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CHLAMYDOMONAS EUGAMETOS (CHLOROPHYTA) STORES PHOSPHATE IN POLYPHOSPHATE BODIES TOGETHER WITH CALCIUM¹

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ABSTRACT

When *Chlamydomonas eugametos* Moewus cells are starved of phosphate, they accumulate $^{32}\text{P}_i$ much faster than before starvation. Phosphate accumulation is stimulated by calcium. Less than 5% of the $^{32}\text{P}_i$ taken up by the cell is present in soluble molecules, suggesting that most is in a metabolically inactive, storage form. Nuclear magnetic resonance and X-ray microanalysis data are presented to show that it is stored as polyphosphate in electron-dense bodies in the cytoplasm. The same bodies accumulate divalent cations, in particular calcium. The P/Ca ratio in the bodies was maintained between 5.4 (1-week-old cells) and 3.3 (5-week-old cells) during cultivation, suggesting that the calcium and phosphorus relations of the bodies are coupled. The possibility that these electron-dense bodies represent calcium stores that can be released to activate calcium signaling is discussed.

Key index words: calcium storage; *Chlamydomonas eugametos*; Chlorophyta; phosphate storage; phosphate uptake; polyphosphate

In the course of a study on the role of polyphosphoinositides in *Chlamydomonas eugametos* Moewus gametes, we noticed that very little of the [^{32}P]- P_i administered to label the phospholipids was incorporated into lipids, proteins, or nucleic acids (Brederoo et al. 1991). A large majority of the label was incorporated into a less soluble fraction, irrespective of the age or phosphate status of the cells. Many organisms including algae, bacteria, and fungi have been shown to store phosphate in electron-dense polyphosphate bodies (for an introduction to this extensive literature, see Harold 1966, Twiss and Nalewajko 1992). Lewin (1952) showed that *C. moewusii* cells contain volutin globules, which we now know to consist largely of polyphosphate (Allan and Miller 1980). Coleman (1978) also claimed that

Chlamydomonas cells contain polyphosphate, based on staining with 4',6-diamidino-2-phenylindole (DAPI), although it was 2 years later that the characteristic yellow fluorescence was shown to be due to polyphosphate (Allan and Miller 1980). More recently, Hebel et al. (1992) used unclear magnetic resonance (NMR) spectroscopy to demonstrate that most of the inorganic phosphate (P_i) fed to P_i -starved *C. reinhardtii* cells is taken up and accumulated as polyphosphate. In this report, we provide the first characterization of P_i uptake and the polyphosphate stores in a *Chlamydomonas* species. Interestingly, polyphosphate is stored in electron-dense bodies together with calcium. The possibility that these major intracellular stores play a role in inositol-1,4,5-trisphosphate/calcium signaling is discussed.

MATERIALS AND METHODS

Chlamydomonas eugametos strains UTEX 9, UTEX 10, 17.17.2, and 5.39.4 (Schuring et al. 1987) were grown in Petri dishes on agar-containing medium (Mesland and van den Ende 1978). Cultures were maintained at 20°C in a 12:12 h LD cycle (30 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Cell suspensions (gametes) were obtained by flooding 3–4-week-old agar cultures with HMCK (10 mM HEPES, pH 7.4, 1 mM MgCl_2 , 1 mM CaCl_2 , and 1 mM KCl). Cells were sedimented by 2 min centrifugation at $1000 \times g$ and then resuspended in HMCK at a density of approximately 2×10^7 cells $\cdot \text{mL}^{-1}$.

HEPES was from Janssen Chimica (Geel, Belgium). [^{32}P]- P_i was from Amersham International (Amersham, UK), and all other reagents were from Merck Nederland B.V. (Amsterdam).

Phosphate uptake and measurements. Cells (2×10^7 mL^{-1}) were incubated in 500 μL HMCK containing 1 μM P_i and 370 kBq [^{32}P]- P_i . To stop the uptake of radioactivity, an equal volume of HMCK containing 1 mM P_i was added, and the cells were sedimented twice for 2 s, each time resuspending the cells in 1 mL HMCK containing 1 mM P_i . The amount of [^{32}P]- P_i in the gamete and first supernatant fractions was determined by liquid scintillation spectrometry.

