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Enzyme-accelerated and structure-guided crystallization of calcium carbonate: Role of the carbonic anhydrase in the homologous system

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ABSTRACT

The calcareous spicules from sponges, e.g. from *Sycon raphanus*, are composed of almost pure calcium carbonate. In order to elucidate the formation of those structural skeletal elements, the function of the enzyme carbonic anhydrase (CA), isolated from this species, during the in vitro calcium carbonate-based spicule formation, was investigated. It is shown that the recombinant sponge CA substantially accelerates calcium carbonate formation in the in vitro diffusion assay. A stoichiometric calculation revealed that the turnover rate of the sponge CA during the calcification process amounts to 25 CO_2 s^{-1} × molecule CA^{-1}. During this enzymatically driven process, initially pat-like particles are formed that are subsequently transformed to rhomboid/rhombohedroid crystals with a dimension of ~50 μm. The CA-catalyzed particles are smaller than those which are formed in the absence of the enzyme. The Martens hardness of the particles formed is ~4 GPa, a value which had been determined for other biogenic calcites. This conclusion is corroborated by energy-dispersive X-ray spectroscopy, which revealed that the particles synthesized are composed predominantly of the elements calcium, oxygen and carbon. Surprising was the finding, obtained by light and scanning electron microscopy, that the newly formed calcitic crystals associate with the calcareous spicules from *S. raphanus* in a highly ordered manner; the calcitic crystals almost perfectly arrange in an array orientation along the two opposing planes of the spicules, leaving the other two plane arrays uncovered. It is concluded that the CA is a key enzyme controlling the calcium carbonate biomineralization process, which directs the newly formed particles to existing calcareous spicular structures. It is expected that with the given tools new bioinspired materials can be fabricated.

1. Introduction

Calcium carbonate ($CaCO_3$) is an inorganic salt that is characterized both by several amorphous polymorphisms (polyamorphism) and by several crystalline polymorphisms [1]. Well-studied examples for polyamorphs are ice and silicon/silica [2,3]. The transition from a poorly ordered amorphous calcium carbonate (ACC) to highly ordered crystalline calcium carbonate (CCC) is well understood [4–7]. The CCC polymorphs exist in three anhydrous crystalline forms – calcite, aragonite and vaterite – and two hydrated crystalline polymorphs – calcium carbonate hexahydrate and calcium carbonate monohydrate. Extensive abiogenic and biogenic $CaCO_3$ deposition can be seen in nature, e.g. the abiogenic travertines, $CaCO_3$ spring deposits [8] or the biogenic coccolith deep-sea deposits [9,10]. The present day $CaCO_3$ precipitation in the ocean is almost exclusively due to an enzymatically controlled intracellular biomineralization process [11], and even the carbonate rocks and caves are mainly formed biogenically, by microorganisms and plants [12].

The level of bicarbonate in solution is crucially important for the rate of carbonate mineral precipitation [13]. Bicarbonate is reversibly formed by hydration of carbon dioxide (CO_2), a reaction which represents the rate limiting step in the process of $CaCO_3$ precipitation in the presence of Ca cations. The conversion (CO_2 and water) to bicarbonate can be drastically upregulated by the carbonic anhydrase (CA), a zinc-containing enzyme (EC 4.2.1.1). The cofactor in this enzyme is involved in the attack of bound OH^- to a CO_2 molecule that is loosely bound in the active center of the enzyme, a reaction during which the coordinated HCO_3^- ion is displaced from the metal ion by H_2O [14]. The CA, one of the fastest catalyzing enzymes, is – in turn – a key catalyst in the fixation of CO_2 and allows an increased deposition of $CaCO_3$ in this biomineralization process [15]. CAs exist in specimens of all living taxa (reviewed in Ref. [16]); the α-CAs in animals, the β-CAs in plant

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chloroplasts and most prokaryotes, γ-CAs in methane-producing bacteria, δ-CAs in diatoms and ε-CAs in bacteria. Focusing on animals, calcitic skeletons are synthesized by calcareous sponges, echinoderms, molluscs and corals [17,18]. Due to the physical association of the carbonic anhydrase with those skeletal elements, the CAs have been implicated in calcite biomineralization in gorgonians [19], molluscs [20], birds [21] and calcareous sponges [22]. The existing causal analytical investigations rely primarily on inhibition experiments, which revealed that inhibition of CAs results in an impairment of calcite formation [23].

Even though calcitic depositions also proceed under physiological conditions in an exergonic way [24], those reactions proceed in vivo much faster than the calcite deposition in vitro if based upon purely chemically driven processes only. As an example, the growth rate of the biogenic calcite spicules in calcareous sponges is very fast: 65 μm h^{-1} [25]. One major biochemical strategy to lower the activation energy of an exergonic chemical reaction in vivo, in a biological system, is the inclusion of enzyme(s) in the reaction chain. A recent example for a biochemically (enzymatically) driven biomineralization reaction is the formation of bio-silica in siliceous sponges, a condensation process accelerated by the enzyme silicatein (reviewed in Refs. [3,26]). The initial process of polycondensation is allowed to proceed at low concentrations and physiological conditions enzymatically, while the product formed is subsequently hardened and formed by non-enzymatically acting polypeptides, e.g. nidogen-like protein [27] or silintaphin-2 [28].

