The enzyme carbonic anhydrase as an integral component of biogenic Ca-carbonate formation in sponge spicules

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text

1. Introduction

Calcium carbonate [CaCO₃] is a biomineral that constitutes the inorganic scaffold of the skeletal elements in non-metazoan (e.g. the single-celled algae coccolithophores) and also metazoan taxa [1,2]. The evolutionary oldest metazoans that invented Ca-carbonate as a scaffold for their skeleton are the calcareous sponges (Class Calcarea) that emerged on Earth, approximately 540 Ma [3]. While the first sponge taxa (Hexactinellida; Demospongia), as the earliest metazoans that diverged from the common metazoan ancestor, the Urmetazoa [4], comprise a siliceous skeleton, the calcareous sponges substitute the inorganic scaffold bio-silica for bio-calcite, very likely due to environmental constraints, the accumulation of Ca-carbonate in the ancient oceans [5]. Even though the present-day oceans are supersaturated with respect to CaCO₃, only very rarely spontaneous abiotic precipitation is seen [6]. In biological systems, e.g. sponges, molluscs or echinoderms, Ca-carbonate is taken up from the aqueous environment as bicarbonate via specific membrane transporters [7] characterized by a Michaelis–Menten constant of around 50 mM [8]. At this concentration, Ca-carbonate precipitates at an extent of around 50% during an incubation period of 20 h in an ammonium carbonate diffusion/dessicator assay at a pH of 7–8 [9]. However, this reaction velocity is too slow to account for the observed Ca-carbonate deposition, measured in vivo, e.g. in the sponge spicule formation in Sycon sp. [10]; those spicules have, with a diameter of around 4 μm, a very fast growth rate of 65 μm/h. Since the calcitic deposition reaction is exergonic [11], an acceleration of the reaction velocity can be reached by lowering the activation energy either allowing the process of Ca-carbonate deposition to proceed on a functionalized organic surface [see Ref. [1]] or by coupling of the membrane-bound bicarbonate transporter with the soluble enzyme, the carbonic anhydrase [CA] [7]. While the accelerating or the decelerating proteinaceous components within biogenic Ca-carbonate skeletal structures, e.g. mollusk shell, have been extensively described (reviewed in Ref. [12]), an enzyme kinetic analysis of the CA during the Ca-carbonate deposition reaction has not been published. CAes form reversibly bicarbonate by

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http://dx.doi.org/10.1016/j.febsopenbio.2013.08.004

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hydration of carbon dioxide (CO₂), a reaction which represents the rate-limiting step in the process of Ca-carbonate precipitation in the presence of Ca cations [13]. The CAeS are among the fastest catalyzing enzymes and – in turn – represent a key catalyst in the fixation of CO₂ during deposition of Ca-carbonate [14].

The enzymes CAeS are found in all living taxa; they catalyze the rate-limiting reaction during Ca-carbonate mineral precipitation [15], i.e. the formation of bicarbonate, which is reversibly formed by a Ca-mediated hydration of carbon dioxide [CO₂]. In the present study we show that in the calcareous sponge spicular system for Sycon raphanus the homologous CA enzyme contributes essentially to the Ca-carbonate deposition by a considerable increase of its reaction velocity. The first experimental evidence that the CA is involved in calcareous spicule formation had been elaborated for S. raphanus [16]. The calcareous sponge S. raphanus reinforces its body both with diactines, two-rayed spicules, and with triactines, three-rayed spicules; the dimension of each ray varies between 100–170 μm in length and 6–10 μm in diameter (Fig. 1A).

2. Materials and methods

2.1. Animals

Specimens of S. raphanus (Porifera, Calcarea, Leucosolenida, Sycettidae) were collected in the Northern Adriatic Sea near Rovinj (Croatia). They were cultivated in the presence of 1 mM CaCl₂. The spicules were isolated from the specimens with 0.5% (v/v) NaOCl [16].

2.2. Expression of carbonic anhydrase cDNA from S. raphanus

The complete cDNA (AMBL Accession No. HE610176), lacking the signal peptide as well as the transmembrane region, but comprising the complete carbonic anhydrase domain was expressed in Escherichia coli [16]. The bacterial cells were grown in Luria broth medium, containing 100 μM ZnSO₄ [17]. The recombinant enzyme was purified by Ni-NTA agarose affinity chromatography. The specific activity was determined to be 2500 units/mg protein, by applying the Wilbur–Anderson assay [18,19].

