Selectivity in biomineralization of barium and strontium

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Abstract

The desmid green alga Closterium moniliferum belongs to a small number of organisms that form barite (BaSO4) or celestite (SrSO4) biominerals. The ability to sequester Sr in the presence of an excess of Ca is of considerable interest for the remediation of 90Sr from the environment and nuclear waste. While most cells dynamically regulate the concentration of the second messenger Ca2+ in the cytosol and various organelles, transport proteins rarely discriminate strongly between Ca, Sr, and Ba. Herein, we investigate how these ions are trafficked in C. moniliferum and how precipitation of (Ba,Sr)SO4 crystals occurs in the terminal vacuoles. Towards this goal, we simultaneously visualize intracellular dynamics of multiple elements using X-ray fluorescence microscopy (XFM) of cryo-fixed/freeze-dried samples. We correlate the resulting elemental maps with ultrastructural information gleaned from freeze-fracture cryo-SEM of frozen-hydrated cells and use micro X-ray absorption near edge structure (micro-XANES) to determine sulfur speciation. We find that the kinetics of Sr uptake and efflux depend on external Ca concentrations, and Sr, Ba, and Ca show similar intracellular localization. A highly ion-selective cross-membrane transport step is not evident. Based on elevated levels of sulfate detected in the terminal vacuoles, we propose a “sulfate trap” model, where the presence of dissolved barium leads to preferential precipitation of (Ba,Sr)SO4 due to its low solubility relative to SrSO4 and CaSO4. Engineering the sulfate concentration in the vacuole may thus be the most direct way to increase the Sr sequestered per cell, an important consideration in using desmids for phytoremediation of 90Sr.

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1. Introduction

The chemical similarity of Ca2+, Sr2+, and Ba2+ leads to indiscriminate transport of these ions by most organisms (Hagiwara and Byerly, 1981). A notable consequence of this in humans is the incorporation of radioactive 90Sr into bone (where Sr2+ substitutes for Ca2+ in apatite), where its decay over a half-life of 30 years is responsible for much increased cancer mortality (Raabe, 1992) in radiation workers and victims of nuclear accidents, warfare, or terrorism. Similarly, phytoremediation approaches to environmental 90Sr cleanup struggle in the presence of Ca due to competition between Ca2+ and Sr2+ for uptake (Singh et al., 2008). Even advanced synthetic chelators and ion-exchange materials (Braun et al., 2002) are grappling with the challenge of selectively separating Sr2+ from ions of similar radius and charge, such as Ca2+, in nuclear waste. Layered sulfide materials with a high affinity for Sr have been reported (Manos et al., 2008); however, while these materials were able to remove 95% of Sr from a mixed ionic solution, nearly 90% of the Ca was also removed. As contaminated waste or surface waters are likely to have Ca concentrations that are several orders of magnitude higher than Sr, there is much room for improvement in Sr/Ca selectivity.

Wilcock and co-workers qualitatively demonstrated that desmid green algae, when cultured in medium containing a high ratio of Sr2+ to Ba2+, form Sr-substituted barite (BaSO4) crystals (Wilcock et al., 1989). We have recently shown that it is possible to create growth conditions under which the desmid Closterium moniliferum precipitates crystals with up to 45 mol% of the Ba lattice positions replaced by Sr (Krejci et al., 2011). Desmids thus belong to a very small number of organisms that are able to selectively sequester Sr and/or Ba in biominerals. Other examples include the radiolarian acantharea (Wilcock et al., 1988) that build their endoskeletons from celestite (SrSO4) and the barite depositing stonewort green algae of genus Chara (Schroter et al., 1975), the ciliate genus Loxodes (Hemmersbach et al., 1999), and a class of deep-sea foraminifera, the xenophyophores (Gooday and Nott, 1982).

These organisms effectively select for Sr and/or Ba over Ca in an environment where the concentration of the latter is between 2 and 5 orders of magnitude higher. In addition, the location of precipitation is carefully controlled. As transport of ions across membranes is frequently highly selective, it is a reasonable proposition...
that cells can control the relative amounts of Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, and Ba\textsuperscript{2+} in the compartment where precipitation occurs, e.g. the vacuole of desmids. The actual precipitation step then may then be dominated by the equilibrium solubility of different inorganic phases (e.g. CaSO\textsubscript{4} \cdot 2H\textsubscript{2}O vs. SrSO\textsubscript{4}), or the more complex binary phase diagrams of solid solutions such as (Ba,Sr)SO\textsubscript{4} for an in-depth analysis of Sr/Ca partitioning in foraminifera see Elderfield et al., 1996). However, as precipitation is rarely near equilibrium, kinetic effects may play an important role as well. The overall process thus depends on a variety of biological and chemical factors. In order to arrive at a mechanistic understanding, it is important to investigate not only the composition of the final biomineral (Krejci et al., 2011), but also the transport of ions through the organism and the actual concentrations where precipitation occurs.

