Cellular calcium pathways and isotope fractionation in *Emiliania huxleyi*

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ABSTRACT

The marine calcifying algae *Emiliania huxleyi* (coccolithophores) was grown in laboratory cultures under varying conditions with respect to the environmental parameters of temperature and carbonate ion concentration [CO_3^{2-}] concentration. The Ca isotope composition of *E. huxleyi*'s coccoliths reveals new insights into fractionation processes during biomineralization. The temperature-dependent Ca isotope fractionation resembles previous calibrations of inorganic and biogenic calcite and aragonite. Unlike inorganically precipitated calcite, the [CO_3^{2-}] concentration of the medium has no significant effect on the Ca isotope composition of the coccoliths. These results indicate a decoupling of the chemical properties of the bulk medium and the calcifying vesicle. Cellular Ca pathways of *E. huxleyi* indicate that fractionation cannot occur at the crystal surface, as occurs during inorganic precipitation. The dominant processes leading to the observed Ca isotope fractionation pattern in *E. huxleyi* are most likely the dehydration of the Ca aquocomplex at the plasma membrane and the attachment of dissolved Ca to proteins of Ca channels. The independence of Ca isotope fractionation from [CO_3^{2-}] and the small temperature dependence of *E. huxleyi* are also important for defining the isotopic signature of the oceanic Ca sink. Since coccolithophores contribute to about half the global $CaCO_3$ production, a relatively uniform isotopic composition of the oceanic Ca sink is further supported.

Keywords: *Emiliania huxleyi*, calcium isotopes, coccolithophores, isotope fractionation.

INTRODUCTION

Coccolithophores are unicellular marine phytoplankton that surround themselves with small calcite plates called coccoliths. Their occurrence in large quantities in the upper surface ocean water makes them important primary producers responsible for approximately half of the global marine carbonate precipitation (Milliman, 1993). Hence, they play an important role for the carbon cycle (and CO_2 balance) as well as for the marine Ca budget. Because their first occurrence dates back to the Triassic, they provide the potential to record long-term changes in ocean chemistry. For these reasons, their Sr/Ca ratios were studied in order to reconstruct growth and calcification rates (cf. Stoll and Schrag, 2000), while their Ca isotopic composition was used to reconstruct the Ca budget of the ocean (De La Rocha and DePaolo, 2000). Despite the important role of coccolithophores with regard to the carbon and calcium cycles in the ocean and proxy applications for paleoenvironmental reconstructions, trace-element and calcium transport as well as their calcification mechanisms are not fully understood.

Calcium isotope analyses might elucidate some of these processes in coccolithophores, because previous work indicates a strong kinetic effect of the carbonate ion concentration [CO_3^{2-}] on calcium isotope fractionation (Lemarchand et al., 2004). Since reduced [CO_3^{2-}] leads to a decrease in calcification rate of *Emiliania huxleyi* (Riebesell et al., 2000), it should also be reflected in the Ca isotopic composition of the coccolith. The apparent temperature dependence on Ca isotope fractionation was also proposed to be caused by [CO_3^{2-}] via the temperature-dependent dissociation of carbonic acid (Lemarchand et al., 2004).

In this study, we investigate the main environmental parameters (temperature and [CO_3^{2-}]) that affect Ca isotope fractionation for coccoliths. Based on these observations, we describe the Ca transport through the cell and the involved Ca isotope fractionation.

METHODS

Coccolithophore Culturing

We cultured the dominant coccolithophore *Emiliania huxleyi* (strain PML B92/11) in sterile filtered (0.2 μm) natural seawater (enriched with 100 μmol L^{-1} nitrate and 6.25 μmol L^{-1} phosphate, and with trace metals and vitamins similar to the description of Guillard and Ryther [1962]) at different temperatures between 5 and 20 °C (Fig. 1A). In addition, we cultured *E. huxleyi* at varying [CO_3^{2-}], between 88 and 600 μ*M* (at variable pH and constant dissolved inorganic carbon [DIC]), at constant temperature (20 °C). Cells were grown in dilute batch cultures with low cell densities and were harvested during the exponential growth phase; in all experiments, less than 5% of the DIC was consumed. The carbonate chemistry of the solution did not change significantly throughout the experiments and between parallel cultures. Coccolithophore culturing and carbonate chemistry analyses followed the descriptions of Zondervan et al. (2002) and Langer et al. (2006). Less than 1% of the dissolved Ca^{2+} was precipitated; therefore, changes of the Ca isotope composition of the fluid due to removal of light Ca isotopes, as observed in clathrites by Teichert et al. (2005), can be neglected for our experiments.

