost half of the bicarbonate entering the rumen comes from saliva (5), where it is most likely produced by active carbonic anhydrases. One such enzyme is the secreted salivary CA-II, which is abundant in cattle and other ruminating animals such as camels or goats, whereas animals not necessarily relying on cellulose-fermentation like baboons and humans have only small amounts of CA-II in their saliva (6).

Most interestingly, apart from ruminants other herbivorous animals have developed chambered stomachs with foregut-fermentation or special fermentation chambers in the gut. Both compartments show equal pH milieus of slightly acidic up to slightly basic in order to support microbial, cellulolytic symbionts (Table 1).

For example, Colobus monkeys, which are unique among primates, share with ruminants a large, multi-chambered stomach containing microbes that enable them to use structural carbohydrates such as cellulose as energy sources (14). The pH of their foregut varies between 5.0 and 8.0, depending on the grade of concentrate food that is fed to the captive animals and used to study the stomach pH (15). Nonetheless, nothing is known about the enzymes involved, or the degree to which they may help to regulate the pH in the fermentation chamber (15;16).
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The connection of certain pH values and cellulose-fermenting symbionts cannot only be found in mammals but also in insects. Although termites show a wide range of food-preferences from soil to grass and wood, many of them depend on the fermentation of cellulose as an energy source, which requires an obligate symbiosis with microorganisms analogous to the bovine rumen (11; 12). And as with ruminants and Colobus monkeys the microbe-packed region of the intestinal tract in termites has a well-regulated pH between 6.0 and 7.5 or up to 10 in some species (11; 12; 13). The fermentation chamber is very well defined by its pH and there is an abrupt drop of pH when reaching the next gut segment in termites (12).

Because of the similarities of the pH milieu in the fermentation chambers in ruminating animals, Colobus monkeys and termites, a prominent expression of salivary or mucosal carbonic anhydrases to regulate this pH is very likely.

Therefore, a comparative study of salivary and mucus carbonic anhydrases in four distinct animal lineages (ruminants, camelids, primates, insects), all characterized by using cellulose-rich food, would hold the potential to discover CA-II as a key enzyme involved in the evolutionary development of cellulose fermentation in animals with the help of symbiotic microorganisms.

The hypothesis to test

Ruminants, camels and Colobus monkeys share the multi-chambered stomach as a common feature as well as the development of severe acidosis, when the pH is reduced due to fermentation of rapidly degraded carbohydrates such as sugars and starch. Thus, pH regulation in the fore-stomach or rumen is crucial for animal well-being and the capability to ferment fibrous, cellulose-rich food with the help of symbiotic micro organisms. The same pH conditions are found in termite guts, equally providing a suitable environment for symbiotic micro organisms. As an adaptation to keep the pH at a constant level for microbial fermentation, it is hypothesized that all ruminants, Colobus monkeys and termites secrete carbonic anhydrases with their saliva or mucus, with CA-II and CA-VI already having been described in ruminating animals such as cattle, goats and camels (Fig. 1).

The amount of salivary CA-II expressed in ruminating animals is much higher in comparison to non-ruminants, such as baboons or humans (6; 17). This implies a key enzymatic role of CA-II in rumination. This idea will gain additional support if a secreted CA-II is present in foregut fermenters originating from three different evolutionary lineages – ruminants, camelids and primates – as well as in termites.

Table 1 | Termites and grass or leave-eating mammals share characteristic pH values in the specialized compartments of their rumen, fore-stomach or gut that contain symbiotic microorganisms fermenting cellulose and thus providing an essential energy source for their hosts.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cellulose from</th>
<th>pH/fermentation chamber</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camel</td>
<td>grass</td>
<td>6.7/fore-stomach</td>
<td>7</td>
</tr>
<tr>
<td>Cattle</td>
<td>grass</td>
<td>6.7/rumen</td>
<td>2; 8</td>
</tr>
<tr>
<td>Hairy sheep</td>
<td>grass</td>
<td>6.12/rumen</td>
<td>9</td>
</tr>
<tr>
<td>Impala</td>
<td>grass</td>
<td>6.30/rumen</td>
<td>9</td>
</tr>
<tr>
<td>Massai goat</td>
<td>grass</td>
<td>6.10/rumen</td>
<td>9</td>
</tr>
<tr>
<td>Thomson's gazelle</td>
<td>Grass</td>
<td>6.04/rumen</td>
<td>9</td>
</tr>
<tr>
<td>Colobus monkey</td>
<td>Leaves</td>
<td>6.5-8/fore-stomach</td>
<td>10</td>
</tr>
<tr>
<td>Higher termites</td>
<td>grass, wood</td>
<td>6.0-7.5; 10/gut</td>
<td>11-13</td>
</tr>
</tbody>
</table>