2.3. Ca-carbonate precipitation assay

For preparation of Ca-carbonate precipitates, the ammonium carbonate diffusion method/“dressicator method” had been used [20,21]. The CO₂ vapor was generated from a NH₄HCO₃ (Sigma; ≥99.0%) solution, placed in the lower compartment, which diffuses into the upper compartment of the dressicator, where a Petri dish with 5 mL of 50 mM CaCl₂ (Sigma; ≥99.9%) was put. This solution was buffered with 10 mM HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid] to pH 7.5. Routinely the assays were performed for up to 30 h at a temperature of 25 °C. Where indicated, 3 units/mL of the recombinant Ca from S. raphanus was added to the CaCl₂ solution. For the determination of the pH dependence of reaction, the assays were buffered with HEPES and 100 mM MES [2-(N-morpholino)ethanesulfonic acid] and then pH was adjusted. Where indicated the CA activity was inhibited by addition of 3 μM acetazolamide (A177 Sigma).

To follow up quantitatively the generation of Ca-carbonate, the free Ca²⁺ concentration in the CaCl₂ solution was determined by EDTA titration [22]. Six parallel determinations were performed. The analyses given, including the hardness tests, were performed immediately after taking the samples.

2.4. Carbonic anhydrase esterase assay

The colorimetric assay using 4-nitrophenylacetate (NPA; Sigma) as a substrate was applied as described [23]. The assay (total volume of 750 μl) was composed of 15 mM Tris/SO₄ buffer (pH 7.4), 3 mM 4-nitrophenylacetate, and 25 μl of recombinant enzyme sample (10 units of enzyme). The reaction was carried out at 22 °C for 3 min. The enzyme activity is given in mmoles·ml⁻¹·min⁻¹.

2.5. Kinetic studies (Lineweaver–Burk plot)

The apparent Michaelis–Menten constants (Kₚ) were calculated from Lineweaver–Burk plots [24].

2.6. Mechanical studies

Mechanical properties of the Ca-carbonate deposits formed were determined with a NanoTest Vantage system (Micro Materials Ltd., Wrexham, UK), equipped with a Berkovitch diamond indenter, allowing continuous depth-sensing indentation [25,26]. Ten indents were performed for each measurement at 25 °C; maximum depth of an indentation was limited to 300 nm.

2.7. Microscopic inspections

Light microscopy (LM) was performed with a light digital microscope (VHX-600 Digital Microscope) from KEYENCE (Neu-Isering, Germany), equipped with a VH-225 zoom lens. For the scanning electron microscopic (SEM) analyses, a HITACHI SU 8000 (Hitachi High-Technologies Europe GmbH, Krefeld; Germany) was employed at low voltage (<1 kV; analysis of near-surface organic surfaces) [27].
2.8 Statistics

The results were statistically evaluated [28].

3 Results

3.1 Acceleration of bio-calcite deposition by CA

Using a starting concentration of 50 mM CaCl₂, about 80% of this soluble salt was converted to insoluble Ca-carbonate after an incubation period of 24 h (at pH 7.5 and 25 °C) by using the ammonium carbonate diffusion process [28]. The mineralization process (based on the decrease of free Ca²⁺ concentration measured) started after an initial lag phase of 5 h (Fig. 2). Addition of the homologous recombinant CA (3 units/mL) significantly increased the reaction velocity and accelerated the mineralization process; after 5 h already 26% of the CaCl₂ had been precipitated, in the presence of CO₂, to Ca-carbonate (Fig. 2). An extent of 80% of precipitated Ca-carbonate was reached after 16 h.

The morphology of the Ca-carbonate crystals formed in the diffusion assay, irrespectively of the presence of the enzyme CA, changes with the progression of the incubation period. Initially, round shaped, pat-like precipitates are formed. In the absence of CA, those deposits are visualized during the first 12 h of incubation, while in the presence of the enzyme the aggregates appear already after 4–8 h (Fig. 1B and C). The sizes of the deposits formed in the absence of the CA are larger, 73 ± 25 μm, than those developed in the presence of CA with 42 ± 28 μm. Subsequently the round shaped pat’s remold to crystal-like prisms morphology (Fig. 1D and E). That dominant morphology of the crystallites is characteristic for calcite crystals [29]. The crystalline arrangement of these components has been established by X-ray diffraction. The energy-dispersive X-ray (EDX)-based elemental analyses revealed that the crystals are composed of the elements calcium, oxygen and carbon (to be published).

