

Characterization of the Selenite Uptake Mechanism in the Coccolithophore *Emiliana huxleyi* (Haptophyta)

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The marine coccolithophore *Emiliana huxleyi* (Haptophyta) requires selenium as an essential element for growth, and the active species absorbed is selenite, not selenate. This study characterized the selenite uptake mechanism using ^{75}Se as a tracer. Kinetic analysis of selenite uptake showed the involvement of both active and passive transport processes. The active transport was suppressed by 0.5 mM vanadate, a membrane-permeable inhibitor of H^+ -ATPase, at pH 8.3. When the pH was lowered from 8.3 to 5.3, the selenite uptake activity greatly increased, even in the presence of vanadate, suggesting that the H^+ concentration gradient may be a motive force for selenite transport. [^{75}Se]Selenite uptake at selenite-limiting concentrations was hardly affected by selenate, sulfate and sulfite, even at 100 μM . In contrast, 3 μM orthophosphate increased the K_m 5-fold. These data showed that HSeO_3^- , a dominant selenite species at acidic pH, is the active species for transport through the plasma membrane and transport is driven by ΔpH energized by H^+ -ATPase. Kinetic analysis showed that the selenite uptake activity was competitively inhibited by orthophosphate. Furthermore, the active selenite transport mechanism was shown to be induced de novo under Se-deficient conditions and induction was suppressed by the addition of either sufficient selenite or cycloheximide, an inhibitor of de novo protein synthesis. These results indicate that *E. huxleyi* cells developed an active selenite uptake mechanism to overcome the disadvantages of Se limitation in ecosystems, maintaining selenium metabolism and selenoproteins for high viability.

Keywords: Active selenite transport • Coccolithophorid • *Emiliana huxleyi* • Phosphate transporter • Selenite transporter • Selenite utilization.

Abbreviations: CHI, cycloheximide; ESM, Erd–Schreiber medium.

Introduction

Selenium (Se) is an essential micronutrient that is required in a very narrow nutritional concentration range in various

organisms. Many organisms, including microalgae, require Se for the synthesis of selenoproteins, which are thought to be the functional forms of Se (for a review, see Araie and Shiraiwa 2009). Using ^{75}Se labeling techniques and bioinformatic approaches, >40 selenoprotein families have been identified in diverse organisms, including bacteria, archaea and eukaryotes (Kryukov et al. 2003, Kryukov and Gladyshev 2004). In animals, Se is generally ingested via organic seleno compounds that are metabolized to selenide, which is then used for the synthesis of selenoproteins in the cytoplasm. In contrast, inorganic seleno compounds are directly absorbed by cells through the cell membrane, especially in microorganisms, as nutrients from the environment. The mechanism of Se transport is well characterized in land plants, which do not require Se, since higher plants have no ability to synthesize selenoproteins (Novoselov et al. 2002). Se is present in the environment in various forms, including the elemental form [Se (0)], selenide [Se^{2+} (II)], selenite [SeO_3^{2-} (IV)] and selenate [SeO_4^{2-} (VI)]. Selenate is the major form of Se in the soil, while selenite is a very minor component. Land plants mainly absorb the selenate ion (Asher et al. 1977, Zayed et al. 1998), and its transport is thought to be non-specifically mediated by the sulfate transporter (Arvy 1993, Honda et al. 1998). A few recent reports have examined the mechanism of the selenite transporter protein. In wheat and rice, selenite uptake is mediated by the phosphate transporter (Li et al. 2008) and the silicon transporter (Zhao et al. 2010) in a competitive manner to phosphate and silicate ions, respectively. The unicellular green alga *Chlamydomonas reinhardtii* can utilize Se but shows no essential requirement. Also, a pH-dependent competition occurs between phosphate and selenite uptake (Riedel and Sanders 1996).

In contrast, the concentration of selenite is very high in seawater, with a ratio of selenite to selenate of approximately 1 : 1 (Cutter and Bruland 1984). Some marine phytoplankton species are known to require Se as an essential element for growth (Hu et al. 1996, Doblin et al. 1999). Both selenate and selenite are accumulated by algae, although selenite is more readily transported into algal cells, particularly at low concentrations (Price et al. 1987). Moreover, selenite was reported to be 1,000 times more toxic than selenate in marine haptophycean algae

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when the concentration was around $1\ \mu\text{M}$ (Danbara and Shiraiwa 1999). These reports suggested the possibility that marine phytoplankton require Se and possess a selenite-specific transport system. The toxicity of and resistance to Se have been well studied in the green algae *C. reinhardtii* and *Scenedesmus quadricauda*, respectively (Morlon et al. 2005a, Morlon et al. 2005b, Fournier et al. 2010) and Se resistance was expected to be due to the mutation of the selenite transport system (Umysová et al. 2009).