One reason why the mechanisms of Ba/Sr sequestration in these organisms have remained largely unexplored is again related to selectivity: it is difficult to simultaneously quantify and visualize subcellular distributions of multiple ions (e.g. Ca, Sr, Ba) by conventional ratiometric fluorescence imaging using ion-selective fluorophores, by autoradiography, or by X-ray techniques such as micro-PX\textsubscript{E} (Brook et al., 1988), and SEM-EDS (Wilcock et al., 1989). While the latter have been used to demonstrate the presence of Ba in desmids and the relative distribution of Sr and Ba in crystals isolated from desmids, quantitative chemical imaging of trace metals at micron–submicron resolution has seen dramatic advances with the advent of X-ray fluorescence microscopy (XFM) at third-generation light sources. We utilize XFM here to investigate the basic mechanisms of strontium and barium selectivity and transport in the desmid *C. moniliferum*, by mapping the intra-cellular concentrations of relevant ions (including Sr, Ca, Ba, and S) throughout Sr/Ba exposure and pulse-chase experiments. Cells were cryo-fixed and freeze-dried to maintain ultrastructure while preventing loss of diffusible ions. Simultaneous observation of the distributions and/or dynamics of other elements (including Fe and Zn), in addition to phase-contrast imaging, allow for correlation between elemental maps and cellular ultrastructure. The goal of this analysis is to identify at which stage(s) (uptake, intracellular transport, and/or precipitation) selectivity occurs, as well as the relative contributions of the biology of the cell and the chemistry of the precipitate. While it may be possible to engineer desmids for environmental remediation directly (Krejci et al., 2011), we aim to understand the basic molecular mechanisms at play, which could then inspire the next generation of materials for the selective separation of \textsuperscript{80}Sr.

2. Materials and methods

2.1. Algae culture

*C. moniliferum* cultures were obtained from The Culture Collection of Algae at the University of Texas at Austin (UTEX). Cultures were maintained in Bold's Basal Medium (Bold, 1949) with 3-fold nitrogen and vitamins (3N-BBM+V) at 20–25 °C in a 12 h light/dark cycle under 20 W/m\textsuperscript{2} of daylight spectrum fluorescent light (Krejci et al., 2011). Algae were transferred to fresh medium every 1–3 months. “Deprived” medium that was not supplemented with Ba\textsuperscript{2+} or Sr\textsuperscript{2+} contained a baseline of 0.007 μM Ba\textsuperscript{2+} and 0.06 μM Sr\textsuperscript{2+}. All cultures were slowly dividing (i.e. doubling times were at least several days), such that cell division on average did not occur during the course of exposure experiments.

2.2. Light and electron microscopy

Live cell confocal microscopy was performed on cells cultured in Ba-supplemented medium. Cells were stained with 1 mg/mL DiOC\textsubscript{6}(3) (Sigma–Aldrich Co., St. Louis, MO), a general membrane stain with excitation and emission at 484 and 511 nm, respectively. Imaging was performed with an SP\textsubscript{2} Confocal Microscope (Leica Microsystems, Wetzlar, Germany) with excitation from Ar (457, 476, 488, and 514 nm) and green HeNe (543 nm) lasers.

For crystal isolation and analysis, cells were allowed to settle on a silicon wafer and ashed in a box furnace at 450 °C for 1.5–2 h. Samples were analyzed uncoated using an 5–4800-II field emission scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

Samples of cells cultured in Ba-supplemented and deprived media were cryofixed and analyzed by cryo-scanning electron microscopy (cryo-SEM) at the Electron Microscopy Center of the ETH Zurich (EMEZ). Samples were concentrated by centrifugation and vitrified by high pressure freezing using a Bal-Tec HFP\textsubscript{010} freeze-liquider (Leica Microsystems, Wetzlar, Germany) in order to sublimate some of the vitrified ice from the fracture surface. Samples were coated with tungsten prior to cryo-SEM imaging in a field emission SEM Leo Gemini 1530 (Carl Zeiss Inc., Oberkochen, Germany) equipped with a cryo-stage.

2.3. Sr\textsuperscript{2+} uptake in bulk cell populations

Cells were incubated in either normal medium (0.17 mM Ca\textsuperscript{2+}) or Ca-deprived medium for 2 h. Following the incubation period, 0.17 mM Sr\textsuperscript{2+} was added to the samples for 0, 1, 2, 6, and 24 h. Cells were then washed three times with Millipore water and incubated in enzyme digestion media containing 2% (w/v) cellulase, 1% (w/v) pectinase, and acetate buffer (pH = 4.96) for 2 h. Samples were homogenized in liquid nitrogen with the addition of 0.3% (w/v) Triton X-100 (G-Biosciences, St. Louis, MO). After homogenization, the remaining solids, primarily undigested cell walls, were pelleted by centrifugation, and the supernatant was collected. The pellet was washed three times with Millipore water, saving the supernatant each time. The supernatant was acid digested overnight in 17% (w/v) HNO\textsubscript{3} at room temperature. The samples were then diluted to 3.5% (w/v) HNO\textsubscript{3} for inductively coupled plasma optical emission spectroscopy (ICP-OES).

Elemental concentrations were determined using a Vesta-MPX ICP-OES (Varian Inc., Palo Alto, CA) at the Integrated Molecular Structure Education and Research Center (IMSERC) of Northwestern University. Quantification was based on calibration curves from six standards ranging from 100 to 300 ppb for Ca\textsuperscript{2+} and from 1 to 50 ppb for Ba\textsuperscript{2+} and Sr\textsuperscript{2+}. The content per cell was calculated by normalizing to cell counts acquired using a Sedgewick–Rafter counting cell.