NMR measurements. Three- and 4-week-old 17.17.2 cells from 20 Petri-dish cultures were harvested and sedimented in a bench centrifuge. The cell pellet was extracted with 30 mL 14% perchloric acid while stirring the mixture continuously for 30 min (Pick et al. 1990). After centrifugation, the supernatant was neutralized by adding excess KHCO_3 . The precipitate of perchlorate

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was sedimented, and the clear supernatant was freeze-dried. Samples of the material were taken up in EDTA (1 mg·mL⁻¹) for NMR measurements.

Phosphorus spectra of the extracts were performed in a Bruker AC-200 spectrometer. All measurements were recorded at room temperature. A 90° pulse of 7 ms was associated with an acquisition time of 0.41 s and a repetition time of 2 s. To achieve a satisfactory signal-to-noise ratio, 528 accumulations were necessary. The pH of the solution was 9.5, so the chemical shift of inorganic phosphate was set at 5.8 ppm relative to methyldiphosphonate at 19.5 ppm.

To perform measurements on living cells (3-week-old 17.17.2), a dense suspension of approximately 2×10^8 cells·mL⁻¹ was loaded into a 20-mm tube in a Bruker AMX-400 WB spectrometer at room temperature. Spectra were accumulated in blocks of 1 h over an 18-h period. Inorganic phosphate was the only detectable peak in the spectrum.

Transmission electron microscopy. For sectioning, cells were fixed for 30 min in a mixture of 3% formaldehyde and 1% glutaraldehyde. Cells were washed by sedimenting them for 2 s at $16,000 \times g$ and resuspending them in water. After recentrifugation, the pellet was mixed into a drop of liquid 2% agarose (50° C). On setting, the agarose was cut into blocks that were washed in water and dehydrated in ethanol, final concentration 70%. Individual blocks were embedded in capsules of LR-White that was polymerized for 24 h at 50° C. Sections were made on an LKB microtome and left unstained or stained with lead hydroxide. Sections or nonfixed intact cells were mounted on copper grids covered with parlodion film and examined in a Philips EM410 electron microscope.

Whole cells were mounted by pipetting drops of cells onto parlodion-covered grids. All fluid was then removed by making contact with blotting paper. The grids were dried in the air and viewed in the electron microscope.

X-ray microanalysis. X-ray microanalytical spot analyses and X-ray microanalysis (XRMA) mappings were performed with a Tracor (TN) 2000 X-ray microanalyzer attached to a Philips EM 400 scanning transmission electron microscope. The system geometry of this instrument was described elsewhere (de Bruijn et al. 1987). Sections collected on copper grids were placed in a beryllium low-background holder that was tilted at an angle of 108° relative to the electron beam (specimen was tilted 18° from horizontal) to enhance the X-ray yield. X-ray point analyses were performed at 80 kV, with a spot diameter of 400 nm during 50 or 100 s lifetime. X-ray maps were acquired at 80 kV. The spot size for the latter measurements was 100 nm. Data were collected from 256×256 pixel points. The dwell time per pixel point was 0.5 s.

RESULTS

Phosphate uptake. In this study, sexually competent *C. eugametos* gametes were used because our work is concerned with intracellular signaling during sexual reproduction. Gametes can only be produced in large numbers by growing them on agar plates and then flooding them with a nonnutrient medium. After 2–3 h, the cells become flagellated and swim into the medium. In previous studies using [³²P]-P_i to label the phospholipids of *C. eugametos* (Brederoo et al. 1991), the mt⁺ strain 17.17.2 was used because older, starved cultures took up phosphate faster than other strains regularly used in our laboratory. For example, when 2-week-old cells were harvested from agar plates, washed, and transferred to 1 μM P_i containing 370 kBq [³²P], the sexually competent cells accumulated label very slowly (Fig. 1). If they were harvested 2 weeks later, the population had not