We previously reported that a haptophycean calcifying alga, the coccolithophore *Emiliana huxleyi*, requires nanomolar levels of Se for growth and that the selenite ion is the predominant species (Danbara and Shiraiwa 1999). We also found that *E. huxleyi* possesses an active transport system that exhibits high affinity for selenite ($\leq 1\ \text{nM}$) (Obata et al. 2004) and has at least six selenoproteins (named EhSEP1–EhSEP6) (Obata and Shiraiwa 2005). The transport mechanism is the key to revealing the mechanism of Se utilization and coccolithophore growth as regulated by Se. However, no information currently exists regarding a selenite-specific transporter mechanism. We studied the effect of several inhibitors on selenite uptake, competitive inhibition by analogous ions and the pH effect on the induction of the selenite transporter. We found that the selenite-specific transport system of *E. huxleyi* showed efficient HSeO_3^- transport activity driven by ΔpH energized by H^+ -ATPase. In addition, *E. huxleyi* cells induced the highly efficient selenite uptake mechanism when Se was limited in the medium. The induction of selenite transport activity was immediately suppressed by the addition of sufficient selenite.

Results

Kinetic analysis of [^{75}Se]selenite uptake activity

Kinetic analysis of [^{75}Se]selenite uptake activity in *E. huxleyi* showed the involvement of two components, a saturable component exhibiting a Michaelis–Menten-type profile and a linear profile (Fig. 1A). The data suggest that selenite transport is driven by two mechanisms, an active transport system mediated by a transporter and simple diffusion. The active transport was dominant when the extracellular selenite concentration was extremely low, and the rate saturated at around $0.7\ \mu\text{M}$. Passive transport was dominant above about $1\ \mu\text{M}$ selenite (Fig. 1A). The K_m (selenite), calculated from the double reciprocal plot, for active selenite transport was $142\ \text{nM}$ (Fig. 1B).

Active species of selenite ions and the driving force for selenite uptake activity

[^{75}Se]Selenite uptake activity in the presence of 1.4, 5.4 and $21.8\ \text{nM}$ selenite at pH 8.3 was inhibited 55–80% by $0.5\ \text{mM}$ vanadate, an inhibitor of the plasma membrane-type H^+ -ATPase (Fig. 2). These results suggest that the selenite transport process is driven by the H^+ concentration gradient.

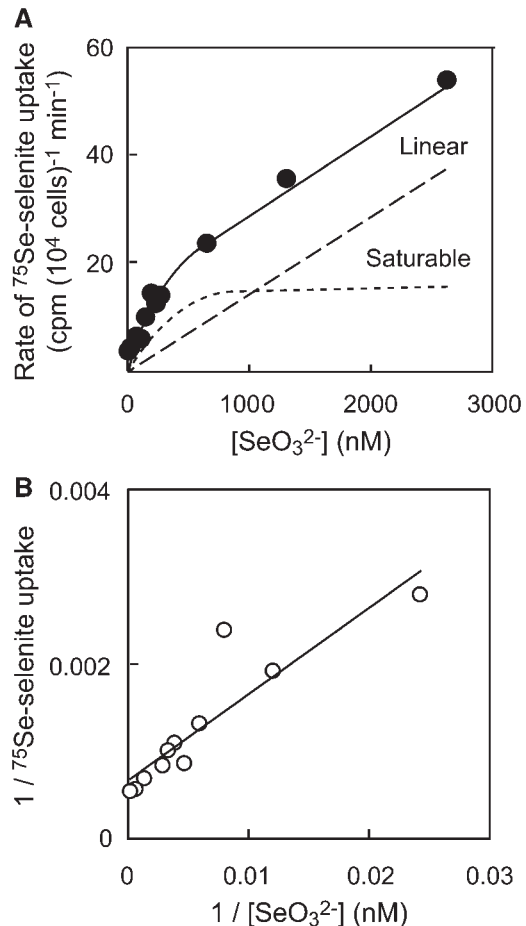


Fig. 1 Kinetic curves of [^{75}Se]selenite uptake activity in *E. huxleyi*. (A) Kinetic curves; (B) a double-reciprocal plot of the saturable component in A. The experimental kinetic curve was treated by component analysis according to our previous report (Obata et al. 2004). The K_m of the saturable component was $142\ \text{nM}$.