2.4. Metal ion exposure

For the Sr\textsuperscript{2+} exposure, cells that were cultured in medium supplemented with 0.1 μM Ba\textsuperscript{2+} were transferred to medium supplemented with 0.17 mM SrCl\textsubscript{2}. Cells were incubated in the high-Sr medium for 1, 2, and 28 h and moved to deprived medium for 45 min prior to cryo fixation. For the Sr/Ba pulse-chase, cells that were cultured in deprived medium were transferred to medium supplemented with 0.35 μM BaCl\textsubscript{2} and 0.17 mM SrCl\textsubscript{2}. Cells were incubated for 30 min in the supplemented medium (the pulse) and returned to deprived medium to incubate for 45, 80, 145 min, 6.5, and 28 h (the chase). For the Ca-free chase, the chase was performed in Ba–Sr– and Ca-free medium, in which an equivalent concentration of Mg\textsuperscript{2+} (0.17 mM) had been substituted for Ca\textsuperscript{2+}. For Zn dynamics, cells that were cultured in deprived medium (0.22 μM Zn\textsuperscript{2+}) were incubated in Zn-free medium overnight (15.5 h), followed by a 6 h pulse in high-Zn medium (0.45 μM). Cells were returned to deprived medium for 45 min prior to cryo fixation.
2.5. Sample preparation for X-ray microanalysis

For X-ray imaging, cells were allowed to settle on silicon nitride windows (area, 1.5 × 1.5 mm; thickness, 500 nm; Silson, Blisworth, UK) for 15 min and processed by cryofixation. Windows were blotted once (0.5 s, 0 blot force) with filter paper to remove excess water and plunge-frozen into liquid ethane using a Vitrobot Mark IV (FEI Company, Hillsboro, OR). Samples were then freeze-dried in an EMS 775 turbo freeze-dryer (Electron Microscopy Sciences, Hatfield, PA) during slow warming from −140 to 20 °C over 12 h.

2.6. X-ray fluorescence microscopy (XFM)

Samples were analyzed by XFM using the X-ray microprobe at beamline 2-ID-E of the Advanced Photon Source (Argonne, IL). A crystal monochromator was used to select the energy of the beam, and a Fresnel zone plate (320 μm diameter, 100 nm outermost zone width, X-radia, Concord, CA) focused the beam to a spot size 0.5–1 μm in diameter. The sample was then raster scanned through the beam, and a full fluorescence spectrum was acquired at each point with a 1 s dwell time using an energy dispersive fluorescence detector (Ultra-LE Ge detector, Canberra, Meridian, CT or Vortex EM, SII NanoTechnology, Northridge, CA). An incident energy of 16.7–17.5 keV was used, with a step size of 1–1.25 eV.

2.7. Sulfur X-ray absorption near edge structure (XANES)

Sulfur K-edge μ-XANES measurements were performed with the scanning X-ray microscope at the 2-ID-B beamline at the Advanced Photon Source. The 2-ID-B beamline is optimized for imaging in the energy range of 1–4 keV and uses a multilayer spherical grating monochromator to select the X-ray energy. The scanning microscope uses a 160 μm diameter Fresnel zone plate with a 50 nm outermost zone to focus the X-ray beam to a spot size of approximately 60 nm (McNulty et al., 2003). Cells of interest were located on silicon nitride windows using an in situ visual light microscope, and specific cell features (e.g., pyrenoid, vacuole) were identified by scanning transmission X-ray microscopy using a PN-type diode detector. Scanning XFM was used to acquire corresponding sulfur elemental maps of the cells at an incident energy of 2535 eV using a silicon drift-diode detector (Vortex EX-60, SII NanoTechnology, Northridge, CA). XANES fluorescence spectra were acquired at selected regions of interest by scanning the incident energy over the sulfur K-edge (typically 2460–2520 eV), with 0.25 eV steps and 2 s dwell time. XANES spectra of reference standards were acquired with an unfocused beam, with dwell times of 1–5 s: standards included cysteine (thiol), methionine (organic sulfide), oxidized glutathione (disulfide), chondroitin sulfate (organic sulfate), barium sulfate (inorganic sulfate), and zinc sulfate (inorganic sulfate).

The XANES data was analyzed using the ATHENA software (Ravel and Newville, 2005). Methionine and zinc sulfate standard spectra were calibrated in energy by aligning the main peak to expected energies for each sulfur species (2470.55 eV for organic disulfide and 2479.58 eV for sulfate) (Pickering et al., 2008), and sample spectra were manually aligned to these standards. All spectra were background-subtracted and normalized in ATHENA, and one iteration of a three-point data-smoothing algorithm was applied to increase signal to noise without disturbing spectral features. Quantification was carried out using the GCF method described by (Prietz et al., 2011); for plots see Fig. S3.

3. Results and discussion

3.1. Ultrastructure and crystals

The unicellular C. moniliformis has a crescent shape and consists of two symmetrical semi-cells (Fig. 1). The nucleus is centralized between the two semi-cells, and a small (6–7 μm diameter) spherical vacuole that usually contains barium sulfate (BaSO₄) crystals is visible at each end of the cell (Brook et al., 1988, 1980; Wilcock et al., 1989). Most of the volume of the cell is occupied by two large chloroplasts with lobes extending along their length.

Using cryo-SEM to examine freeze-fracture surfaces of frozen hydrated cells (Fig. 2), we observe a vesicle-dense region between the chloroplast and the cell membrane. Larger structures that appear to be vacuoles are also visible in this region, as are Golgi bodies. In the center of the cell, finger-like projections of the chloroplast surround the nucleus and are separated by vacuoles. Near the ends of the cell, vesicles surround the terminal vacuoles and densely fill the cytoplasmic space between the vacuole and the cell tip.