FIG. 1. Uptake of [³²P]-P_i and its incorporation into lipids by *C. eugametos* cells. Suspensions of 17.17.2 cells (10^7 cells in 1 mL HMCK) were incubated in 1 μM P_i containing 370 kBq [³²P], and the uptake into 2- (○) and 4-week-old (□) cells was measured with time, together with the incorporation of radioactivity into the phospholipids of 4-week-old cells (■). Uptake is shown as the percentage of the total P_i added to the cells. Note that the difference in scale between the two axes emphasizes that only a small fraction of the P_i taken up is incorporated into the lipid fraction.

grown but the same number of cells now took up most of the radioactivity within 10 min. During the first 2 weeks of culture, while the population grew from 10^6 to 4×10^9 cells per agar plate, the P_i content of the medium fell from 2.5 to 0.5 mg·mL⁻¹ but thereafter remained constant. Presumably the P_i remaining in the medium after 2 weeks was not available for uptake. Thus, 4-week-old cells, in contrast to those at 2 weeks, had been starved of P_i and had developed the ability to rapidly take up P_i. The kinetics of accumulation were closely reflected in the kinetics of ³²P incorporation into the phospholipids, emphasizing that less than 1% of the label taken up was incorporated. Thus, most of the [³²P]-P_i entering the cell was not immediately available for metabolism, suggesting that it could be stored.

To test whether cells indeed respond to P_i starvation by enhancing their ability to accumulate P_i, 2-week-old cultures were suspended in HMCK medium (10 mM HEPES, pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, and 1 mM KCl) supplemented with 1, 10, or 100 μM P_i. At intervals, the cells were washed, and their net uptake rates were tested. The rapid accumulation of [³²P]-P_i was observed after 1, 2, and 4 days, respectively (Fig. 2). Because the cells did not grow under these conditions, the length of time taken to deplete the medium of phosphate and respond by enhancing their P_i-uptake activity was dependent on the starting concentration of phosphate.

The presence of calcium affected the accumulation of phosphate. When cells were incubated in 100 μM EDTA in 10 mM HEPES, pH 7.4, the net uptake rate was strongly reduced (Fig. 3). On adding 1 mM

Fig. 2. Influence of P_i treatment on uptake of $[^{32}P]-P_i$. Four-week-old 17.17.2 cells were inoculated in liquid medium containing 1 ($\square$), 10 ($\circ$), and 100 ($\triangle$) $\mu M P_i$. Samples were taken from these cultures, washed three times, and incubated (10^7 cells in 1 mL HMCK) in 1 $\mu M P_i$ containing 370 kBq $[^{32}P]-P_i$ for 10 min. Uptake is represented as percentage of total added P_i taken up by the cells in a 10-min period.

calcium to the same medium, the uptake rate recovered completely, while magnesium and potassium were much less effective.

Phosphate storage. Less than 5% of the radioactivity in the cells could be readily solubilized, irrespective of the strain or its age. Apparently much of the P_i was stored in a less soluble form. Coleman (1978) suggested that *Chlamydomonas* accumulates polyphosphate, based on the presence of yellow fluorescent DAPI-staining bodies. Similar fluorescent bodies were found in live *C. eugametos* cells treated with DAPI (not shown). The same bodies could also be stained with Toluidine blue, a stain previously used for phosphate-rich volutin granules in *Chlamydomonas* (Lewin 1952), and neutral red that colors acidic vesicles. More convincing evidence for polyphosphate in *C. eugametos* cells was obtained by ^{31}P -NMR spectroscopy. Three-week-old 17.17.2 cells were concentrated (2×10^8 cells $\cdot mL^{-1}$) in a 20-mm NMR tube without taking measures to illuminate or aerate them. Cultures then become anaerobic due to the limited surface area for gas exchange and, under these conditions, Hebel et al. (1992) showed that *C. reinhardtii* converts polyphosphate to P_i .

