Native-state imaging of calcifying and noncalcifying microalgae reveals similarities in their calcium storage organelles

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Calcium storage organelles are common to all eukaryotic organisms and play a pivotal role in calcium signaling and cellular calcium homeostasis. In most organelles, the intraorganellar calcium concentrations rarely exceed micromolar levels. Acidic organelles called acidocalcisomes, which concentrate calcium into dense phases together with polyphosphates, are an exception. These organelles have been identified in diverse organisms, but, to date, only in cells that do not form calcium biominerals. Recently, a compartment storing molar levels of calcium together with phosphorous was discovered in an intracellularly calcifying alga, the coccolithophore *Emiliania huxleyi*, raising a possible connection between calcium storage organelles and calcite biomineralization. Here we used cryo-imaging and cryospectroscopy techniques to investigate the anatomy and chemical composition of calcium storage organelles in their native state and at nanometer-scale resolution. We show that the dense calcium phase inside the calcium storage compartment of the calcifying coccolithophore *Pleurochrysis carterae* and the calcium phase stored in acidocalcisomes of the noncalcifying alga *Chlamydomonas reinhardtii* have common features. Our observations suggest that this strategy for concentrating calcium is a widespread trait and has been adapted for coccolith formation. The link we describe between acidocalcisomal calcium storage and calcium storage in coccolithophores implies that our physiological and molecular genetic understanding of acidocalcisomes could have relevance to the calcium pathway underlying coccolithophore calcification, offering a fresh entry point for mechanistic investigations on the adaptability of this process to changing oceanic conditions.

acidocalcisome | biomineralization | intracellular calcium store | coccolithophore | cryo–X-ray tomography

Among the list of elements essential for life, calcium deserves a unique place (1). Calcium ions are of vital importance to numerous cellular processes in all organisms (2). Most prominent is the usage of calcium as intracellular messenger. Critical for such usage is a tight control of its cytosolic concentration, which is usually maintained in the submicromolar range. Several cellular organelles, such as the endoplasmic reticulum, Golgi apparatus, vacuoles, and lysosomes, are known to facilitate dynamic control over the cytoplasmic calcium concentration, releasing and sequestering calcium to and from the cytoplasm (1). Acidocalcisomes are highly remarkable calcium-containing organelles (3). They have been documented in bacteria, protists, and mammalian cells, and have been proposed to represent the earliest form of an intracellular calcium pool (4). Among microalgae, acidocalcisomes have been identified in a few species, among them the model green alga *Chlamydomonas reinhardtii* (5–7). The calcium concentration in acidocalcisomes can surpass millimolar levels, which is much higher than in any other organelle (4, 8). Acidocalcisomes are rich in phosphorous, stored in the form of polyphosphate, and also contain other monovalent and divalent cations (6, 9). Little is known about the chemical environment that facilitates these very high calcium concentrations within acidocalcisomes. This is because, until recently, the analytical approaches available for characterization of acidocalcisomes have usually employed sample preparation techniques that have limited preservation power.

A very different use of calcium, but also widespread throughout the kingdoms of life, is in the formation of mineralized body parts such as shells, skeletal elements, and scales (10). It has been shown, for several calcifying organisms, that the mineralization pathway involves the formation of membrane-bound granules, packed with a precursor mineral phase, which are deposited at the site of mineral growth (11). The mineral precursors, most commonly calcium carbonates or calcium phosphates, are dense phases with calcium content in the molar range (12, 13). The physiology and biochemistry of these precursor-rich compartments remains largely elusive.

Coccolithophores (Calcihaptophycidae, Haptophyta) are a group of unicellular marine algae that cover their cells with mineralized scales called coccoliths. Understanding coccolithophore physiology, and specifically coccolith formation, is of upmost importance for understanding the ecological dominance of these organisms in modern oceans (14), and interpreting the

Significance

Coccolithophores are abundant unicellular marine algae that produce calcified scales via a controlled intracellular process. Understanding the cellular controls over the calcification process is a pressing need to predict the influence of changing oceanic conditions on these major contributors to global marine calcification and carbon fluxes. Using several microalgae, and a combination of state-of-the-art cryoelectron and cryo soft X-ray microscopy, we demonstrate that the recently discovered calcium stores of coccolithophores are similar to the common calcium storage organelles of noncalcifying organisms. These results relate questions of environmental and evolutionary significance to a large body of physiological and molecular genetic findings of better-characterized organisms, and therefore provide fresh entry points for understanding calcification in coccolithophores.

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geochemical past of our planet (15, 16). It is known that the calcium carbonate crystals that form the coccolith nucleate and grow within a specialized compartment, called the coccolith vesicle, which is located close to the center of the cell, and formation can be as fast as one per hour (17). This rapid process requires high fluxes of calcium into the coccolith vesicle.