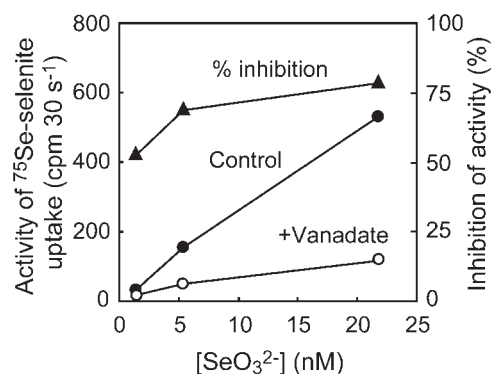


Fig. 2 Effect of vanadate on [^{75}Se]selenite uptake activity in *E. huxleyi*. [^{75}Se]Selenite uptake activity was determined in cells incubated for 30 s with [^{75}Se]selenite after 30 min incubation of cells without (filled circles) or with (open circles) vanadate ($0.5\ \text{mM}$, final concentration) at pH 8.3. The concentrations of [^{75}Se]selenite were 1.4, 5.4 and $21.8\ \text{nM}$. Filled circles, control; open circles, +vanadate; filled triangles, percentage inhibition by vanadate.

We tested whether the H^+ concentration gradient mediates selenite transport through the plasma membrane in *E. huxleyi* cells. The relationship between [^{75}Se]selenite uptake activity and external pH was examined at various selenite concentrations, shown in Fig. 3. The percentage incorporation of selenite, calculated as the ratio of radioactivity transported into the cells to that in the medium, was also determined (Fig. 3). The value was higher at acidic pH than at alkaline pH, i.e. it increased with increasing H^+ concentration. Such a trend was very similar to the percentage distribution of the protonated form of selenium, HSeO_3^- . In addition, the percentage incorporation of [^{75}Se]selenite increased along with decreases in selenite concentration. These trends were also observed with the addition of vanadate (Fig. 3). Thus, increases in the $[H^+]/[\text{selenite}]$ ratio may be effective in driving the active transport process. These results indicate that selenite can be co-transported with H^+ and released by H^+ -ATPase through the plasma membrane in *E. huxleyi*, suggesting that HSeO_3^- may be the active species.

Effect of anions analogous to selenite on [^{75}Se]selenite uptake activity

To examine substrate specificity, [^{75}Se]selenite uptake activity was assayed in the presence of anions analogous to selenite, such as selenate, sulfate, sulfite and phosphate. Selenite activity was not inhibited by the addition of sulfate (Fig. 4A). Competitive inhibition was observed after the addition of sulfite (Fig. 4B), and non-competitive inhibition was seen after the addition of selenate (Fig. 4C). Fifty percent of [^{75}Se]selenite uptake activity was inhibited at a concentration of $100\ \mu\text{M}$ (Fig. 4B, C). However, the analogous anion concentrations were extremely high at micromolar levels, whereas the selenite

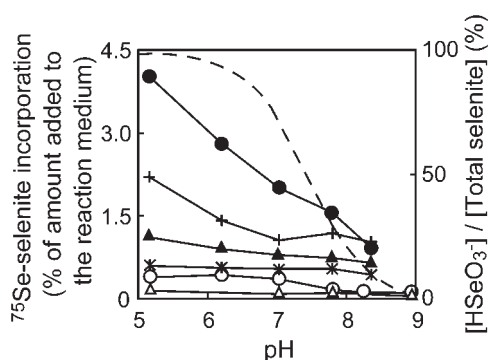


Fig. 3 pH-dependent curve of [^{75}Se]selenite uptake in *E. huxleyi* in the absence or presence of vanadate. [^{75}Se]Selenite uptake activity was determined for 30 s at various concentrations of [^{75}Se]selenite in cells not treated (filled circles, plus signs, filled triangles, asterisks) or treated with (open circles, open triangles) vanadate (final concentration, $0.5\ \text{mM}$) after 30 min of incubation at various pH values. The concentrations of [^{75}Se]selenite were the following: filled circles, $20.6\ \text{nM}$; open circles, $21.8\ \text{nM}$; plus signs, $41.1\ \text{nM}$; filled triangles, $82.3\ \text{nM}$; open triangles, $87.1\ \text{nM}$; asterisks, $329.2\ \text{nM}$. The cell concentration was 10^7 . The dotted line shows the ratio of $[\text{HSeO}_3^-]$ to total inorganic selenium calculated using the dissociation constant.