In the absence of Sycon spicules in the Ca-carbonate forming assay, the crystal-like prisms associate to each other and form rope-/bundle-like aggregates (Fig. 1D). However, after addition of Sycon spicules to the precipitation assay, the crystallites associate perfectly with their smaller planes along opposing surfaces of the spicule ray (Fig. 1D and E). The two arrays of crystals, the overgrowth, are facing each other along the spicules and leave the two remaining surface areas uncovered.

3.2 Dependence of CA-mediated calcite formation on temperature and pH

The extent of Ca-carbonate deposition at 10 °C is independent on the presence of CA and amounts to 24 ± 3 mM·15 h⁻¹ (pH 7.5) (Fig. 3). At higher incubation temperatures, the reaction velocity of CA-driven Ca-carbonate formation is significantly higher than that in the absence of CA. At 15 °C, the extent is 20.9 ± 3.1 mM·15 h⁻¹ in the absence of CA while it amounts to 30.4 ± 4.6 mM·15 h⁻¹ in the presence of 3 units/mL of CA; at 20–30 °C, the quantities of Ca-carbonate formed even doubles in the presence of CA, for example, at 25 °C the values are 49.6 ± 5.1 (presence of CA) and 23.4 ± 3.1 (absence of CA). In the absence of CA, the extent of Ca-carbonate precipitation does not change with the rising of temperature. Varying the pH value in the precipitation assay shows that in the absence of CA the precipitation of Ca-carbonate increases only slightly from pH 6.0 with 8.4 ± 0.9 mM·15 h⁻¹ (at 50 mM CaCl₂) to 17.2 ± 2.9 mM·15 h⁻¹ at pH 8.0. In contrast, the CA-driven reaction velocity increases markedly from pH 6.0 with 8.1 ± 1.0 mM·15 h⁻¹ to 42.1 ± 4.6 mM·15 h⁻¹ (pH 8.0); Fig. 4. In a parallel study it was found that the enzyme CA-driven reaction has an optimum activity between pH 7.5 and 8.0 (to be published). To support the discovery that the Ca-carbonate deposition reaction is driven by the enzyme, a CA-specific inhibitor (3 μM acetzolamide [30]) had been added to the mineralization assay. These inhibition studies revealed that in the presence of 3 μM acetzolamide the amplification of the Ca-carbonate deposition reaction due to the presence of CA was almost completely abolished (Fig. 4).

3.3 Determination of the Michaelis–Menten constant for the CA-driven mineralization

The Ca-carbonate formation reaction follows substrate saturation kinetics. Under the assay conditions used here (50 mM CaCl₂, pH 7.5, 25 °C), the linear increase of the reaction velocity is seen between
inhibitor experiments. The assay was binant CA substrate hydratase for and approached 0.32 mM CO
subrate 4-nitrophenylacetate (using CO\textsubscript{2} as substrate [32]) and for the esterase (with the substrate 4-nitrophenylacetate [33]). The \( K_m \) constant for the sponge CA/esterase was determined by using the method of Lineweaver and Burk [34].

The apparent Michaelis–Menten constant for the sponge recombiant enzyme and using 4-nitrophenylacetate as esterase substrate was calculated from a Lineweaver–Burk plot [34]; Fig. 6. Varying the substrate concentration between 0.25 and 3 mM, a \( K_m \) constant of 6.2 ± 1.0 mM was found, under a maximal reaction velocity of 0.32 ± 0.05 mmolmes·m\(^{-1} \cdot \text{min}^{-1} \). Using the same approach, the Michaelis–Menten constant had been determined in the CO\textsubscript{2} diffusion assay using 10 and 50 mM CaCl\textsubscript{2} (Fig. 7). The Lineweaver–Burk plot was computed from which the apparent \( K_m \) value was determined (9.9 ± 2.1 mM with respect to CaCl\textsubscript{2}) together with the corresponding \( V_{max} \) (24.9 ± 3.7 mM Ca-carbonate formed during 5 h incubation period).