Recent state-of-the-art high-resolution cryoimaging of the dominant coccolithophore species *Emiliania huxleyi* revealed the presence of an intracellular compartment containing a calcium-rich phase, with calcium concentrations in the order of 10 M (18). Calcium in this compartment is colocalized with phosphorous, most probably stored in the form of polyphosphates (18). This compartment appears to participate in the supply of the coccolith vesicle with calcium (19). The discovery of this concentrated calcium pool in a calcifying organism raises many questions, including how widespread such pools are in coccolithophores and how their chemical and anatomical features compare with those of concentrated calcium pools in noncalcifying organisms. Here, we examined several algal species, both mineral-forming and nonmineralizing, for the presence of concentrated calcium stores. We characterize highly concentrated Ca stores in native state conditions using high-resolution imaging of vitrified cells. The results show that acidocalcisomes and calcium-rich compartments in coccolithophores share many anatomical and chemical features, suggesting an ancestral mechanism for the formation of a dynamic calcium pool that was recruited by coccolithophores to support calcification. Interestingly, we found no calcium-rich compartments in a noncalcifying coccolithophore mutant and a noncalcifying haptophyte with calcifying ancestor, which could be the cause of the absence of calcification activity in these cells.

Results

We chose the model coccolithophore species *Pleurochrysis carterae* (20) as a starting point for an in depth investigation of intracellular calcium pools among the Calcihaptophycidae. *P. carterae*, like most other coccolithophores, belongs to a different order than the previously investigated coccolithophore *E. huxleyi*, is found in coastal habitats compared with the global distribution of *E. huxleyi*, and is known to have marked differences in the coccolith formation process (20, 21). Thus, we expect differences and similarities between *E. huxleyi* and *P. carterae* regarding calcium pools to provide clues about the common mechanisms that underlie coccolithogenesis.

Detecting soluble Ca phases other than free Ca^{2+} inside cells is incompatible with traditional light, fluorescence, and electron microscopy, and requires the use of specialized techniques (22). We used synchrotron-based cryo–soft-X-ray tomography (cryoSXT), as it allows the 3D imaging of cryopreserved cells, unfixed and unstained, with a spatial resolution in the range of tens of nanometers (23). We used *P. carterae* cells that had the external coccolith shell dissolved, because shell dissolution triggers formation of new coccoliths and enhances the visibility of intracellular materials (24). The cells were vitrified by plunging into liquid ethane 4 h after shell dissolution, a time point where the cells had recovered from the dissolution treatment and started coccolith production to rebuild the coccolith shell. A few new coccoliths were already secreted to the cell surface (Fig. 1). Tilt series images of selected cells were acquired at a photon energy of 520 eV, where concentrated organic and inorganic matter appears darker than water-rich environments, and the entire volume of the cell was reconstructed in three dimensions (Fig. 1 *A*–*C*). The tomographic data were rich in structural details and enabled the segmentation of coccoliths and intracellular organelles such as the chloroplasts, nucleus, vesicles, and other membrane-bound compartments (Fig. 1*C*).

Several bodies with strong contrast were prominent within the cells (Fig. 1 *A* and *B*). To identify Ca-rich entities, we acquired images at two specific photon energies, before the Ca L-edge (342 eV) and exactly at the Ca L_2-edge peak maximum (353.2 eV), where Ca is highly absorptive (Fig. 1*D*). Because the X-ray absorption of the other elements is nearly the same at these energies, the differences between these two images yield a calcium distribution map. This 2D Ca map shows multiple Ca-rich entities within the cells (Fig. 1*D*). By convolving the 2D Ca distribution map with the 3D tomography data, we reconstructed the spatial organization of the Ca-rich entities in the cell (Fig. 1*E*). These can