Sample Preparation and Ca Isotope Analysis

Prior to Ca isotope analysis, coccolith samples were bleached in a sodium-hypochlorite solution (~1% active chlorine) to remove organic components. Afterwards, the samples were washed in ultrapure water (pH 8–9 using added NH_4OH to prevent dissolution), in methanol (CH_3OH), and finally six times in ultrapure water. The $^{44}Ca/^{40}Ca$ ratio of the coccolith $CaCO_3$ was measured using thermal ionization mass spectrometry (TIMS) double-spike technique at the Leibniz Institute of Marine Sciences (IFM-GEOMAR), Kiel (Heuser et al., 2002). Isotope values are reported as $1000 \cdot \ln \alpha$ [where $\alpha = (^{44}Ca/^{40}Ca_{solid})/(^{44}Ca/^{40}Ca_{fluid})$] as well as $10^6 \cdot \ln(\alpha_{mu})$, providing the isotope fractionation per 1 atomic mass unit (amu) (with $\alpha_{mu} = \alpha^{0.2683}$; Gussone et

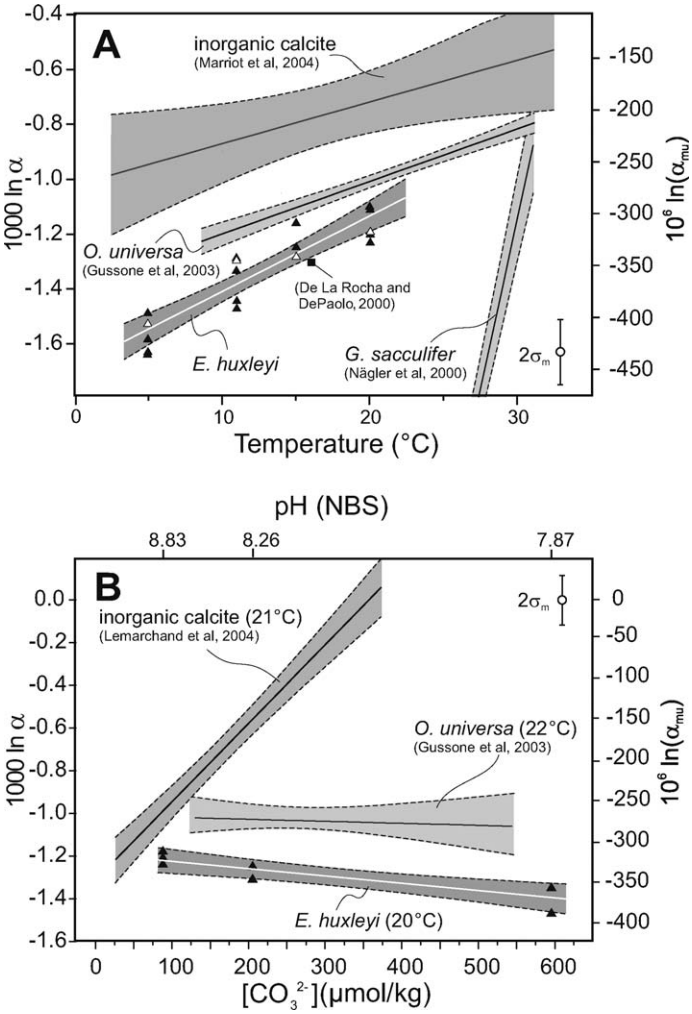

Figure 1. A: Ca isotopes of cultured *Emiliania huxleyi* as a function of temperature: $1000 \cdot \ln\alpha = -1.68 \pm 0.08 + (0.027 \pm 0.006) \times T(°C)$, with $P < 0.0001$, $R^2 = 0.84$, and $N = 19$. Shaded area is 95% confidence band. Observed absolute fractionation is in accordance with published data of *E. huxleyi* (De La Rocha and DePaolo, 2000). Samples that were not bleached prior to analysis (open triangles), representing the total of organically and inorganically bound Ca, exhibit identical fractionation as the bleached pure $CaCO_3$ of the coccolith. Temperature dependence is similar to *Orbulina* (*O.*) *universa* and inorganic calcite, but is considerably smaller than *Globigerinoides sacculifer*. B: Ca isotopes as a function of $[CO_3^{2-}]$ in ambient solution: $1000 \cdot \ln\alpha = -1.18 \pm 0.06 + [CO_3^{2-}(\mu mol/kg)] \times (-3.4 \times 10^{-4} \pm 1.5 \times 10^{-4})$, where $P = 0.004$, $R^2 = 0.77$, and $N = 8$. Shaded area is 95% confidence band; $1000 \cdot \ln\alpha$-$[CO_3^{2-}]$ dependence of *E. huxleyi* is similar to *O. universa* but small compared to inorganic calcite, indicating a decoupling of internal and external chemistry of fluids; pH (NBS) scale applies only to *E. huxleyi* data.