### 3.4. Abiogenic Ca-carbonate deposition, followed by enzymatic, biogenic Ca-carbonate synthesis

Cleaned Sycon spicules having a smooth surface (Fig. 8A) had been incubated in the CO\textsubscript{2} diffusion chamber in the absence of CA for 10 h. During this period an approximately 40 nm thick layer of newly synthesized Ca-carbonate, composed of irregularly accumulated crystallites, are deposited onto the spicular surface (Fig. 8B and C). In contrast, if the spicules are incubated at first for 5 h in the absence of CA and then for additionally 5 h in the presence of CA the region of the spicules exposed to the enzyme solution become covered with orderly arranged Ca-carbonate deposits (Fig. 8D and E). During this process the new calcitic mantle around the spicule increases in size from about 40 nm to 7 \( \mu \)m. At a higher magnification the difference
in morphology between the abiotically formed irregular initial deposits and the biogenically formed regular prisms are becoming evident (Fig. 8G).

3.5. Mechanical properties of the Ca-carbonate deposits

The calcitic crystals formed in the CO₂ diffusion assay were analyzed for their hardness using a Berkovich diamond indenter. After the recording of the load-displacement curves the corresponding Martens hardness had been determined. All the in vitro synthesized crystals show hardness values varying insignificantly between 4.2 ± 1.3 GPa and 4.7 ± 1.7 GPa. As a reference the hardness of the Sycon spicules had been determined with 5.3 ± 1.8 GPa.

4. Discussion

The results reported here demonstrate that – as established for the siliceous sponge spicules [35,36] – the biomineralization process of the calcareous sponge spicules are also decisively driven enzymatically. Using the calcareous spicules from S. raphanus as an example, it is shown that the enzyme CA isolated from this animal and subsequently prepared in a recombinant way contributes essentially to the extent of the Ca-carbonate synthesis in vitro. As schematically outlined in Fig. 9, the enzyme CA is crucially important for the trapping of CO₂/bicarbonate within an organism in general and a given cell in particular. The enzyme CA both provides bicarbonate to the respective transporters, and after translocation into the compartment, removes again bicarbonate from this site. During the synthesis of Ca-carbonate this enzyme again provides the required bicarbonate anion as a substrate for the formation of Ca-carbonate in the presence of Ca²⁺. Importantly, during this reaction again CO₂ and water is released that served again as a substrate for the CA to initiate a further round of Ca-carbonate deposition. This implies that for one mole of Ca-carbonate two enzymatic CA-mediated steps are required to facilitate and accelerate the Ca-carbonate deposition. The experiments show an acceleration of the calcification process in the presence of the CA, following the kinetics of this enzyme with respect to the reaction temperature and pH as well as the Michaelis–Menten (Kₘ) affinity constant. Remarkable is the finding that a rapid association of the newly formed crystals with the homologous spicules occurs in a highly ordered pattern.

In ongoing studies we could determine that the CA-driven Ca-carbonate formation in vitro starts with the synthesis of vaterite. This crystalline form is then converted to calcite. The conclusion comes from experiments using Fourier transform infrared spectrometry under determination of the characteristic absorption bands for vaterite and 745/744 cm⁻¹ and calcite at 713/711 cm⁻¹. These new data allow now the fabrication of calcitic structures along a calcitic “template” and opens new horizons for a biotechnological application of calcareous structures. In turn, the data reported also underscore again the utilization of sponge spicules as a molecular blueprint for the manufacturing of novel materials with hitherto unknown properties, like those recently shown for the fabrication of calcite spicules [37] along the structure-determining silicatein protein [38].

Acknowledgements

W.E.G.M. is a holder of an ERC Advanced Investigator Grant (No. 268476 BIOSILICA). This work was supported by grants from the Deutsche Forschungsgemeinschaft (Schr. 277/10–2), the European Commission (“BIOMINTEC” No. 215507; Industry-Academia Partnerships and Pathways “CoreShell” No. 286059; “SPECIAL”; No. 266303; “MarBioTec EU-CN” No. 268476; and “BlueGenics” No. 311848), and the International Human Frontier Science Program.