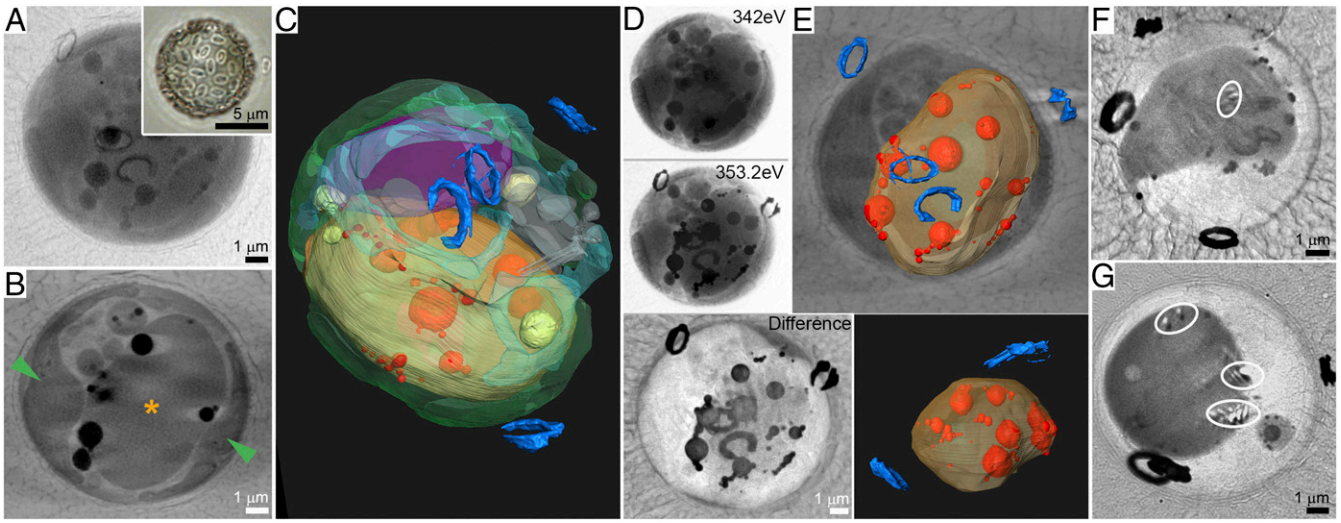

Fig. 1. CryoSXT images of vitrified *P. carterae* cells producing coccoliths. (*A*) Raw X-ray image of the cell at 0° tilt and 520 eV. (*Inset*) A light microscopy image of a living cell with a coccolith shell. (*B*) A slice in the 3D reconstructed volume of the cell showing the chloroplasts (green arrowheads), a large membrane-bound compartment (*), several vesicles, and X-ray–absorbing bodies (dark). (*C*) Surface rendering of the segmented structures showing the chloroplasts in green, the nucleus in purple, the large vacuole-like compartment in brown, lipid bodies in yellow, several vesicles in gray, and coccoliths in blue. For more details, see Movie S1. (*D*) Spectromicroscopy at the Ca L-edge, with an image before the edge (342 eV), one at the edge (353.2 eV), and the corresponding difference image showing in dark contrast areas where Ca is abundant. (*E*) The 3D segmentation of Ca-containing entities. Coccoliths are in blue, Ca-rich bodies are in red, and the compartment they are enclosed in is depicted in brown. A second view at a different angle demonstrates that the Ca-rich bodies are located inside a single compartment. (*F* and *G*) CryoSXT Ca maps showing *P. carterae* cells with high luminal Ca concentrations in the vacuole-like compartment and only a few and small Ca-rich bodies. White ellipses depict direct beam artifacts, which cannot be avoided for such thick samples.

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be divided into coccoliths, extracellular as well as intracellular, which are at various stages of maturity, and spherical Ca-rich bodies. Notably, all of the Ca-rich bodies are localized inside a membrane-bound compartment that makes up a large proportion of the cell's volume and is clearly distinct from the chloroplast and nucleus. The Ca-rich bodies inside the compartment were positioned in close proximity to the compartment's membrane (Fig. 1*E*). Bodies with high contrast outside this compartment do not show up in the Ca map and are most likely lipid bodies. Among the various cells we imaged, the calcium in the compartment showed varying appearances. In some cells, calcium was detected only in the spherical Ca-rich bodies (Fig. 1*D*). In other cells, homogenous and more dilute calcium distributions were observed, sometimes containing a few Ca-rich bodies and sometimes none (Fig. 1 *F* and *G*). These observations suggest that this compartment could be a dynamic calcium store.

To further investigate the ultrastructure and chemical composition of the Ca-rich bodies, we used cryoscanning electron microscopy (cryoSEM). *P. carterae* cells were high-pressure frozen and then freeze-fractured to expose the cell interior for cryoSEM imaging. The fracture planes traversed the cells randomly, sometimes following the contours of organelles and sometimes cutting through their interior. In many cells, the fracture exposed a large compartment with an intact surface (Fig. 2*A*). From its smooth surface, round protrusions were observed bulging out (Fig. 2 *B*–*F*). Imaging with an electron backscatter detector that is sensitive to the atomic mass of the sample showed a bright contrast for the protrusions, suggesting that they contain elements heavier than carbon and oxygen at high concentrations (Fig. 2*C*). We used energy dispersive spectroscopy (EDS) to investigate the elemental composition of the protrusions. Mapping the elemental distribution in the sample, as well as collecting spectra from designated areas, showed that the protrusions are rich in phosphorus, calcium, and potassium (Fig. 2 *D* and *E*). This composition is similar to that reported for the Ca–P bodies of *E. huxleyi* except for the higher potassium content and the absence of magnesium and sodium (18). However, as the Ca–P bodies of *E. huxleyi* were characterized in resin-embedded cells, their elemental composition may have been subjected to quantitative alterations. The Ca content and the spherical nature of the protrusions suggest that they are the Ca-rich bodies seen in the cryoSXT datasets.