The calcitic spicules from the calcareous sponges are almost exclusively composed of $CaCO_3$ [29–31], and represent an (almost) single crystalline morphology [17,32,33]. However, the solid calcareous spicules are not entirely free of proteins; an intraspicular organic matrix had been detected [32–34]. Special cells within the sponges, the sclerocytes, form the calcareous spicules, a process which occurs completely extracellularly, according to published data [35,36].

Our recent studies, based on the application of molecular biological techniques, disclosed genes that encode for candidate proteins involved in the control of biomineralization; furthermore we identified peptides by a phage display technique that are functioning in the recognition of the calcareous spicular structures [22]. The results obtained prompted us to elucidate the role of the CA from the calcareous sponge *Sycon raphanus* during the formation of calcitic deposits. Our in vitro approaches presented here revealed that the CA accelerates the $CaCO_3$ mineralization process and guides the growing calcitic crystals to the calcareous spicules in a highly ordered manner.

2. Materials and methods

2.1. Sponge specimens and their spicules

Specimens of *Sycon raphanus* (Porifera, Calcarea, Leucosolenida, Sycettidae) were collected in the Northern Adriatic Sea (Limski Chanel near Rovinj, Croatia). There, they grow at a depth of between 2 and 7 m as epibionts onto the mussel *Mytilus galloprovincialis*, together with algae class of the Rhodophyceae. The specimens were cultivated in aquaria in artificial sea water (Tropic Marine, Tropic Marine Centre Ltd, Rickmansworth, UK) in Mainz (Germany) at 17 °C [22,37]. The specimens used for the experiments, summarized here, were cultivated under Ca^{2+} deprivation (in the presence of 1 mM $CaCl_2$) for 5 days. The specimens from *Suberites domuncula* (Porifera, Demospongiae, Hadromerida) were likewise collected in the Northern Adriatic and kept in aquaria.

The spicules both from *S. raphanus* (calcareous spicules) and from *S. domuncula* (siliceous spicules), used for the induced crystallization experiments were isolated and purified with 5% (v/v) NaOCl for 15 min [22]. The *S. raphanus* spicules, used for co-localization experiments with carbonic anhydrase by immunoanalysis, were obtained from tissue samples by submersing them into distilled water, supplemented with Na-azide (NaN_3 0.2% (w/v)); after vigorous shaking the spicules were collected by sequential sedimentation.

2.2. Expression of carbonic anhydrase cDNA and its activity

The procedure for the identification and completion of the *S. raphanus* CA cDNA (SRCA) and the deduced protein have been described [22]. Likewise the procedure for the expression of the cDNA, performed in *Escherichia coli*, is partially published [22]. For the study here, we expressed the cDNA comprising the complete carbonic anhydrase domain (aa_{17} to aa_{286}) by using the *E. coli* Gateway-Technology (Invitrogen, Carlsbad, CA, USA); the signal peptide (aa_1 to aa_{16} (of the deduced complete polypeptide)) as well as the transmembrane region (aa_{287} to aa_{312}) has been omitted. The *pPET161-DEST* vector was used. The positive transfected clones of *E. coli* (strain BL21Star; DE3) were grown in LB medium; expression was induced with 1 mM IPTG (isopropylthio-β-galactoside) for 12 h at 37 °C. The bacterial pellets were lysed with BugBuster according to the instructions of the manufacturer (Novagen/Merck KGaA, Darmstadt, Germany). After sonication the suspension was solubilized with the lysis buffer (50 mM KH_2PO_4, pH 8.0; 6 M urea, 300 mM KCl and 5 mM imidazole). Finally, the polyhistidine-tagged protein was purified by Ni–NTA agarose affinity chromatography [38]. The purified recombinant protein (aa_{21} to aa_{283} of the complete polypeptide) had the calculated size of 29,052 Da.

The signal peptide of the carbonic anhydrase from *S. raphanus* was predicted by using the described algorithm ("SMART" http://smart.embl-heidelberg.de/); the Motif Scan analysis was performed with the SIB Bioinformatics Resource Portal programs (http://expasy.org/).

The specific activity of the recombinant carbonic anhydrase was determined to be 2500 units mg^{-1} protein, by using the Wilbur–Anderson assay [39,40].

2.3. Raising of antibodies and immunohistology

The polyclonal antibodies were raised in female rabbits (White New Zealand), as described [22]. The recombinant sponge CA was injected into the animals; after the third boost the serum was collected and prepared; it is termed PoAb-aCA. The titer was determined to be 1:10,000. In controls, adsorbed sera were used which were prepared by incubating 10 ml of PoAb-aCA with 1 mg of the recombinant CA.

Immunohistological analysis was performed with 8 μm tissue slices [41]. The samples were fixed in paraformaldehyde and incubated with the primary antibodies PoAb-aCA (dilution: 1:5000) in blocking solution and incubated while shaking at 4 °C overnight. The immunocomplexes were visualized with Cy5-conjugated goat anti-rabbit immunoglobulin G (IgG). Parallel slices were stained with DAPI (4′,6-diamidino-2-phenylindole). Then, the slices were inspected with an Olympus AHBT3 microscope under immunofluorescence light at an excitation light wavelength of 647 nm (Cy5-stained structures) or of 490 nm (DAPI).

2.4. SDS–PAGE and Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed as follows. Samples of 5 μg of protein were mixed with loading buffer (Roti-Load; Carl Roth, Karlsruhe, Germany), boiled and subjected to SDS–PAGE (15% acrylamide and 0.1% SDS) as described [42]. The gels were stained with