These results are consistent with ones observed previously in freeze-substituted/embedded transmission electron microscopy (TEM) sections of other Closteriaceae species (Baylson et al., 2001; Domoycz, 1999; Linde et al., 2001). The vesicle-dense region around the periphery of the cell is known to exhibit cytoplasmic streaming, where vesicles are transported along the cell periphery on an extensive network of actin filaments (Linde et al., 2001). Many of these vesicles contain polysaccharide mucilage that is carried to the cell tips and secreted through pores in the cell wall (Baylson et al., 2001). The vacuoles that are situated in the grooves of the chloroplast are thought to comprise an extensive vacuolar network.

Endoplasmic reticulum (ER) has been observed by TEM to be adjacent to the chloroplast membrane and in the cytoplasmic region at the tips of the cell (Linde et al., 2001). Although we did not see these structures with cryo-SEM, one fracture through the cell tip near the vacuole did reveal a vesicle-poor region that may correspond to ER (Fig. 2F). There has been speculation that the ER-rich polar region is related to the “spitzenkorper” found in the tips of stonewort green algae; interestingly, stoneworts are among the few organisms other than desmids that mineralize BaSO₄. In stoneworts, BaSO₄-containing statholiths and the spitzenkorper have both been shown to be involved in gravity perception (Braun, 2002), but a similar gravity-sensing mechanism has not been observed in desmids.

3.2. Cellular origins of selective sequestration

Among organisms that mineralize Ba and/or Sr, desmids are unique in that they can be deprived of Ba, and thus of BaSO₄ crystals. This is in contrast with organisms that require Ba or Sr and offers a significant experimental advantage to study uptake and selectivity in the desmid system. In actively dividing cultures, cells that are virtually free of crystals can be obtained by growth in Ba- and Sr-deprived medium for several weeks to several months (Brook et al., 1988). This is most likely the consequence of diluting the existing crystals during many cell divisions while preventing the formation of new crystals, rather than a dissolution of crystals inside the cells due to low concentrations of Ba in the medium (Wilcock et al., 1989).
When crystal-free cells were exposed to medium supplemented with 0.35 μM Ba2+, we observed by optical microscopy crystal formation in the terminal vacuole of most cells after 1–2 h. Within 24 h, several crystals were apparent in the vacuoles of almost all cells. In cells cultured long-term (several months or more) in medium supplemented with 0.1 μM Ba2+, these crystals occasionally formed large clusters in the vacuole and were additionally found migrating throughout the cell. This was most often the case in low cell density conditions (less than 4000 cells/mL), presumably due to high Ba2+ availability per cell, whereas usually 1–3 crystals were present in higher density cultures where cells compete for available Ba2+.

Sr incorporation into desmid crystals (Wilcock et al., 1989; Krejci et al., 2011) occurs by Sr2+ substitution for Ba2+ in the barite crystal lattice to form a (Ba,Sr)SO4 solid solution (Brower, 1973). Due to the high solubility of SrSO4 relative to BaSO4 (3.81 × 10−7 M2 for SrSO4 and 1.08 × 10−10 M2 for BaSO4, Bolz and Tuve, 1973) the precipitation of Sr-rich crystals requires a higher aqueous Sr/Ba ratio and a higher activity product in the vacuole than Sr-poor or pure BaSO4 crystals (Krejci et al., 2011). Neither pure celestite (SrSO4) nor calcium sulfate dihydrate (CaSO4·2H2O) crystals are formed under any conditions. It thus appears that the cell selectively precipitates barite and strontium-substituted barite. An ion-selective transmembrane transport step could explain how the cell separates Ba and Sr from Ca. There are a number of membranes over which partitioning of Ba2+ and Sr2+ could possibly occur, including the plasma membrane and the membranes of the ER, vesicles, and vacuoles. Mitochondria, which are known to transport and sequester Ca, may also play a role (Gunter et al., 1994).

In the context of optimizing the sequestration of 90Sr from the environment or nuclear waste (Krejci et al., 2011), it is critical to understand the relative contributions of uptake, transport, and precipitation processes to the overall observed selectivity for Ba and Sr vs. Ca ions. This requires knowledge of the spatial distributions, absolute concentrations, and time-dependent changes of all relevant ions within the cell.

### 3.3. Elemental analysis and mapping

Ratiometric imaging using ion-selective fluorescent probes is widely used to localize and quantify the concentrations of Ca2+ and other metal ions (Domaille et al., 2008; Dunn et al., 1994). However, there are no fluorescent dyes selective for either Sr2+ or Ba2+ in the presence of physiological levels of Ca2+. XFM, on the other hand, combines extremely low minimum detection limits on the order of 10−20 mol/μm² (Twining et al., 2003) with subcellular (~150 nm) resolution, without the need for exogenous probes (Fahrni, 2007). Unlike optical microscopy, it can be used to quantify multiple chemically similar elements at the same time, independent of whether they are dissolved, bound to biomolecules of any kind, or in crystalline form. A growing number of studies have taken advantage of these unprecedented capabilities, for example to quantify and visualize platinum cancer drugs (Hall et al., 2006), magnetic resonance contrast agents (Endres et al., 2006), and endogenous metals during angiogenesis and macrophage differentiation (Finney et al., 2007; Glesne et al., 2006).