The presence of multiple, randomly distributed Ca–P-rich bodies inside a single large compartment of *P. carterae* cells raises the question of whether each body is separated from the bulk of the compartment by a membrane. This information may have consequences for understanding what defines the place where the bodies form. We addressed this question on cells in which the fracture went through the Ca–P-rich bodies and the compartment membrane could be seen. A featureless transition from the compartment solution to the Ca–P-rich body was observed in these images (Fig. 3). Therefore, we conclude that the Ca–P-rich body is in direct contact with the compartment solution and not engulfed by a membrane.

The identification of Ca–P-rich bodies in calcifying cells of *P. carterae* and *E. huxleyi*, both representing different orders of the coccolithophores (21, 25), prompted us to investigate whether Ca–P-rich bodies are also present in recently diverged noncalcifying descendants. First, we analyzed with cryoSXT cells of *Isochrysis galbana*, a noncalcifying haptophyte species that is a closer relative of *E. huxleyi* than *P. carterae* (21, 25). The 3D reconstruction of *I. galbana* tomograms shows the cell anatomy to be similar to that of *E. huxleyi* (Fig. 4*B*). Interestingly, the calcium distribution maps of cells in logarithmic growth phase from independent cultivations did not show any significantly concentrated calcium pools (Fig. 4*A*). Second, we imaged an *E. huxleyi* strain (CCMP2090) that lost the ability to calcify. These cells also lacked Ca–P bodies (Fig. 4 *C* and *D*). This tentative correlation between the inability to calcify and the lack of Ca–P-rich bodies within the Calcihaptophycidae