concentration was at a nanomolar level. Therefore, these results suggest that selenite is taken up by the selenite-specific transport system in *E. huxleyi*, but not by non-specific selenate, sulfate and sulfite transport systems. In contrast, competitive inhibition was observed by the addition of $3\ \mu\text{M}$ phosphate and the K_m was increased 5-fold (Fig. 4D). This suggests the possibility that selenite is taken up by a transport system similar to phosphate transport systems.

Regulation of the induction of the selenite transporter

Changes in the kinetic parameters of [^{75}Se]selenite uptake activity were examined using cells grown for 7 d in Se-sufficient medium transferred to either Se-deficient or Se-sufficient conditions (Fig. 5). The K_m (selenite) rapidly decreased under Se-deficient conditions but it remained almost constant at high levels under Se-sufficient conditions (Fig. 5A). In contrast, the V_{max} of [^{75}Se]selenite uptake activity was almost constant under both Se-sufficient and Se-deficient conditions (Fig. 5B), suggesting that cells induce the selenite transport mechanism to adapt to Se-deficient conditions. The effect of selenite on changes in selenite uptake activity was examined by the addition of selenite to Se-deficient culture (Fig. 6). After 7 d of incubation in Se-deficient conditions, $10\ \text{nM}$ selenite was added to create Se-sufficient conditions. The rates of [^{75}Se]selenite uptake under limiting concentrations of selenite were measured below or around the K_m (selenite) values, which depend on the saturable component (active transport system). The rates decreased within 24 h after the addition of $10\ \text{nM}$ selenite. The decrease in selenite uptake activity indicated the down-regulation of the induced selenite transport mechanism. To determine the induced selenite transport mechanism, whether by newly synthesized proteins or some modifications such as phosphorylation, we added cycloheximide (CHI), an inhibitor of de novo protein synthesis. When the Se-sufficient culture was transferred to a Se-deficient culture, selenite uptake activity increased, indicating induction of the selenite transport mechanism, which was then blocked by the addition of $50\ \mu\text{g}\ \text{mL}^{-1}$ CHI (Fig. 7). Selenite uptake activity remained at the same level in cells treated with CHI. Therefore, the decrease in K_m values may have been related to the proton pump, and the increases in the H^+ concentration gradient or another active selenite transport system was synthesized de novo in Se-depleted cells.

Discussion

Only a few studies have examined selenite transport in non-photosynthetic organisms. Red blood cells and keratinocytes show selective and efficient selenite uptake following reduction by glutathione (Suzuki et al. 1998, Ganyc and Self 2008). *Saccharomyces cerevisiae* shows selenite uptake by *Jen1P*, a proton-coupled monocarboxylate transporter, with a K_m of $0.91\ \text{mM}$, which is comparable with the K_m for lactate (McDermott et al. 2010). Lazard et al. (2010) reported selenite