al., 2005). The average $2\sigma_m$ standard error of our data was 0.12‰ (30 ppm/amu).

RESULTS

Calcium Isotopes in Coccolith of *E. huxleyi*

The temperature dependence of Ca isotope fractionation in *E. huxleyi* is 0.027 ± 0.006‰/°C (Fig. 1A), similar to the planktic foraminifera *Orbulina universa* (0.019 ± 0.003‰/°C) (Gussone et al., 2003), but considerably smaller than the planktonic foraminifera *Globigerinoides sacculifer* (0.24‰/°C) (Nägler et al., 2000), which allowed the use of Ca isotopes as a temperature proxy (Zhu and Macdougall, 1998; Gussone et al., 2004). The Ca isotope fractionation observed for *E. huxleyi* was in good agreement with a previously published value of cultured *E. huxleyi* at 16 °C ($1000 \cdot \ln\alpha = -1.3$‰) and several carbonate oozes (De La Rocha and DePaolo, 2000). In contrast, Quaternary coccolith oozes of Zhu and Macdougall (1998) showed considerably lighter values ($1000 \cdot \ln\alpha = -1.9$ ‰ to -2.6 ‰). The calcium isotope fractionation of cultured *E. huxleyi* samples that were processed without bleaching (open triangles in Fig. 1A), representing the total Ca in the cells (bound to organic compounds and carbonate), was identical to the fractionation of the bleached pure $CaCO_3$ samples.

Similar to *O. universa*, we observed only a minor dependence of Ca isotope fractionation in *E. huxleyi* on changes in the carbonate chemistry (Fig. 1B), which is in contrast to the strong $[CO_3^{2-}]$ dependence observed for inorganic calcite precipitation (Lemarchand et al., 2004).

DISCUSSION

Calcium Isotope Fractionation Mechanisms

Our culturing experiments show that *E. huxleyi* exhibits a small but significant temperature-dependent Ca isotope fractionation. For other marine species, as well as inorganically precipitated carbonates, Lemarchand et al. (2004) suggested that the small temperature dependence can be explained by the temperature-dependent speciation of the carbonic acid (Millero, 1995), leading to an increase in $[CO_3^{2-}]$ with increasing temperature. The increase in $[CO_3^{2-}]$ then leads to a higher saturation state of calcite and to increasing precipitation rates, resulting in reduced Ca isotope fractionation in the calcite.

While the observed temperature-dependent Ca isotope fractionation in *E. huxleyi* appears to be in agreement with the model by Lemarchand et al. (2004), we observed no significant dependence of Ca isotope fractionation on $[CO_3^{2-}]$. This might imply that the observation that pCO_2 alters coccolith morphology and calcification rate (Riebesell et al., 2000), does not simply reflect the correlation between the extracellular and coccolith vesicle calcite saturation state. This finding is coherent with more recent data of Schulz et al. (2004), which show that under zinc limitation, the detrimental effect of changes in the carbonate system is significantly attenuated. This indicates that there is a more subtle biochemical system regulating the extent of calcification and coccolith morphology.

Further support for this reasoning is provided by the observation that coccolith production is a highly regulated process, in which the coccolith morphology strongly depends on the functioning of coccolith-associated polysaccharides (Henriksen et al., 2004; Young et al., 1999).

It is reasonable to assume a decoupling of the coccolith vesicle chemistry from the bulk solution because the ionic composition of the vesicle is defined by transmembrane ion transport of, e.g., HCO_3^-, H^+, and Ca^{2+}. A decoupling of the carbonate chemistry in the coccolith vesicle and the bulk solution is also required because carbon is transported into the vesicle as HCO_3^- (due to a pH of 7–7.2 in the cytoplasm; Brownlee and Taylor, 2004; Dixon et al., 1989), while H^+, which is generated during $CaCO_3$ formation, has to be removed from the vesicle to keep the pH value inside the vesicle favorable for carbonate precipitation. In principle, it might be possible that the temperature-dependent speciation of the carbonate system (i.e., between CO_2, HCO_3^-, and CO_3^{2-}) inside the coccolith vesicle is responsible for the observed temperature-dependent Ca isotope fractionation, but a closer look at the Ca budget in *E. huxleyi* reveals that Ca isotope fractionation cannot occur during $CaCO_3$ precipitation inside the coccolith vesicle.