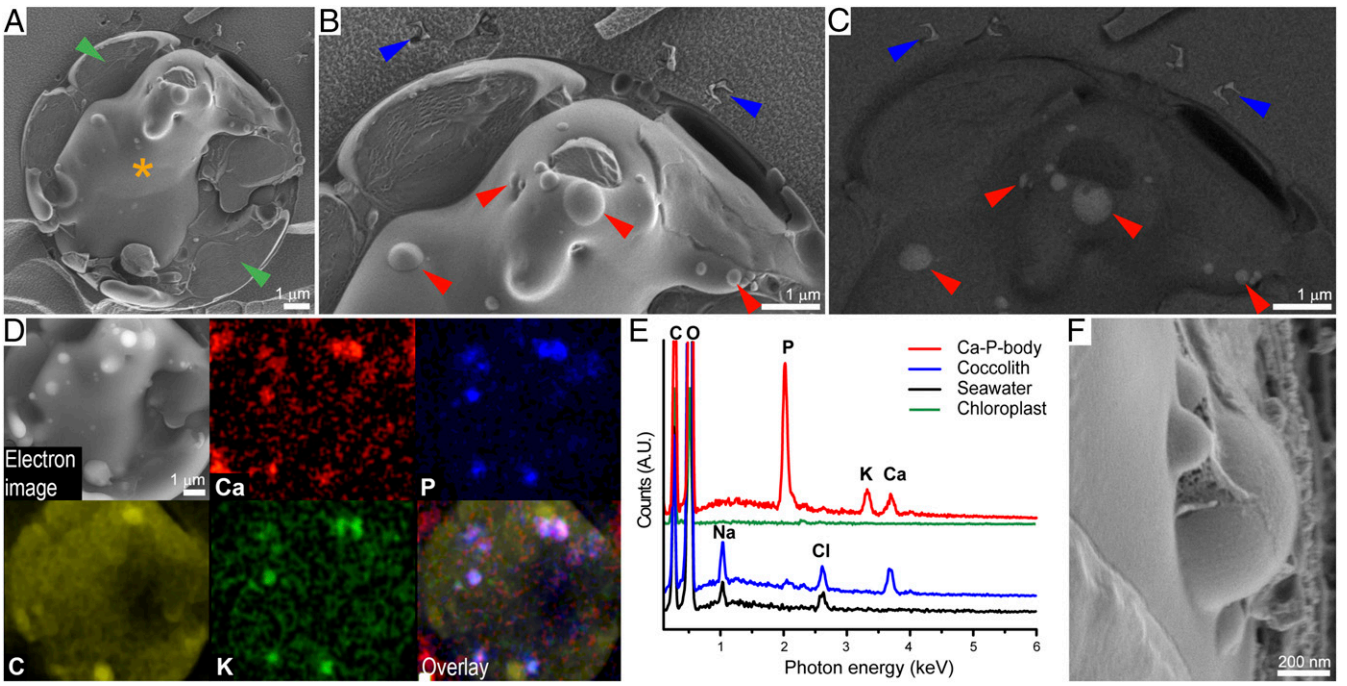

Fig. 2. CryoSEM micrographs and elemental composition of a high-pressure frozen and freeze-fractured *P. carterae* cell. (*A*) A fracture surface showing the inside of a cell. The chloroplast (green arrowheads) was fractured open, while a large organelle in the cell middle remained unfractured (*). (*B*) Higher magnification of *A* showing the surface of the organelle with bulging protrusions (red arrowheads) and the cross-section of two extracellular coccoliths (blue arrowheads). (*C*) Back-scattered electron image of the same area as in *B*. The coccoliths and the protrusions exhibit bright contrast. (*D*) EDS maps of the most abundant elements in the cell. (*E*) EDS spectra taken at the indicated positions. Note that the coccoliths are smaller than the EDS resolution. Therefore, NaCl from the seawater medium (0.5 M NaCl) can be seen in the coccolith spectrum. (*F*) High-magnification image showing several Ca–P-rich vacuolar protrusions in profile.

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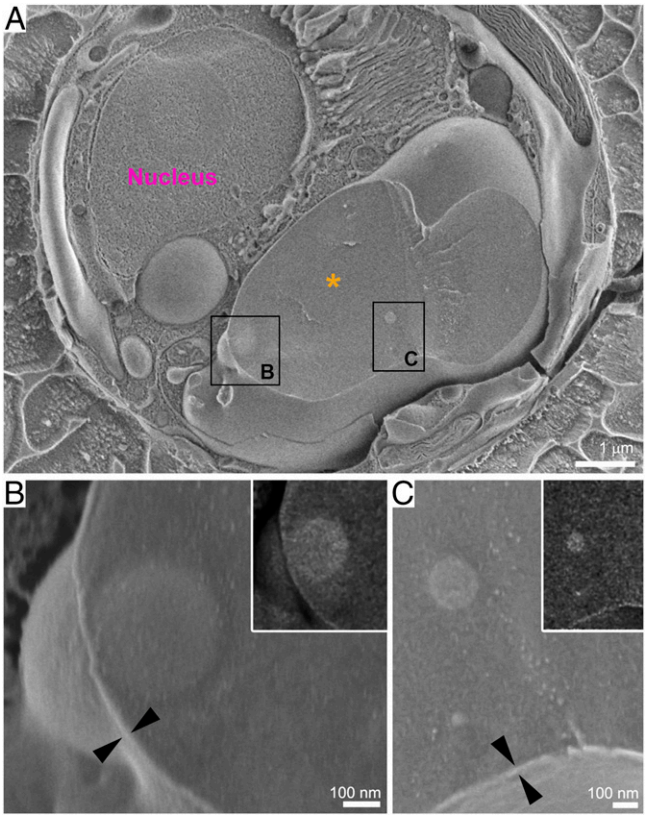

Fig. 3. Ultrastructure of the Ca–P-rich bodies. (*A*) CryoSEM image of a freeze-fractured *P. carterae* cell with the Ca storage compartment (*) cut open, showing two Ca–P-rich bodies (in rectangles). (*B* and *C*) Higher-magnification images of the framed areas in *A* showing Ca–P-rich bodies and the nearby compartment membrane (arrowheads). The contrast of the membranes is different from that of the material surrounding them. The boundary of the Ca–P bodies does not show a membrane-characteristic contrast pattern, suggesting the body material to be in direct contact with the compartment solution. (*Insets*) The corresponding backscattering images in which the brightness of the Ca–P bodies corresponds to their richness in calcium. The membranes are bright due to the steep topography.

may be a second line of evidence for the involvement of Ca–P-rich bodies in coccolith formation.

Cryoimaging techniques are perfectly suited for characterizing the ultrastructure and composition of highly concentrated Ca pools and the subcellular compartments involved in their formation. In coccolithophores, the molecular biology and biochemistry of such organelles is difficult to study. In other organisms, the situation is reversed, with the biology of concentrated calcium phosphate stores being well investigated (26, 27), but the native ultrastructure and mineralogy remaining largely uncertain, since the methodology used for characterization involved dried or chemically fixed and/or resin-embedded cells. If commonalities exist in the mineralogy of Ca–P-rich phases in coccolithophores and biochemically better-characterized organisms, it would imply that the underlying cellular machinery may be related, which would help with elucidating the molecular underpinnings of coccolith formation.

C. reinhardtii is a unicellular, noncalcifying, green alga, which is known to store Ca and P in acidocalcisomes (6). We used cryoSXT to analyze intracellular Ca pools in *C. reinhardtii*. The tomography data acquired at 520 eV showed the known organellar components of *C. reinhardtii* cells in native organization and the presence of bodies containing strongly X-ray absorbing material (Fig. 5 *A* and *B* and Movie S2). Spectromicroscopy at the Ca L-edge showed this material to be a calcium-rich phase (Fig. 5*C*). This result demonstrates the importance of spectromicroscopy for the interpretation of soft X-ray tomography data, as these strongly X-ray–absorbing bodies were previously interpreted to be lipid bodies (28). The calcium-rich bodies are located inside larger membrane-bound compartments (Fig. 5 *D* and *E*). Based on their Ca content and morphological characteristics, we interpret these compartments to be the acidocalcisomes seen in earlier studies by electron microscopy (6).