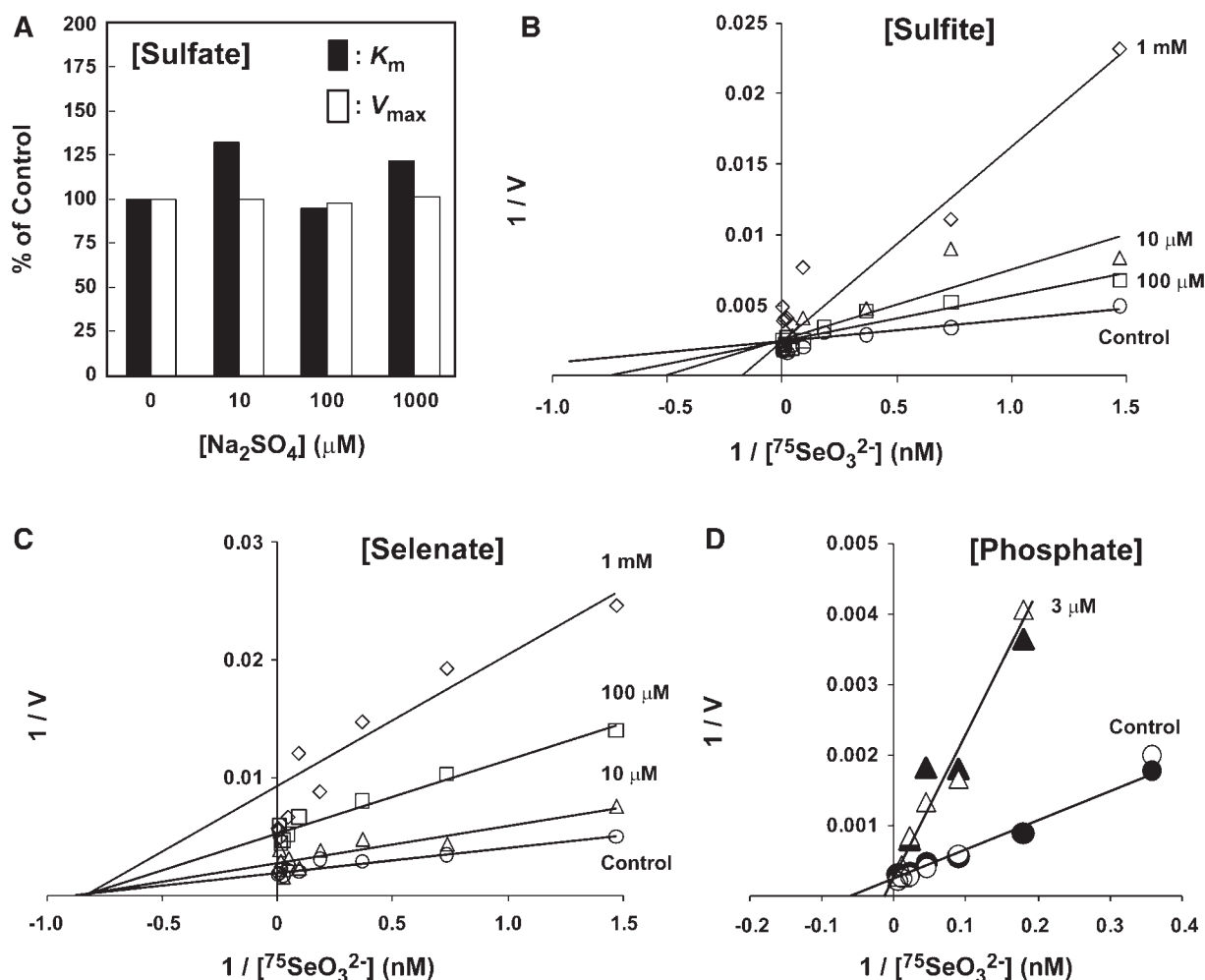


Fig. 4 Effect of various selenite analogs such as sulfate (A), sulfite (B), selenate (C) and phosphate (D) on $[^{75}\text{Se}]$ selenite uptake activity in *E. huxleyi*. The activity was determined at a saturated concentration of selenite in the absence or presence of competitors for 30 s. The concentrations tested were 0.01, 0.1 and 1 mM for sulfate, sulfite and selenate, and 3 μM for phosphate. To avoid contamination of sulfate or phosphate from the culture medium, cells were washed by centrifugation and resuspended in sulfate- or phosphate-free medium, respectively, before assaying. (A) The ratios of the K_m and V_{\max} calculated with sulfate to those without sulfate. (B) A double-reciprocal plot of a saturable component. Open circles, without the addition of sulfite (control); open triangles, with 10 μM sulfite; open squares, with 100 μM sulfite; and open diamonds, with 1 mM sulfite. (C) A double-reciprocal plot of a saturable component. Open circles, without the addition of selenate (control); open triangles, with 10 μM selenate; open squares, with 100 μM selenate; and open diamonds, with 1 mM selenate. (D) A double-reciprocal plot of a saturable component. Open and filled circles, without the addition of phosphate (control); open and filled triangles, with 3 μM phosphate.

uptake by high- and low-affinity orthophosphate transporters (K_m values of 19 μM and 4 mM, respectively) in *S. cerevisiae*. Ghariieb and Gadd (2004) reported two selenite transport systems (K_m values of 54 and 435 μM) that were measured by using $[^{75}\text{Se}]$ selenite in *S. cerevisiae*. Thus, several variations in Se utilization exist among organisms.