For a period of 18 h, spectra were made by accumulating scans every hour. Within the first 4 h, no signal was detected, but thereafter a single P_i peak appeared that increased linearly with time (Fig. 4, insert). This illustrated that cells contained a large source of phosphate, even though the P_i signal may have been due to phosphate lost to the medium. Cells were therefore extracted in 14% perchloric acid to solubilize short-chain polyphosphates (Pick et al. 1990). After freeze-drying, the extract was taken up in EDTA solution and analyzed. The dominant form of ^{31}P was polyphosphate, with the major signal arising from central phosphate residues (Fig. 4). Because long-chain polyphosphates (>10 resi-

Fig. 3. Stimulation of phosphate uptake by calcium. Four-week-old cells (17.17.2) were suspended in HMCK (=10 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM KCl) or in 10 mM HEPES, pH 7.4, alone. $[^{32}P]-P_i$ uptake was then measured with or without addition of the components listed. Uptake is presented as the percentage of $[^{32}P]-P_i$ in the cells after a 10-min period.

dues) are not readily extracted into perchloric acid, the relative amount of polyphosphate could be much greater than presented in these data. Other prominent resonance signals were attributed to sugar phosphates, di-esters, and P_i . The unidentified peaks at -2.68 and -16.58 ppm may represent tripolyphosphate. These data establish the presence of polyphosphate in *C. eugametos* and suggest that it is present in bodies that can be stained yellow by DAPI.

This conclusion was supported by transmission electron microscopy combined with XRMA. Both intact, nonfixed cells and sections of fixed material were analyzed. Electron micrographs show prominent electron-dense bodies (Figs. 5, 6). XRMA of these bodies (Fig. 5c) made it clear that they specifically accumulate phosphorus together with cations, in particular calcium. Similar analyses of the cytoplasm (Fig. 5b) or any part of the cell protoplast or wall revealed much lower concentrations. Quantitative data are difficult to produce using this technique, so the relative concentrations of elements in the electron-dense bodies compared with the neighboring cytoplasm were calculated for 18 cells using an analysis period of 5 min. In eight of the cells, the cytoplasmic phosphorus concentration was too low to register. In the other ten, the average phosphorus ratio for electron-dense body to neighboring cytoplasm was 45 to 1. Because of the nonregistering concentrations, this must be seen as a lower limit

FIG. 4. ^{31}P -NMR analysis of *C. eugametos* cells and extract. When cells (17.17.2) were subjected to *in vivo* ^{31}P -NMR, no signal was detected within the first 4 h, but then a single peak corresponding to P_i was detected (insert a) that increased in time (insert b), indicating that a polymeric form of phosphorus was present in the cell. After extracting gametes in 20% perchloric acid, the supernatant was freeze-dried, taken up in EDTA (1 mg mL $^{-1}$) and subjected to NMR spectroscopy. The spectrum shows that polyphosphate is the major phosphate source in the extract.

ratio. The major mineral elements found in the cytoplasm were potassium, chlorine, and sulphur. Calcium was not registered in any of the analyses of the cytoplasm and is therefore more concentrated in the electron-dense bodies than phosphorus. Therefore, polyphosphate bodies represent the major source of intracellular calcium. Although data are presented for 17.17.2 cells, equivalent bodies were also found in strains UTEX 9, UTEX 10, and 5.39.4. Electron-dense bodies were found irrespective of age or whether cultured in liquid or agar-containing medium, or whether the cells were gametes or vegetative cells. The best way to observe them was in whole-cell, nonfixed mounts (Fig. 6) because they were immediately visible as round, electron-dense, vesicle-like bodies. They had a liquid nature in as much as they sometimes expanded rapidly under an intense electron beam. In living cells, DAPI-stained bodies were sometimes seen to fuse into larger round bodies. In sections of fixed material, the electron-dense bodies were more angular (Fig. 5), often associated with lacunas as if the polyphosphate/cation complex had been precipitated by glutaraldehyde fixation. Perhaps the complex is normally stabilized by protein that prevents calcium phosphate from crystallizing.