We characterized the acidocalcisome content by collecting images varying the energy across the Ca L-edge in 0.1-eV steps. The complete X-ray absorption near-edge structure (XANES) at the Ca L-edge were extracted from different areas of interest in the images of the energy scan. The spectrum of the Ca phase in the acidocalcisomes closely resembles the spectrum of a synthetically prepared calcium polyphosphate precipitate, and is markedly different from that of soluble calcium in solution (Fig. 5*F*). This demonstrates that most of the acidosomal calcium is not stored in the form of soluble ions but is precipitated by the polyphosphates into a distinct chemical phase. The spectrum of the acidosomal Ca phase also shows high similarity to the spectrum of the Ca–P-rich bodies we previously reported for the coccolithophore *E. huxleyi* (*SI Appendix*, Fig. S1), suggesting that this phylogenetically widespread way of storing calcium is also suited to fulfill the specialized requirements of coccolith formation.

Discussion

Dense Ca–P-rich intracellular granules were initially observed more than 50 y ago, but, until recently, technical difficulties rendered the determination of their native characteristics highly uncertain (29). Recently developed cryotechniques now allow an accurate determination of the concentration and chemical phase of dense Ca–P-rich pools in situ. Our cryoimaging and cryospectroscopy study has shown the presence of Ca–P-rich bodies in the coccolithophore *P. carterae* and the noncalcifying green alga *C. reinhardtii*. In previous cytological studies of *P. carterae*, Ca–P-rich

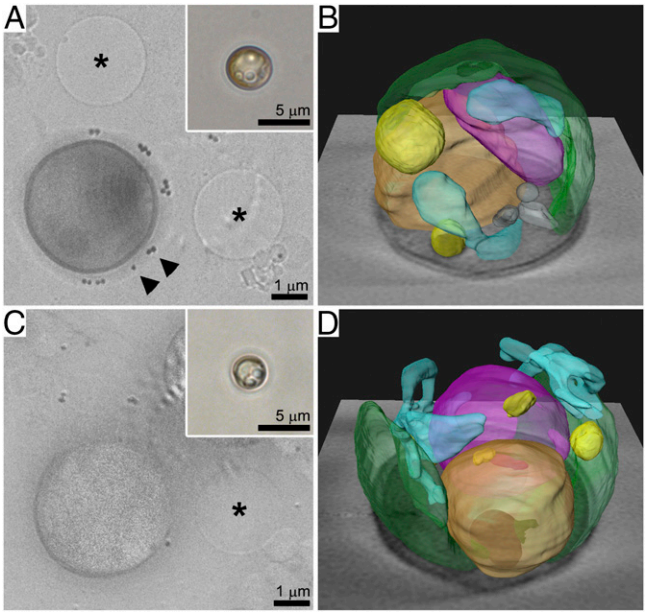

Fig. 4. Calcium pools in noncalcifying haptophytes: (*A* and *B*) *I. galbana* and (*C* and *D*) noncalcifying *E. huxleyi*. (*A* and *C*) Calcium distribution maps derived from cryoSXT spectromicroscopy. The circular holes in the support film are indicated with an asterisk (*), and gold nanoparticles that serve as fiducial markers are marked with arrowheads. (*Insets*) Light microscopy images of the cells before freezing. (*B* and *D*) A 3D volume rendering of the intracellular compartments identified with cryoSXT. The chloroplast is shown in green, the nucleus is purple, a large compartment with unknown content is brown, vesicles are gray, a filamentous membrane system is cyan, and lipid bodies are yellow.

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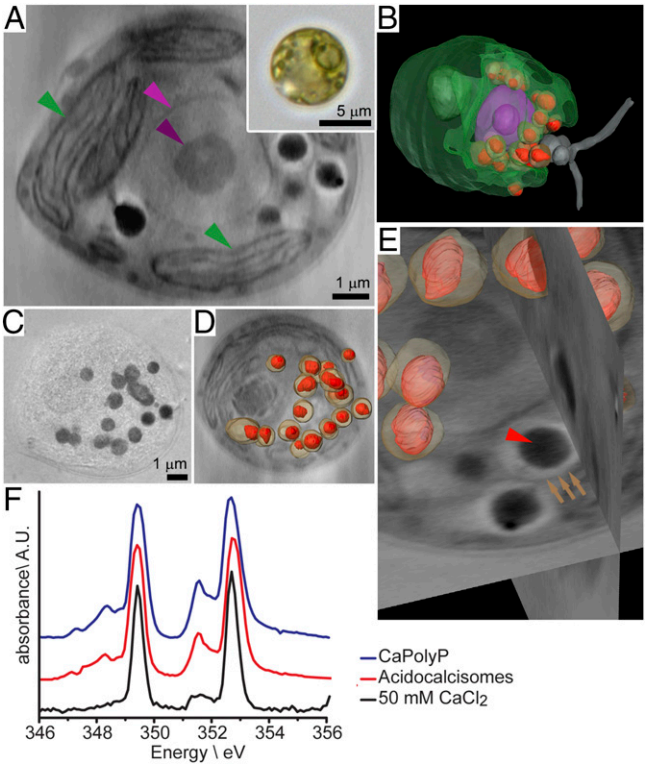