Cellular Ca Transport

The proposed Ca pathways in *E. huxleyi* are visualized in Figure 2: Ca is present in the bulk solution ($[Ca^{2+}] = 10$ m*M*) as Ca^{2+}-

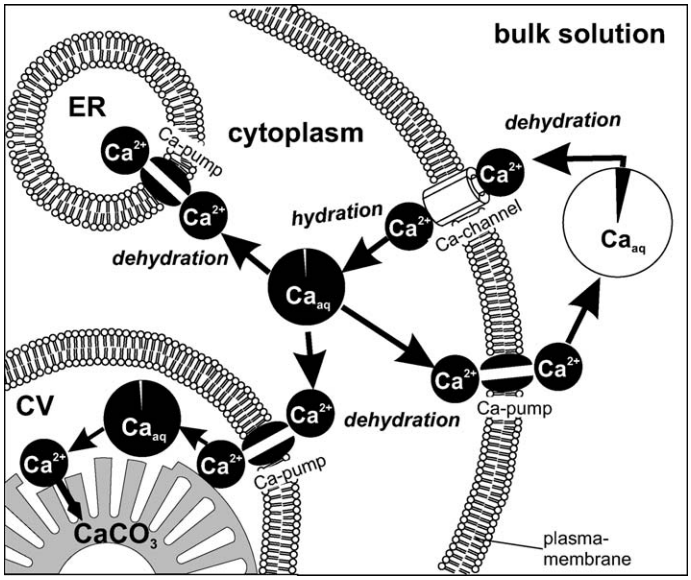

Figure 2. Proposed calcium transport pathways in *Emiliania huxleyi* from seawater to site of calcification: Dissolved Ca (Ca_{aq}) is dehydrated at cell surface and transported through Ca-selective channels into cell. Ca^{2+} ion that enters cytoplasm is rehydrated and removed from cytoplasm by Ca pumps and transferred into cell organelles (e.g., endoplasmic reticulum [ER] or Golgi apparatus), in coccolith vesicle or out of cell. Ca entering coccolith vesicle (CV) is nearly completely incorporated into $CaCO_3$ of coccolith. Calcium reservoirs are shown as pie charts, and dimension of black pie slice indicates portion of Ca removed from respective Ca reservoir (not to scale).

aquocomplex (Ca_{aq}) (cf. Schwenk et al., 2001). At the cell membrane, the water-shell of the Ca is stripped off, and Ca enters the cell through Ca-selective channels (Brownlee and Taylor, 2004). Within the cytoplasm, Ca is rehydrated. Calcium has to be removed quickly from the cytoplasm to keep its concentration very low (~0.1 μM Ca) (Brownlee et al., 1995) and constant, because important cell processes like photomorphogenesis (Shacklock et al., 1992) and hormone signal transduction (McAinsh et al., 1990) are regulated by Ca signals. Hence, we can assume that practically all Ca that enters the cytoplasm is immediately removed from the cytoplasm by Ca pumps. The portion of Ca that is transported is indicated by the black slices of the pie charts in Figure 2. We distinguish three important pathways: (1) Ca is removed from the cytoplasm and transported back to the bulk solution. (2) Ca is transported into cell organelles like the ER (endoplasmic reticulum) or the Golgi apparatus. (3) Ca is transported into the coccolith vesicle, where $CaCO_3$ precipitation takes place. The Ca that enters the vesicle is probably hydrated again (because $CaCO_3$ is believed to be precipitated from an aqueous solution inside the vesicle; Young et al., 1999), before it is dehydrated at the crystal surface and incorporated into the coccolith calcite.