Selenite uptake by *E. huxleyi* was shown to be driven by active and passive transport processes that play significant roles at low and high selenite concentrations (Fig. 1). These results clearly confirmed our previous results (Obata et al. 2004), although the K_m value in this study was slightly higher than that in the previous study. The selenite uptake was

strongly pH dependent and the active species of selenite in active transport was HSeO_3^- (Fig. 3). The process was found to be driven by ΔpH energized by H^+ -ATPase (Fig. 2). In general, the proton pump (H^+ -ATPase) is a H^+ transporter forming a H^+ concentration gradient across the membrane. Three types of H^+ -ATPase, types F, V and P, are localized in plastids and mitochondria, the vacuole and the cell membrane, respectively. Anions are thought to be unable to pass freely through biomembranes because of their negative charge (Konings et al. 1995). Therefore, sulfate and phosphate are known to be co-transported with H^+ , released by the P-type H^+ -ATPase, into the cell (Breton and Surdin-Kerjan 1977,

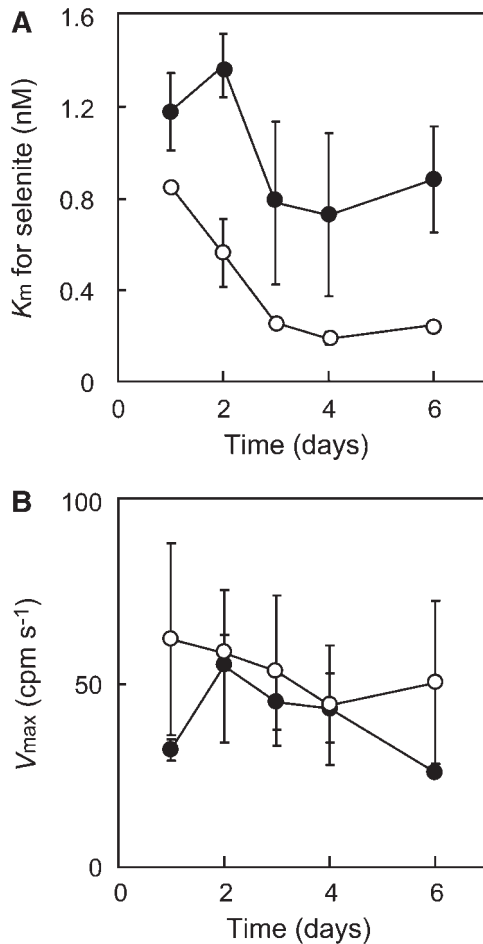


Fig. 5 Changes in the K_m (selenite) and V_{max} of [⁷⁵Se]selenite uptake activity in *E. huxleyi* after transferring cells grown under Se-sufficient (10 nM) conditions to Se-sufficient (filled circles) and Se-deficient (open circles) medium. To determine kinetic parameters, [⁷⁵Se]selenite uptake activity was determined for 30 s at 11 different concentrations (0.3–348.3 nM) at each sampling time. The experiment was repeated twice in independent cultures, and an average result is shown. The values were calculated as $OD_{750} = 1$. (A) K_m (selenite); (B) V_{max} . Filled circles, Se-sufficient conditions (control); open circles, Se-deficient conditions.

Hawkesford et al. 1993, Mitsukawa et al. 1997, Honda et al. 1998, Mimura 1999). Like sulfate and phosphate transport processes, selenite uptake was shown to be associated with P-type H^+ -ATPase (Fig. 2). In addition, we tested the pH dependency of selenite uptake in the presence of vanadate. The results showed the same tendency as the results in Fig. 3, while the ratio was lower than that observed without vanadate (data not shown). Therefore, we concluded that selenite is actively absorbed depending on the H^+ concentration gradient and co-transported with H^+ produced by P-type H^+ -ATPase.

Some transporters carry ions non-specifically. For example, the sulfate transporter competitively moves selenate in land plants (Arvy 1993, Honda et al. 1998), the phosphate transporter