Fig. 5. *C. reinhardtii* acidocalcisomes analyzed by cryoSXT. (*A*) A slice in the 3D reconstructed data. Color-coded arrowheads indicate the chloroplast (green) and the nucleus and nucleolus (pink and purple, respectively). (*Inset*) A light micrograph of a live cell. (*B*) A 3D volume rendering of an entire cell. The nucleus (pink) and the acidocalcisomes (red) are partially enclosed by the cup-shaped chloroplast (green). (*C*) Spectromicroscopy at the calcium L-edge revealed the X-ray dense bodies to be Ca-rich and not lipid bodies. (*D*) A 3D representation of the cell in *C* showing only the Ca-rich bodies and the enclosing membranes. (*E*) High magnification showing the organization of a Ca-rich body (red arrowhead) in the acidocalcisome (brown arrows). (*F*) Ca L-edge XANES spectra extracted from different regions of image stacks traversing the energy range around the Ca L-edge.

bodies were not observed (30, 31), probably because standard cell imaging protocols caused their dissolution. In *C. reinhardtii*, each Ca–P-rich body resides within an individual organelle called acidocalcisome, whereas, in *P. carterae*, all Ca–P-rich bodies are contained inside a single compartment. In both algae, multiple Ca–P-rich bodies per cell have been observed. For the coccolithophore *E. huxleyi*, we recently observed that the cells contain primarily one Ca–P-rich body, which we always found enclosed in a compartment much larger than the body itself (18). Despite the differences in the organization of the Ca stores between the three algae, the mineralogy of the Ca–P-rich bodies is highly similar. Considering that the mechanism of concentrating and releasing Ca in Ca–P-rich bodies is rooted much deeper in the evolution of organisms than coccolith calcification, it is likely that this pathway for the concentration of calcium was available to the haptophyte ancestor of coccolithophores and then adapted to serve calcification (8, 25). However, given the current dearth of molecular information on the pathway underlying the formation of Ca–P-rich bodies in coccolithophores, the possibility that body formation is unrelated to other organisms cannot be completely ruled out. To clarify this point, further studies are required.

Our X-ray images of *I. galbana* RCC1353 and *E. huxleyi* CCMP2090, a mutant that has lost the ability to calcify, show that both lack Ca-rich pools. This may be related to the lack of the organelle for Ca body formation or the absence of polyphosphate for Ca sequestration, resulting from either unfavorable growth conditions or a constitutive inability to synthesize polyphosphate. In general, the polyphosphate pool of cells is known to be dynamic (32). Under phosphate-replete conditions, when phosphate availability exceeds the demand by growth, polyphosphates usually accumulate. Under phosphate depletion, polyphosphates will then be hydrolyzed to phosphate. Previous imaging of a calcifying *E. huxleyi* strain, grown in artificial seawater medium supplemented with 10 μM phosphate, demonstrated that *E. huxleyi* accumulates polyphosphate under our growth conditions (18). As the noncalcifying mutant was sampled at a very similar cell density and the cultivation conditions were virtually identical, the absence of a detectable Ca pool points to a physiological problem in Ca and/or polyphosphate metabolism. Considering that stable noncalcifying mutants frequently arise in laboratory cultures of *E. huxleyi*, and the current lack of knowledge on the molecular basis of this shutdown, our finding provides an entry point for deciphering this phenomenon (33, 34). For *I. galbana*, the situation is less clear, as conditions under which *I. galbana* accumulates polyphosphate are elusive. Further research is required to discover the genetic and mechanistic reasons underlying our observations for both species.

Even though we documented several types of calcium distribution in *P. carterae*, ranging from a homogenous distribution within the compartments to localization only in Ca–P-rich bodies, it is impossible to derive the dynamics of the process from these snapshots. For example, we do not know if the Ca–P-rich bodies form from the more diluted Ca phase (Fig. 1 *F* and *G*) or if the diluted phase is a result of dissolution of the bodies. The localization of the Ca–P-rich bodies inside the Ca-rich compartment (Fig. 2) and the lack of internal membranes within the compartment suggest that the bodies nucleate and grow at the inner surface of the compartment membrane, fed by material delivered through it. It is likely that a membrane-associated polyphosphate polymerase plays an important role in this process (35). Expression of such polymerase by *P. carterae* is documented by the available transcriptome data (36). In the future, the combination of advanced imaging and spectroscopy with biochemical tools may yield a comprehensive, detailed understanding of these processes.