From the XRMA maps (Fig. 6), it is obvious that high concentrations of phosphorus accumulated with

FIG. 5. Electron micrographs and X-ray microelement analyses of *C. eugametos* cells. Cells (17.17.2) were fixed, embedded, sectioned, and stained with lead hydroxide before photographing in a transmission electron microscope. a) X-ray microanalysis was performed on equivalent nonstained sections. b) Spot analysis of the cytoplasm. c) Spot analysis of an electron-dense body (see * in a). c = chloroplast, n = nucleus, p = pyrenoid, w = cell wall. Scale bar = 0.83 μm .

FIG. 6. Electron micrograph and XRM maps of a *C. eugametos* cell. a) Transmission electron micrograph of a nonfixed, nonstained intact 17.17.2 cell. b) The distribution of phosphorus (red), calcium (green), potassium (blue), and magnesium (yellow) in the same cell. c) The distribution of phosphorus is shown in detail. The relative intensity increases from red to white. d) Colocalization of calcium and phosphorus in the electron-dense bodies. Where the red of phosphorus and the green of calcium coincide, the signal is represented as orange/white. The X-ray analyses were made at an angle of 18° relative to that of the electron beam, explaining why the element concentrations do not coincide exactly with the electron-dense bodies. Scale bar = $2\ \mu\text{m}$.

calcium and, to a lesser extent, magnesium. Potassium was present in the electron-dense bodies at more or less the same level as in the cytoplasm. The map of calcium distribution in *C. eugametos* resembles the X-ray map presented by Kaska et al. (1985) for *C. reinhardtii* and suggests that the calcium bodies they visualized are equivalent to the phosphorus bodies described here, although they did not present electron micrographs to make the comparison more decisive. The electron-dense bodies varied greatly

in size and number per cell. Of 60 individual 17.17.2 cells analyzed, the average per cell was 16, with the number ranging from 4 to 52. The diameter was between 0.02 and $2\ \mu\text{m}$, although they were seldom more than $1\ \mu\text{m}$. Smaller bodies may have been present, but their electron density was too low to qualify them as electron-dense bodies. By photographing ten cells and measuring relative sizes, the total volume of electron-dense vacuoles was calculated to be about 1% of the total cell volume. From the relative

difference in phosphorus signal in the electron-dense bodies compared with the cytoplasm, we estimate that at least 40% of cellular phosphate was in the bodies. Because calcium could not be detected in the cytoplasm, the strong signal from the electron-dense bodies could mean that they contained essentially 100% of the calcium in the cells. The polyphosphate bodies of other algae have also been shown to accumulate calcium (e.g. Adamec et al. 1979, Sicko-Goad and Stoermer 1979, Daniel and Chamberlain 1981, Jensen et al. 1982, 1986, Rachlin et al. 1984). The relative composition of the bodies was consistent. Of 36 cells ranging from 1 to 5 weeks old that were analyzed, the average P/Ca ratio was 4.6 (SD 0.9). There was a tendency for the composition to change with age, because the P/Ca ratio in 1-week-old cells was 5.4 but declined to 3.3 after 5 weeks, perhaps reflecting the fact that phosphorus became limiting in older cultures. Nonetheless, even cells in 7–13-week-old cultures contained many electron-dense bodies of the usual size that had all accumulated phosphorus and calcium. In these cultures, the P/Ca ratio was maintained at about 3, indicating that the phosphorus and calcium relations of the bodies was coupled.

Interestingly, we encountered some bodies that were devoid of mineral content, but their electron-dense appearance was unaltered, indicating that this property was not due to the high concentration of phosphate, calcium, and so forth.