The X-ray fluorescence microprobe operates on similar principles as the electron microprobe (Fahrni, 2007; Ingram, 1999); incident X-rays (or electrons) are focused on a sample, leading to the ejection of inner-shell electrons. Higher-shell electrons then fill the vacancies, resulting in the emission of X-rays that are characteristic of elements within the sample. An advantage of the electron microprobe is the relative ease of generating and focusing electrons, and nanometer resolution is routinely achieved for very thin samples with this technique. However, multiple scattering in samples thicker than 1 μm degrades the spatial resolution to >1 μm (Kirz, 1980) and makes the quantification of elemental content difficult (Ingram, 1999). Electrons also generate a large bremsstrahlung background, which decreases the signal-to-noise ratio and increases the radiation dose required for trace element analysis (Kirz et al., 1978). While the electron microprobe may be advantageous for thin samples, the lack of multiple scattering and limited generation of bremsstrahlung by X-rays makes X-ray...
microscopy optimal for thicker samples. In addition, the energy of the incident X-rays can be scanned through elemental absorption edges to obtain local chemical state information via XANES spectroscopy. Because the goal of this work is to obtain an overall quantitative picture of ion uptake and transport in *Closterium*, the ability to image whole cells without sectioning is a significant advantage of XFM.

For imaging by XFM, *C. moniliferum* cells were cryofixed by plunge-freezing into liquid ethane and freeze-dried. This preparation minimizes relocation of diffusible ions (Cheng and Caffrey, 1996; Malm et al., 2009) while preserving structure. While an incident X-ray energy of 10 keV is often used for XFM due to excellent sensitivity for many elements, including Ba (L-edges: 5.2–6 keV) (Thompson and Vaughan, 2001), Sr imaging (K-edge: 2.5 keV) requires a higher incident energy (e.g. 17.5 keV), which leads to reduced Ba-sensitivity. Best sensitivity to lighter elements such as S is obtained by using an incident energy just above the S K-edge (e.g. 2.5 keV).

### 3.4. Quantitative XFM imaging of crystals in cells

XFM of cells cultured in Ba-enriched (0.1 μM Ba^{2+}) medium for an extended period of time revealed BaSO_4 crystals in the terminal vacuoles and throughout the cells (Fig. 3). In cells additionally exposed to 0.1 μM Sr^{2+} in the medium, Sr co-localizes with both Ba and S in hotspots, due to co-precipitation of Sr with barite (Krejci et al., 2011; Wilcock et al., 1989). The medium supplemented with 0.1 μM Ba^{2+} and Sr^{2+} is undersaturated with respect to (Ba,Sr)SO_4.
For precipitation to occur in the vacuoles, the cell needs to increase the concentration of Ba\textsuperscript{2+}, Sr\textsuperscript{2+} and/or SO\textsubscript{4}\textsuperscript{2−} by active transport into the vacuole. Elevated levels of SO\textsubscript{4}\textsuperscript{2−} could be the base of a “sulfate trap” mechanism of selectivity, where the less soluble BaSO\textsubscript{4} and (Ba,Sr)SO\textsubscript{4} precipitate while the more soluble CaSO\textsubscript{4}2H\textsubscript{2}O (\(K_{sp} = 3.14 \times 10^{-5}\) M\(^{2}\), Haynes and Lide, 2010) does not. Depending on the exact concentrations maintained in the vacuole, the precipitation of pure SrSO\textsubscript{4} could be prevented. Alternatively, and possibly in addition to such a sulfate trap mechanism, sequestration could involve a Ba/Sr-selective transport step across the plasma membrane or the vacuolar membrane. To differentiate between these mechanisms it is necessary to determine the localization and relative and absolute concentrations of Sr, Ba, Ca, and sulfate in the different cellular compartments.

### 3.5. Correlating XFM elemental maps with cellular ultrastructure

Identification of organelles in XFM images is frequently possible directly from elemental maps. This is aided by the unique morphology and organization of Closterium cells – for example, the nucleus is always in the center between the two semi-cells, terminal vacuoles are always near the tip, and the chloroplasts fill most of the space in between. There is little cell-to-cell variation in the placement of these organelles. The relatively large size of Closterium cells allows for easy visualization of some organelles by light microscopy for correlation with XFM images, as well as high-definition XFM with many pixels per organelle. Comparison of the XFM elemental maps to a simultaneously acquired differential phase contrast image can also provide direct correlation of elemental information and cell structure (de Jonge et al., 2007).

In XFM images of Closterium (Fig. 4), the lobed chloroplast is clearly visible in Fe maps, as has been observed in diatoms (Twining et al., 2003). Zn appears elevated in the nucleus of the cell, similar to yeast (Ortega et al., 2003). Regions of high sulfur concentration within the chloroplast correspond to pyrenoids and are likely a consequence of high local concentrations of RuBisCO (Kaplan and Reinhold, 1999). The pyrenoids can often also be distinguished in XFM phase images, indicating their high mass density. Fe (and Mn, not shown) levels in the cell tip are strongly elevated over cellular background, possibly in association with terminal pores (Lorch, 1978).