There are several suggested functions for acidocalcisomes in general, and for dense Ca–P-rich phases in noncalcifying cells (4). In the case of intracellularly calcifying organisms, it seems likely that this reservoir participates in the pathway supplying the mineral-forming compartment with calcium. If not functionally linked, the Ca storage compartment and the coccolith vesicle must be high-capacity Ca sinks which run in parallel. The sink redundancy in this case would require a sophisticated regulatory system for partitioning the Ca flux according to the receiving status of each compartment. In *P. carterae*, the storage compartment is the dominant dynamic Ca reservoir, but, even though we investigated actively calcifying cells, we never observed spatial proximity between it and the coccolith vesicles. This implies an additional step in the transport of calcium from this reservoir into the coccolith vesicle if such a transfer occurs. The reason we do not detect this intermediate step could be that calcium is transported in a more diluted form, or in smaller particles below our detection limit. Previous studies have reported vesicles, loaded with ~20-nm calcium-loaded particles (37), which were shown to fuse with the coccolith vesicle and may represent this missing step in the calcium delivery pathway.

The here-reported similarity between the Ca–P-rich bodies of coccolithophores and the Ca deposits in acidocalcisomes provide an entry point for an exciting crosstalk between the biomineralization field, where the physicochemical fundamentals of calcium handling and mineralization are addressed, and researchers addressing the molecular biology of acidic cellular calcium stores and calcium handling in noncalcifying organisms. The microscopy and spectroscopy tools used for studying transient Ca-rich phases in biomineralization processes may allow for fresh insights on the in situ chemistry and anatomy of concentrated calcium stores, such as acidocalcisomes. By analogy, the immense biochemical and molecular biology knowledge available for model organisms such as *C. reinhardtii* offers a promising

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starting point for elucidating the biochemistry and functionally of the Ca–P-rich bodies in coccolithophores.

Conclusions

The ability to precipitate a dense phase rich in Ca and P within an intracellular compartment is present in distantly related algae. In coccolithophores, and likely also in many other organisms, such Ca–P-rich phases were mostly overlooked, most likely because standard sample preparation protocols for ultrastructural imaging cause their dissolution. Cryoimaging and cryospectroscopy of vitrified cells is the approach of choice to observe such elusive Ca phases and to determine their native characteristics. The dense Ca–P-rich phase has direct contact with the compartment membrane. In coccolithophores, where calcium seems to be transferred to the mineralization site over a Ca–P-rich phase, it is essential to consider these complex transport regimes when aiming to understand the effects of physiological and environmental conditions on coccolith formation.

Materials and Methods

Algae and Cultivation. *P. carterae* CCMP645, *I. galbana* RCC1353, and *E. huxleyi* CCMP2090 were grown in artificial seawater medium at 18 °C and a 12 h/12 h light/dark cycle. *C. reinhardtii* cc620 and cw15 were grown in Tris-acetate-phosphate medium at 22 °C and continuous illumination.

CryoSXT and Spectromicroscopy. *P. carterae* cells were decalcified with 10 mM EDTA for a few minutes and then allowed to resume coccolith formation in fresh medium for 4 h. Cells were placed on top of Quantifoil holey film copper TEM grids and plunge-frozen in liquid ethane. X-ray imaging was performed at the MISTRAL beamline (ALBA Synchrotron) (38). A tilt series at 520-eV X-ray energy was collected to three-dimensionally reconstruct the internal cell architecture. Alignment and reconstruction of the tilt series was done with IMOD. The visualization and segmentation of the final volumes were carried out using Amira 3D software. For a Ca $L_{2,3}$-edge energy scan, the same field of view was repeatedly imaged under changing X-ray energies starting at 344 eV and going up to 360 eV in 0.1-eV steps. Calcium localization in the samples was visualized by subtracting the 342-eV image from the 353.2-eV image.

CryoSEM and EDS. Four-hour recalcifying *P. carterae* cells were pelleted and resuspended in 10% Dextran in seawater. Then 2 μL of cells were sandwiched between two metal discs (3-mm diameter, 0.1-mm cavities), high-pressure frozen (HPM10; Bal-Tec), and freeze-fractured (BAF 60; Leica Microsystems). Samples were imaged at −120 °C in an Ultra 55 SEM (Zeiss) by using a secondary electron in-lens detector and a backscattered electron in-lens detector. Samples analyzed with an EDS detector were coated with 6 nm of carbon.

Detailed experimental procedures can be found in *SI Appendix*.

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