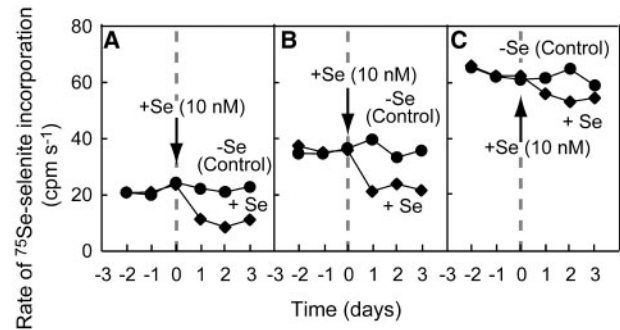


Fig. 6 Effect of selenite on the induction of [⁷⁵Se]selenite uptake activity in *E. huxleyi* during culture. *Emiliania huxleyi* cells were grown in Se-deficient medium for 7 d, and then selenite (10 nM, final concentration) was added to the medium at time 0 days (filled diamonds) or no addition selenite was made (filled circles). After harvesting cells from the culture, they were washed with Se-deficient medium to remove selenite. Then, [⁷⁵Se]selenite uptake activity was determined for 30 s in the presence of 1.4 (A), 7.7 (B) or 44.4 nM (C) [⁷⁵Se]selenite. The experiment was repeated twice in independent cultures, and an average result is shown. The values were calculated on the basis of $OD_{750} = 1$.

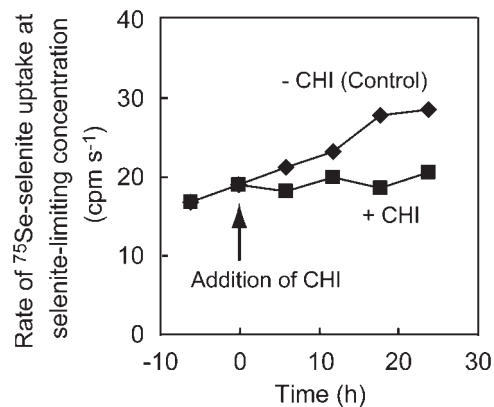


Fig. 7 Effect of CHI on the induction of selenite uptake activity in *E. huxleyi* under Se-deficient conditions. After 7 d incubation with 10 nM selenite, *E. huxleyi* cells were transferred to Se-deficient medium. After 30 h incubation, CHI (50 $\mu\text{g ml}^{-1}$, final concentration) was added to the medium at time 0 h (filled squares) or was not added (filled diamonds). [⁷⁵Se]Selenite uptake activity was determined in the presence of 5 nM [⁷⁵Se]selenite for 30 s at 6 h intervals.

competitively carries selenite in wheat and *S. cerevisiae* (Li et al. 2008, Lazard et al. 2010), and the monocarboxylate transporter competitively transfers selenite in *S. cerevisiae* (McDermott et al. 2010). However, in *E. huxleyi*, selenite was specifically absorbed in the presence of divalent anions that were expected to be competitors (Fig. 4). Selenite uptake was inhibited only when a high concentration of anions was added. Although the K_m value was five times higher after the addition of 3 μM phosphate compared with no addition (Fig. 4C), this did not change after adding 300 nM phosphate (data not

shown). Therefore, specific selenite transport is thought to occur in *E. huxleyi* and the transporter may function as, and be similar to, the phosphate transporter. These results suggest that the selenite transport system in *E. huxleyi* is highly specific for the selenite ion. Such high specificity for selenite is unique. Zhao et al. (2010) reported that selenite is taken up non-specifically through the silicon transporter in rice, which requires silicon to strengthen itself. *Emiliana huxleyi* is not yet known to require silicon, and thus silicon would not act as a competitor of selenite in this coccolithophore. However, other algae, such as diatoms that require both selenite and silicon, may absorb selenite through the silicon transporter.

When cells were incubated in Se-deficient medium, the K_m (selenite) rapidly decreased (Fig. 5A) while the V_{max} of [^{75}Se]selenite uptake activity was almost constant (Fig. 5B). Furthermore, when 10 nM (final concentration) selenite was added to the Se-depleted cells, [^{75}Se]selenite uptake activity decreased, indicating the down-regulation of the selenite transport mechanism (Fig. 6). These results suggest that *E. huxleyi* newly induces the selenite transport mechanism, which has a high affinity for selenite. If the number of selenite transport systems increases, the K_m (selenite) will remain unchanged while the V_{max} will increase. The addition of CHI, an inhibitor of de novo protein synthesis on 80S ribosomes, to the Se-depleted cells decreased selenite uptake activity (Fig. 7). This result suggests that the selenite transport mechanism is induced by de novo protein synthesis under Se-deficient conditions. The induction of the selenite transporter is very important to utilize selenite efficiently from seawater containing 0.1–0.2 nM selenite. The high-affinity sulfate transporter is known to be up-regulated under S-limiting concentrations (Yoshimoto et al. 2007). Like the sulfate transporter, the selenite transport system is thought to be induced to acclimate to Se-limiting conditions. Moreover, the K_m values reportedly decrease in the sulfate transporter depending on the decrease in pH (Hawkesford et al. 1993). Another possibility is thought to be the increase in the synthesis of proton pumps to change the H^+ concentration gradient to provide energy for selenite transport.