The very high water content of the terminal vacuoles led to shrinkage during freeze-drying (Fig. 5), hindering identification
by light microscopy. However, in Zn XFM maps, small regions (~5 μm diameter) of elevated Zn concentrations were found near the tips of most cells. When *Closterium* was deprived of Zn overnight, these high-Zn regions near the tips could no longer be found. However, they did appear again after incubation of deprived cells in Zn-enriched medium (0.45 μM for 6 h). This dependency of Zn levels is similar to Zn dynamics observed in the yeast vacuole (Simm et al., 2007). Consequently, we use the Zn signal to define the vacuole region-of-interest (ROI) in elemental maps.

### 3.6. Ca, Sr, and Ba co-localize in endomembrane system between the chloroplast lobes

The dynamics of uptake and transport of Ba and Sr vs. Ca were investigated by comparing elemental maps recorded at different times after exposing cells to media supplemented with the ions of interest. We used imaging conditions optimized for Sr mapping (17.5 keV). This allowed us to simultaneously image Sr, Ba, and Ca, albeit with reduced sensitivity for Ba. When cells were exposed to 0.17 mM Sr\(^{2+}\), Sr appeared to enter at the same rate everywhere along the cell (Fig. 6). After 1 h, Sr levels in the vacuole appeared elevated above the cellular background. At later time points, a pattern of alternating ridges of high and low Sr concentration became increasingly apparent. Using the signal from Fe as an indicator of the chloroplast, we determined that Sr concentrations were highest in between the lobes of the chloroplast (Fig. 4). When cells were exposed to 0.17 mM Sr\(^{2+}\) and 0.35 μM Ba\(^{2+}\) for 30 min followed by a wash (1 h) in Ca/Sr/Ba-deprived medium, Sr and Ca distributions were remarkably similar (Fig. 6). Both exhibit elevated concentrations in the central region of the cell; comparison with cryo-SEM suggests that these elevated concentrations originate from the vacuoles that interdigitate with the extensions of the chloroplast surrounding the nucleus (Fig. 2D). Sr and Ca concentrations also appear to be high in the terminal vacuole and between the chloroplast lobes. In this latter area, small regions of high concentration are surrounded by lower concentrations, likely due to compartmentalization in vesicles, small vacuoles, and/or ER (Fig. 2A). Ba maps also show elevated concentrations in the vacuole, but the Ba distribution throughout the rest of the cell is more difficult to determine due to lower signal to noise (Fig. S1). We conclude that it is probable that Sr (and possibly Ba) are taken up into the endomembrane system in parallel with the well-known sequestration of Ca in this compartment (Ashby and Tepikin, 2001; Cobbett, 2000).

### 3.7. Interdependence of Ca and Sr transport

In a Sr/Ba pulse-chase experiment, we observed the effects of the presence of Ca\(^{2+}\) in the medium on Sr\(^{2+}\) transport. Cells were subjected to a 30 min pulse of 0.17 mM Sr\(^{2+}\) and 0.35 μM Ba\(^{2+}\) followed by a Sr/Ba-free chase. For one set of samples, the chase medium contained the normal media Ca\(^{2+}\) concentration of 0.17 mM; for the other, the chase medium contained no Ca\(^{2+}\). For both conditions, Sr XFM images showed reversible uptake where an initial spike in intracellular Sr levels was followed by a slow decrease (Fig. 6). However, when the chase medium was Ca-free, the total cellular Sr levels at various time points during the chase were consistently higher than with the Ca-containing chase medium. The absence of Ca\(^{2+}\) in the chase medium thus decreased Sr efflux. Apparently, the cellular response to Ca deprivation, i.e. limiting Ca\(^{2+}\) export (Reuter and Seitz, 1968), also limits Sr\(^{2+}\) export in *Closterium*. This suggests a lack of selectivity in efflux of Sr and Ca.

Further evidence that uptake and transport of Sr and Ca are linked was revealed by ICP-OES elemental analysis of bulk cell populations (Fig. 7). Cells were exposed to 0.17 mM Sr\(^{2+}\) in the presence or absence of 0.17 mM Ca\(^{2+}\). At all time points in the course of the exposure, the total Sr content of cells exposed to Sr\(^{2+}\) in the absence of Ca\(^{2+}\) was higher than in those in Ca-replete conditions. After 24 h, the Sr content of the Ca-deprived cells was nearly twice that of Ca-replete cells. This increase in Sr\(^{2+}\) uptake in the absence of Ca\(^{2+}\) suggests competitive uptake of these ions and is consistent with transport of Sr\(^{2+}\) through Ca\(^{2+}\) channels (Nachshen and Blaustein, 1982).
In conclusion, these results suggest that neither uptake nor efflux across the plasma membrane is highly selective for Sr$^{2+}$ (and likely Ba$^{2+}$) vs. Ca$^{2+}$. These ions further co-localize in the endomembrane system, which indicates that they are stored/transported together in this compartment. This suggests that the terminal vacuoles, where crystals are formed, play the largest role in selectivity.

### 3.8. Uptake of Ca, Sr, and Ba into the terminal vacuoles

The terminal vacuoles were defined using a two-dimensional region-of-interest (ROI) based on Zn maps as described above. Elemental content was determined from these ROIs, and concentrations in the vacuole of the live cell were estimated based on an average vacuole diameter of 6.5 $\mu$m. An XFM image is a two-dimensional projection of a three-dimensional elemental distribution; thus, the vacuolar signal is superimposed with a cellular background, resulting in a slight overestimation of vacuolar concentrations.