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It is further suggested that there is a relation between fore-stomach pH and salivary CA-II expression/activity. A high amount of rapidly degraded sugars in the diet of captive animals causes an excess of short-chain fatty acids (SCFA) such as propionate or acetate, which are produced by endosymbiotic microorganisms in the fore-stomach or rumen (5). Due to this excess of SCFA, the pH in the fore-stomach drops (5). We hypothesize that during developing acidosis the resulting imbalance of ruminal pH triggers an increased expression on the protein level of salivary carbonic anhydrases to cope with the changing pH level. This might be induced by the perception of SCFA, which are known to enter the blood stream (5; 18; 19). In the salivary glands, SCFA from the blood might be recognized by the recently described GPR43 receptors (20), which are known to alter gene expression in cells as a consequence of activation (21). However, it is unknown yet if the expression of the CA gene family in salivary glands might be altered by activated GPR43. Nonetheless, once the maximum in either expression or activity of CA-II has been reached, the surplus of free acids in the stomach can no longer be compensated for. At this time, acidification of the stomach becomes irreversible, leading to the manifestation of acidosis, which has been reported before to occur regularly in ruminants, camels and colobines, respectively (5; 22-24). Although SCFA were also described as products at the end of cellulose fermentation in termites (25), nothing is known about possible acidose-like conditions in these insects. However, the analogy with the processes taking place during ruminal fermentation in cattle suggests that similar regulatory mechanisms might take place also in termites.

Testing the hypothesis

Two experimental approaches are suggested to test the hypothesis of CA-II involvement in the evolutionary establishment of the symbiosis between herbivorous animals and cellulolytic microbials.

The first step is to characterize the potential expression of secretory CA-II protein in the mucus of termites as well as in the saliva of Colobus monkeys in comparison to cattle, goats and camels. Termite hindguts and their fluids will be collected according to the method provided by Odelson & Breznak (25). Salivary sampling will be performed using Salivetten® (Sarstedt, Nümbrecht, Germany) to collect resting saliva in anaesthetized colobines in a zoo setting as previously described for other primate species (26). Immunoblotting is a fast and simple technique to test the expression and activity of CA-II in these samples.
method to detect the enzymes in salivary samples (6). An anti-bovine CA-specific antibody as well as an anti-human CA-II-specific antibody have already been tested and found to be suitable for detection of salivary CA-II in a wide range of mammals including cattle, camels, Hamadryas baboons, Geladas baboons and humans (6). If one or both antibodies detect a protein at 29 kDa, this will be further analysed and identified using mass spectrometry. Describing a CA-II from ruminant and Colobus saliva together with CA from termite mucus will strongly support the idea of CA-II being a key enzyme in the evolutionary development of the symbiosis of herbivorous animals (host) and cellulolytic bacteria (symbionts).

The second step in testing the hypothesis is meant to identify a clear connection between CA-II expression and ruminal pH. Therefore, an animal model using sheep as the ruminating model species will be established. Fistulated sheep are subjected to subacute ruminal acidosis (SARA) according to Brossard et al. (27). The daily hay rations are replaced with a 60:40 mixture of wheat/hay. Due to the increased presence of highly digestible, rapidly fermentable carbohydrates that lead to excessive concentrations of SCFA, ruminal pH will be decreased to an acidosis-like level (sub-acidosis) without dangerous side effects for the animals. After sampling, proteomic analyses (2D gel electrophoresis, immunoblotting, mass spectrometry and LCMS) can be used on the animal samples to follow protein expression changes of CA-VI and CA-II in saliva and ruminal fluid during acidosis induction (pH drop). The results of this experiment will help to clearly support or reject the idea of CA-II being a key regulator of cellulose fermentation. If CA-II is involved in pH regulation, then it is suggested to be increasingly expressed in acidotic environments.

Implications of the hypothesis
The high expression of CA-II in saliva of ruminating animals may be interpreted as an adaptation to maintain a suitable environment for ruminal microbes that are essential for the digestion of tough-structured, fibrous grass diets (3, 6). This would get strong support from the expression of the identical enzyme in Colobus monkeys, known to be the only foregut-fermenting primates, as well as in termites, the hindgut of which is an insect analogue to the bovine rumen. Providing the evidence of a connection between salivary CA concentration and changing ruminal or stomach pH in fore-stomach fermenters will enable us to easily and non-invasively detect and diagnose developing acidosis in a zoo environment before it becomes serious or lethal. Thereby, the overall performance and breeding success of Colobus monkeys or zoo ruminants could be improved.

Studies following the idea and techniques presented here may help to increase our knowledge of the important role that pH regulation plays in the evolution of the symbiosis of herbivorous animals with their symbiotic, cellulolytic microbes. Moreover, they will help us to better understand the mechanisms working between organs (stomach, salivary glands) when species specialize to dietary niches. This hypothesis is thus carefully outlined here in hopes of stimulating a broader interest in further studies concerning the role of salivary or mucus carbonic anhydrases in species that depend on cellulose fermentation by endosymbionts.

Originality statement
Ideas about the involvement of salivary CA-II in pH regulation in ruminants have previously been discussed (6, 17, 20) and are cited properly throughout the manuscript.

Author contributions
MM and KHS drafted this manuscript and its content together. Both authors read and approved its final version prior to submission.

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