DISCUSSION

When *C. eugametos* cells are starved of phosphate, their ability to accumulate P_i increases. This may reflect an increase in uptake into the cell and/or an increase in their ability to store P_i because our results show that much of the phosphate entering the cell is stored as polyphosphate in vacuole-like organelles. Such phosphate-rich bodies seem common in algae (e.g. see Harold 1966, Adamec et al. 1979, Elgavish and Elgavish 1980, Vorisek and Zachleder 1984, Watanabe et al. 1987, Pick and Weiss 1991, Twiss and Nalewajko 1992). Presumably the major function of these storage structures is to help buffer and maintain a critical P_i concentration in the cytoplasm, much as the cell vacuole of higher plants is thought to function (Mimura 1995).

We were able to stain structures in *C. eugametos* with DAPI. They were then visible as yellow fluorescent particles. Allan and Miller (1980) showed that similar fluorescent particles in yeast could be artificially created by mixing polyphosphate and S-adenosyl methionine with DAPI. Cationic S-adenosyl methionine had previously been isolated from yeast vacuoles, and Schwencke and deRobichon-Szulmajster (1976) suggested that it concentrates there by binding to polyphosphate. The polyphosphate bodies represent so-called volutin granules, which were long known in yeast, for the DAPI-staining bodies also stained with neutral red and Tolui-

dine blue, classic volutin stains. The presence of similar bodies in *C. eugametos* with the same staining properties is a strong indication that they are equivalent to the volutin-containing vacuoles of yeast. However, we failed to detect sulphur in most of the electron-dense bodies in *C. eugametos*, which indicates that S-adenosyl methionine is absent or less prevalent than in yeast volutin granules. Nonetheless, the intense characteristic yellow fluorescence of DAPI-treated polyphosphate bodies in live *Chlamydomonas* cells is a most convenient way of detecting them and monitoring their distribution.

The accumulation of phosphorus together with calcium in vacuoles could account for the stimulating effect of the cation on P_i accumulation that we and others have found (Goodenough et al. 1993). The idea that polyphosphate stores are also calcium stores is of great potential significance in relation to the role of calcium as a signal. Membrane receptor activation can lead to the production of inositol-1,4,5-trisphosphate ($InsP_3$) as a second messenger in the cell. It binds to an $InsP_3$ receptor in the membrane of a calcium storage site such as the endoplasmic reticulum (animals) or tonoplast (plants), which then opens to release Ca^{2+} into the cytoplasm. The increase in Ca^{2+} subsequently activates various calcium-dependent processes such as the phosphorylation/dephosphorylation of proteins, eventually producing a biological response. In the dimorphic yeast *Candida albicans*, acidic vacuoles have recently been shown to contain $InsP_3$ -sensitive calcium sources (Calvert and Sanders 1995). In *Chlamydomonas*, several examples of both natural and artificial activation of this signaling system have been presented in the literature (Musgrave et al. 1992, 1993, Quarmby et al. 1992, Yueh and Crain 1993, Quarmby and Hartzell 1994), and the subsequent responses are known to be at least partly caused by an increase in intracellular calcium (Sanders and Salisbury 1989, 1994). This indicates that $InsP_3$ -sensitive calcium stores must be present in this alga. Working with *C. reinhardtii*, Kaska et al. (1985) illustrated XRMA maps of cells showing that calcium originally accumulated in electron-dense bodies became dispersed throughout the cell very soon after sexual fusion. We have been able to reproduce their findings using *C. eugametos*. It is attractive to believe that in both cases the calcium is mobilized by $InsP_3$ because we have recently shown that $InsP_3$ levels rise dramatically when gametes fuse together (Musgrave et al. 1993). These data suggest that the polyphosphate bodies in at least some algae must be seen in a new light.