The membrane of the coccolith vesicle closely surrounds the growing coccolith and does not contain a substantial fluid reservoir. Transmission-electron micrographs reveal that the vesicle volume is at most double the coccolith volume of ~1.8 μm^3 (Young and Henriksen, 2005, personal commun.). Assuming a Ca concentration of ~500 μM in the coccolith vesicle, which is a concentration typically found in the endoplasmic reticulum (Meldolesi and Pozzan, 1998), a Ca content of ~40 ag (4×10^{-17}g) can be calculated. The coccolith, which is built inside this vesicle, has a final weight of ~2.3 pg $CaCO_3$ (0.9 pg Ca) (Young and Ziveri, 2000). Thus, the Ca in the vesicle has to be refilled ~23,000 times during ~0.7 h of coccolith formation (Paasche, 1962). Continuous Ca flux into the vesicle is therefore necessary to allow the coccolith to grow. Since almost all of the Ca entering the vesicle is incorporated into the coccolith, no Ca isotope fractionation is possible at the site of precipitation.

Therefore, the observed Ca isotope fractionation has to take place prior to $CaCO_3$ precipitation, during Ca uptake or cellular Ca transport and storage processes. We will briefly discuss both possibilities, to identify the most likely process responsible for Ca isotope fractionation. In principle, Ca isotope fractionation during cellular Ca transport is possible; however, so far no information exists about the fractionation of Ca isotopes at Ca-ATPase and Ca-binding proteins. As a first approximation, it is reasonable to assume that the Ca pumps at the plasma membrane, in endomembranes, and in the membrane of the coccolith vesicle, are similarly built (Evans and Williams, 1998), using the same basic Ca-binding mechanism, and are thus very likely to introduce the same isotope fractionation to the transported Ca. In addition, the Ca isotopic composition of the coccolithophore cell (including organically bound Ca in the soft tissue) exhibits identical values as the coccoliths (only pure $CaCO_3$; Fig. 1A), indicating that no fractionation occurs between soft tissue and the $CaCO_3$ of the coccolith. Therefore, Ca isotope fractionation most likely takes place at the plasma membrane, when Ca_{aq} is dehydrated and attached to proteins at the entrance of a Ca channel.

Marriott et al. (2004) explained Ca isotope fractionation during calcite precipitation by equilibrium fractionation effects due to a decrease in bond strength and subsequent tendency to enrich the light isotope in the carbonate lattice. In contrast to inorganic precipitation, coccolithophores introduce a spatial separation between the site of dehydration and precipitation. Due to their internal Ca budget and Ca transport system, they prevent Ca isotope fractionation from occurring at the site of precipitation. The Ca isotope fractionation at the Ca channel might be thermodynamically comparable to the fractionation mechanism proposed by Marriott et al. (2004) for inorganic precipitation, since dehydration of Ca_{aq} and attachment of Ca to the crystal surface might energetically resemble attachment of Ca to proteins, considering the similar Ca isotope fractionation and temperature sensitivities of inorganically precipitated calcite and coccoliths. This biologically induced isotope fractionation is expected to decrease with increasing temperature (a common behavior of stable isotope fractionation), leading to the observed positive correlation between Ca isotope fractionation and temperature.

The different Ca isotope fractionation of coccolithophores and foraminifera (Fig. 1) are most likely related to the different mechanisms involved in the Ca uptake. While in *E. huxleyi*, Ca isotope fractionation is mainly controlled by the dehydration of Ca_{aq} at the plasma membrane, additional mechanisms have to be considered in foraminifera, like Ca uptake by endocytose of seawater vesicles, Rayleigh fractionation effects in cellular Ca reservoirs, and mixing of different Ca reservoirs.

CONCLUSIONS

The link between Ca isotope fractionation and cellular Ca pathways in *E. huxleyi* improves our understanding of proxy formation during coccolith biomineralization. The limited Ca reservoir in the coccolith vesicle, preventing Ca isotope fractionation at the site of precipitation, should be considered for the incorporation of trace elements (e.g., Sr) as well. By comparing Ca isotope fractionation of different species or materials, we find that similar temperature dependencies do not necessarily point to the same fractionation mechanisms. Our results have further implications for the application of Ca isotopes as an environmental proxy: The main factor influencing Ca isotopes in coccoliths is the isotopic composition of the seawater. The second parameter that affects Ca isotope fractionation is temperature, with a small

sensitivity of 0.027‰/°C. Changes in ambient [CO_3^{2-}] or pCO_2 introduce only a small variation in Ca isotope composition of coccoliths, which can be neglected for paleoceanographic reconstruction purposes. The result that the isotope fractionation between the coccolith calcite and the seawater is relatively uniform and is not strongly affected by temperature or CO_2 is of particular importance for Ca budget calculations, since coccolithophores are an important marine Ca sink, contributing about half of the marine carbonate production.

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