In this study, we showed that the selenite uptake system in *E. huxleyi* has two components, the active and passive transport processes; HSeO_3^- is the active species of selenite for transport into cells accompanied by H^+ ; the selenite uptake system in *E. huxleyi* cells is highly specific to selenite and not competitively inhibited by analogous anions, such as sulfate, sulfite and selenate, but is inhibited by phosphate; and the high-affinity selenite transport mechanism is induced by de novo protein synthesis in Se-depleted cells but not in Se-sufficient cells. Selenite(IV) is a more reduced form of Se compared with selenate(VI). Thus, for *E. huxleyi* to absorb selenite is reasonable, even if the concentration of selenite is lower than that of selenate in seawater. Moreover, other algae, especially secondary symbionts such as diatoms, dinoflagellates and other prymnesiophytes in seawater, require selenite as a Se source. Therefore, the selenite-specific uptake system may not be unique to

E. huxleyi. This study provides the first evidence of the presence and regulation of a selenite-specific transport mechanism, contributing to the knowledge of selenite transporters and Se utilization in marine algae.

Materials and Methods

Cell growth

The coccolithophore *E. huxleyi* (Lohman) Hay & Mohler (Haptophyta) was obtained from the algal culture collection of the National Institute for Environmental Studies (NIES 837), Tsukuba, Japan. For stock culture, we used natural seawater containing artificial micronutrients in Erd–Schreiber medium (ESM) with 10 nM disodium selenite.

For kinetic analysis, and to test the induction of the selenite transport system, cells were grown in MA-ESM medium. The medium contains synthetic seawater, Marine Art SF (produced by Tomita Pharmaceutical and purchased from Osaka Yakken) containing ESM enriched with 10 nM disodium selenite, according to the method of Danbara and Shiraiwa (1999). The suspension was continuously bubbled with air at a flow rate of 100 ml min^{-1} .

In both stock and experimental cultures, cells were continuously illuminated by fluorescent light at an intensity of $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The temperature was maintained at 20°C .

[^{75}Se]Selenite uptake assay by the silicone–oil layer centrifugation technique

An aliquot of the cell suspension just harvested from culture was pre-incubated under dark conditions for 30 min in an L-shaped glass vessel (volume: 10 ml). A ^{75}Se transport assay was performed using the silicone–oil layer centrifugation technique. Before starting the assay, a tube containing the silicone–oil layer (the tube for termination of the reaction) was prepared as follows: 20 μl of killing solution (1 M glycine containing 0.75% SDS, pH 10.0) was placed at the bottom of a 0.4 ml microcentrifuge tube (No. 72.700; Assist) and 60 μl of silicone oil (mixture of SH 550 and SH 556 at a ratio of 2 : 1; Dow Corning Toray Silicone Co., Ltd.) was placed on it. Then, the reaction was initiated by transferring the cell suspension (300 μl) into a 1.5 ml plastic tube (reaction tube) with 0.01% Tween-20 (Wako Pure Chemicals Co. Ltd.) and an aliquot of various concentrations of [^{75}Se]selenite (University of Missouri). After 30 s incubation, 200 μl of the cell suspension was transferred into the termination tube and layered onto the silicone oil layer. Then, the reaction was terminated by immediate centrifugation at $10,000 \times g$ for 1 min to sediment the cells through the silicone–oil layer. The tube was quickly frozen in liquid nitrogen. To obtain the culture medium and cell pellet separately, the tube was cut with scissors at the position of the silicone–oil layer. The radioactivity of both fractions was determined using a gamma counter. All experiments with radioactive materials were performed at the Radioisotope Center, University of Tsukuba.

Inhibitors and reagents

Sodium vanadate, an inhibitor of H⁺-ATPase in the plasma membrane, sodium selenite and other anions were purchased from Wako Pure Chemicals Co. Ltd. CHI, an inhibitor of de novo protein synthesis on 80S ribosomes, was purchased from Sigma-Aldrich Corporation.

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