When cells were cultured in Ba- and Sr-deprived medium, the vacuole could not be distinguished in Ba, Sr, or Ca XFM maps; i.e. the vacuolar area concentrations were the same as cellular background. However, changes in the Ba, Sr, and Ca concentrations of the vacuole were noticeable during the Sr/Ba pulse-chase (30 min pulse of 0.17 mM Sr$^{2+}$ and 0.35 $\mu$M Ba$^{2+}$ followed by a Sr/Ba-free chase). After a 35 min chase, Ba, Sr, and Ca in the vacuole appeared elevated above cellular background by factors of 2.4, 2.5, and 1.5, respectively. The vacuolar Sr concentration of 0.8 mM was found to be greater than the external Sr concentration, which implies active transport (Krejci et al., 2011), for example by a cation ATPase pump or a cation/proton antiporter. While we are confident of the increase in concentration of all three ions, the relative amounts are probably less accurate due to the low signal to noise ratio for both Ca and Ba. In the case of Ba, this is due to low absolute concentrations. The presence of significant amounts of Ca in the cell wall and the cytosol contributes to a high cellular Ca background, against
which the vacuolar concentrations are difficult to determine precisely. However, the cell appears to respond to the Sr/Ba spike as if it were a Ca spike, by extruding Sr, Ba, and Ca ions to the vacuole, possibly via the same mechanisms. In the green algae *E. viridis*, a similar uptake of Sr\(^{2+}\) into the vacuole as a consequence of high medium levels has been suggested (Bauer *et al.*, 1998). It is possible that the Sr/Ba ions are transported into the vacuole faster than Ca, i.e. with a degree of selectivity, as indicated by different relative increases over cellular background concentration. However, if it exists, the effect seems to be relatively small.

When a Ca-free chase medium was used instead, elevated vacuolar Sr concentrations were observed compared to the regular chase; after a 1 h Ca-free chase, the estimated range of Sr concentration in the vacuole was 1.7–2.3 mM. This is in agreement with our previous observation that Sr retention in the cell is higher when the chase medium is Ca-free, and suggests that reduced efflux in the vacuole was 1.7–2.3 mM. This is in agreement with the protein-rich pyrenoid, the vacuole, and vacuolar BaSO\(_4\) crystals. The pyrenoid XANES spectrum shows a large peak close to 2480 eV that corresponds to sulfates, as well as a small peak near 2470 eV that corresponds to organic sulfur species, such as thiols, thi-ethers and di-sulfides. This is expected; in plants and algae, the chloroplast is the site of primary assimilation of sulfate, where sulfate is reduced and the amino acids cysteine and methionine are biosynthesized (Melis and Chen, 2005). The large sulfate peak is also present in the spectrum from the vacuole, but the peak at 2470 eV is absent. This indicates a lack of significant quantities of sulfur-rich amino acids in this region and suggests that most of the sulfur in the vacuole is in the form of sulfate. Using the GCF method (Prietzel *et al.*, 2011), we found that >92% of sulfur in the pyrenoid and >99% of sulfur in the vacuole was present as sulfate (Fig. S3). This includes sulfated polysaccharides, although the vacuoles are not considered to have a major role in synthesis or transport of sulfated polysaccharides found in the extracellular mucilage sheath of desmids (Domozych *et al.*, 2007; Linde *et al.*, 2001). If we thus consider vacuolar sulfur concentrations (34–160 mM) to be slight overestimations of free sulfate concentrations, these results are high compared to typical reported vacuolar concentrations, i.e. 6–75 mM (Buchner *et al.*, 2004).

3.9. Sulfur speciation and the role of sulfate in the vacuole

Sulfur concentrations in the vacuole consistently appear elevated above cellular background (Fig. 8), ranging from 0.45 to 1.6 fmol/\(\mu\)m\(^2\) and averaging 0.79 fmol/\(\mu\)m\(^2\). This corresponds to vacuolar sulfur concentrations in the range of 34–160 mM, with an average of 76 mM. This measured sulfur concentration is the sum of free inorganic sulfate, sulfated polysaccharides, and sulfur in proteins such as phytochelatins, which are part of the detoxification mechanism of many plant cell vacuoles (Cobbett, 2000; Kiemle *et al.*, 2007).

To investigate sulfur speciation, we performed sulfur K-edge \(\mu\)-XANES on freeze-dried cells (Fig. 9). The submicron spot size of the X-ray beam used in the technique allowed for characterization of the sulfur species present in different regions of the cell, including the protein-rich pyrenoid, the vacuole, and vacuolar BaSO\(_4\) crystals. The pyrenoid XANES spectrum shows a large peak close to 2480 eV that corresponds to sulfates, as well as a small peak near 2470 eV that corresponds to organic sulfur species, such as thiols, thio-ethers and di-sulfides. This is expected; in plants and algae, the chloroplast is the site of primary assimilation of sulfate, where sulfate is reduced and the amino acids cysteine and methionine are biosynthesized (Melis and Chen, 2005). The large sulfate peak is also present in the spectrum from the vacuole, but the peak at 2470 eV is absent. This indicates a lack of significant quantities of sulfur-rich amino acids in this region and suggests that most of the sulfur in the vacuole is in the form of sulfate. Using the GCF method (Prietzel *et al.*, 2011), we found that >92% of sulfur in the pyrenoid and >99% of sulfur in the vacuole was present as sulfate (Fig. S3). This includes sulfated polysaccharides, although the vacuoles are not considered to have a major role in synthesis or transport of sulfated polysaccharides found in the extracellular mucilage sheath of desmids (Domozych *et al.*, 2007; Linde *et al.*, 2001). If we thus consider vacuolar sulfur concentrations (34–160 mM) to be slight overestimations of free sulfate concentrations, these results are high compared to typical reported vacuolar concentrations, i.e. 6–75 mM (Buchner *et al.*, 2004).