Assuming the polyphosphate stores represent a source of calcium that can be mobilized and not just a calcium trap, we envisage the release of Ca^{2+} via an $InsP_3$ receptor being coupled to an influx of K^+ from the cytoplasm to maintain electroneutrality. As Ca^{2+} is pumped back into the stores, K^+ is displaced to the cytoplasm. This implies that poly-

phosphate bodies may preferentially accumulate some cations but can also accumulate others. Because they are often acidic compartments (those in *C. eugametos* stain with neutral red), they could also accumulate basic compounds. Indeed, studies with other algae have shown that polyphosphate bodies accumulate many compounds ranging from heavy metals (Twiss and Nalewajko 1992, and the references therein) to positively charged fluorescent dyes (Greenfield et al. 1987, Pick et al. 1990, 1991, Pick and Weiss 1991, Weiss and Pick 1991). In this way, they can protect cells from toxicity and large pH changes. However, if the accumulation of basic or cationic compounds interferes with the calcium relations of the polyphosphate bodies, as has been reported (Daniel and Chamberlain 1981, Rachlin et al. 1984, Jensen et al. 1986), signaling could be affected with grievous effects for cell development. We are presently studying the effects of NH_4^+ ions that are known to be accumulated in polyphosphate bodies (Greenfield et al. 1987, Pick et al. 1990, 1991, Pick and Weiss 1991) and that drastically reduce the sexual competence of *C. eugametos* gametes (Musgrave et al. 1981).

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MODULATION OF THE HEAT SHOCK UBIQUITIN POOL IN *SKELETONEMA COSTATUM* (BACILLARIOPHYCEAE)¹

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ABSTRACT

Immunoblotting experiments performed with an anti-ubiquitin antibody revealed that *Skeletonema costatum* (Grev.) Cleve cells contained free ubiquitin as well as ubiquitin conjugated to various endogenous proteins. A temperature shift from 18° to 30°C greatly increased the total amount of ubiquitin and particularly the ubiquitin fraction in high molecular mass conjugates. A solid-phase immunoassay indicated values of 0.031 ± 0.004 pmol·10⁻⁶ cells for free ubiquitin and 0.046 ± 0.004 pmol·10⁻⁶ cells for conjugated ubiquitin for cells grown at 18°C, and 0.056 ± 0.008 pmol·10⁻⁶ cells and 0.21 ± 0.03 pmol·10⁻⁶ cells, respectively, after a temperature increase from 18° to 30°C. Cell-free extracts of *S. costatum* were equally able to form thiol ester linkages with ¹²⁵I-ubiquitin in an adenosine triphosphate-dependent manner at 18°C and at 30°C. Cell-free extracts were also able to conjugate ¹²⁵I-ubiquitin to endogenous proteins, but the ubiquitin conjugation rate at 30°C was lower than at 18°C. Incubation of *S. costatum* for 3 h at 30°C and then for 3 h at 18°C resulted in the formation of high amounts of ubiquitin conjugates, suggesting that partially inactive or denatured proteins accumulate during heat stress. These denatured proteins are then conjugated to ubi-

quitin very efficiently when the physiological temperature is restored. Thus, *S. costatum* cells contain ubiquitin and an active ubiquitin conjugation system responding to stress conditions (temperature stress). The intracellular concentration of ubiquitin conjugates is most likely limited by the availability of protein substrates to be conjugated rather than by ubiquitin-conjugating activity.

Key index words: *Bacillariophyceae*; heat stress; *Skeletonema costatum*; ubiquitin; ubiquitin-conjugating activity

Ubiquitin is a highly conserved protein of 8.5 kDa that occurs in all eukaryotes and differs in only three amino acid substitutions between mammalian and plant cells (Vierstra et al. 1986). It is located intracellularly either free or linked to a variety of cytoplasmic, nuclear, or integral membrane proteins. Ubiquitin is conjugated to specific target proteins by the action of an adenosine triphosphate (ATP)-dependent multienzyme system (Hershko et al. 1983), which includes E₁, a ubiquitin-activating enzyme (Mr 105 kDa) that hydrolyzes ATP to form a thiol ester with the carboxyl terminus of ubiquitin; E₂, a family of proteins (Mr 14–230 kDa) responsible for ubiquitin transthiolation; and E₃ (Mr 180 kDa), a protein ligase that catalyzes the formation of an isopeptide bond between the c-terminal glycine of

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