3.10. Sequestration of Ba and Sr by sulfate trap

With the observation that Sr\(^{2+}\) and Ca\(^{2+}\) seem to be transported into and out of the vacuole indiscriminately, selective trans-membrane transport steps appear to play no major role in selectivity in *Closterium*. Instead, the observed sulfur accumulation in the terminal vacuoles points to a “sulfate trap” mechanism, where Ba\(^{2+}\), Sr\(^{2+}\), and Ca\(^{2+}\) are pumped into the vacuole non-selectively (with possibly a slight bias against Ca\(^{2+}\)) and the lower-solubility (Ba,Sr)SO\(_4\) precipitates preferentially over the more soluble SrSO\(_4\) and Ca-SO\(_4\)2H\(_2\)O. This is consistent with the fact that the Sr/Ba ratio in the medium is a good predictor of the ratio in the crystals if the Lippmann diagram for (Ba,Sr)SO\(_4\) is consulted (Krejci *et al.*, 2011). Interestingly, concentration estimates reveal that the total

![Fig 8](image-url) Composition of the terminal vacuole. XFM maps of (A) calcium, (B) sulfur, (C) strontium, and (D) zinc of a cell exposed to 0.17 mM Sr\(^{2+}\) and 0.35 \(\mu\)M Ba\(^{2+}\) for 30 min, followed by a 6.5 h Sr/Ba-free chase. White arrows in the Zn map indicate the vacuole. Sr, S and Zn concentrations in the vacuole are significantly elevated above cellular background levels. The vacuole can be distinguished in the Ca map, although to a lesser extent due to higher cellular background. Imaging was performed at 17.5 keV, 1 s dwell time, and 1.25 \(\mu\)m step size.
3.11. Conclusions and outlook

In *C. monilferum*, we observed little evidence for a major role of selective transport proteins in uptake, efflux, or transport of Ba\(^{2+}\) and Sr\(^{2+}\) vs. Ca\(^{2+}\). Desmids instead seem to maintain a condition where the vacuole is just undersaturated or slightly supersaturated with respect to CaSO\(_4\)-2H\(_2\)O and supersaturated for SrSO\(_4\), yet nucleation is suppressed such that only (Ba,Sr)SO\(_4\) and BaSO\(_4\) precipitate. The question remains why the sulfate trap does not “snap shut” to form sulfate biominerals in all other cells that sequester SO\(_4^{2-}\) in vacuoles (Kataoka et al., 2004), especially in view of the comparable levels in the desmid vacuole. One way would be to exclude Ba\(^{2+}\) from the vacuole, as the solubility of (Ba,Sr)SO\(_4\) decreases dramatically compared to that of SrSO\(_4\) as the Ba\(^{2+}\) mole fraction in the aqueous solution increases. Indeed, there are some reports on ion transporters selecting against Ba\(^{2+}\) and/or Sr\(^{2+}\) (Krichok et al., 2004; Schumaker and Sze, 1986). Alternatively, or in addition, there may be a mechanism for preventing nucleation and/or inhibiting crystal growth. For example, cation-binding macromolecules in the vacuole could limit the availability of free ions and thus lower supersaturation, or proteins could bind and prevent the growth of crystal nuclei. The apparent mobilization of Ba (that is not bound in crystal form) from the vacuole in pulse-chase experiments may be an indication that such a mechanism still operates in desmids, if less effectively than in other organisms. More puzzling still is the question of how the radiolarian acanthareae are able to precipitate the intermediate solubility SrSO\(_4\). A comparative analysis of macromolecules from Sr/Ba-mineralizing organisms and other plants/algae with respect to their influence on nucleation and growth could ultimately help engineer hyper-acumulating organisms for \(^{90}\)Sr remediation.

Desmids themselves are attractive candidates for bioremediation of low-level radioactive effluents as they are robust in culture, needing only sunlight and a few nutrients, and with a small amount of Ba\(^{2+}\) in the environment could act as Sr\(^{2+}\) sinks through the sulfate trap mechanism. In fact, the apparent lack of highly selective biological transport in desmids can be utilized to increase Sr incorporation in crystals to 45 mol\% by increasing the Sr/Ba ratio and activity product in the medium (Krejci et al., 2011). Conceivably, Sr\(^{2+}\) sequestration could be further optimized, for example by engineering the sulfate concentration in the vacuole, or by reducing Sr\(^{2+}\) influx across the plasma membrane, e.g. by inhibiting Ca\(^{2+}\)-ATPase activity (Avery et al., 1999). Notably, desmids have been found to have a high level of induced radiation tolerance (Howard and Cowie, 1975, 1976). With recent events emphasizing the need for alternatives to fossil fuels, a broad investigation of the mechanisms at play in desmids and other Sr/Ba-mineralizing organisms is warranted and may provide new inspiration for selective separation of \(^{90}\)Sr.

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Appendix A. Supplementary data

Baylson, F., Stevens, B., Domozych, D., 2001. Composition and synthesis of


