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Carbon fixation by the peculiar marine diatom *Haslea ostrearia*

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Abstract

During batch culture of *Haslea ostrearia* the highest carbon (^{14}C) fixation rate was found *in vivo* in cells that did not accumulate the blue pigment marennine (green form). This fixation rate decreased concomitantly with the accumulation of marennine. *In vitro*, no phosphoenolpyruvate carboxylase (PEPC) activity was detected, but nearly equivalent activities of ribulose-1,5-bisphosphate carboxylase (RuBPC) and phosphoenolpyruvate carboxykinase (PEPCK) were found in the green form. However, the activity of RuBPC was lower than that of PEPCK during marennine accumulation. *In vitro* carboxylase activities were strongly inhibited by the addition of a marennine extract. A full description of this inhibition could not be confirmed within the cells because marennine accumulates in small cytoplasmic vesicles.

Additional key words: chlorophyll; phosphoenolpyruvate carboxykinase; phosphoenolpyruvate carboxylase; photosynthesis; ribulose-1,5-bisphosphate carboxylase.

Introduction

Haslea ostrearia is a pennate diatom that synthesises a water-soluble blue pigment, marennine. The structure and physiological functions of this pigment within the cells are not known yet. This diatom is used in France during the breeding of green-gilled oysters. In oysters-ponds, conditions of the diatom bloom and the parameters that induce marennine synthesis are not fully understood. However, some hypotheses have been proposed on the role of external factors such as nutrient depletion (Neuville and Daste 1972) or high irradiance (Mouget *et al.* 2000). In batch culture, iron and manganese enrichment increases algal growth (Moreau *et al.* 1994), but in static cultures the blue pigment accumulates quickly at the end of exponential growth phase (Robert 1978).

Processes related to the light-harvesting mechanisms for this microalga were studied by Schubert *et al.* (1995), Tremblin and Robert (1996), and Mouget *et al.* (2000). Using fluorescence during photosynthesis, Schubert *et al.* (1995) found that marennine is not directly involved in light harvesting for either photosystem (PS) 1 and PS2, but filters the long-wavelength absorbance of chlorophyll (Chl). Nassiri *et al.* (1998) showed that marennine is located in some cytoplasmic vesicles particularly concentrated in the apical areas but also located around the periphery of the cell (thus above and beneath plastids) which could confirm a shadowing effect of this blue pigment observed by Schubert *et al.* (1995). Using the production of oxygen, Tremblin and Robert (1996) also showed that photosynthesis of *H. ostrearia* is related to bluing of the cells.

We studied characteristics of inorganic carbon fixation during batch growth and marennine accumulation. Total fixation ^{14}C was measured in batch culture during the entire cell development (*i.e.*, in the absence and presence of marennine) from the non-blue stage (cells without marennine accumulation) to the blue-stage (with marennine accumulation). The global measurement of inorganic carbon fixation did not differentiate between the relative contributions of the two metabolic pathways: Calvin-Benson cycle and β-carboxylation.

In photoautotrophic marine phytoplankton as in other plants, inorganic carbon may be metabolised through the Calvin cycle *via* the ribulose-1,5-bisphosphate carboxylase (RuBPC) and by β-carboxylation *via* the enzymes

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phosphoenolpyruvate carboxylase (PEPC) and phosphoenolpyruvate carboxykinase (PEPCK) or by the enzyme pyruvate carboxylase. As concerns the carbon budget, the two pathways are not equivalent. CO_2 fixation by RuBPC uses photons as energy source and is responsible for gross production (Descolas-Gros and Oriol 1992). β-carboxylation also fixes inorganic carbon but as energy source uses metabolites previously synthesised in the cell or from the external medium. In addition for marine phytoplankton, the ratio of β-carboxylase activity to RuBPC activity is usually different during the growth phases of a culture and also between species at the same stage of growth (Beardall 1989, Descolas-Gros and Oriol 1992). Therefore, the total inorganic carbon fixation and variations in activities of RuBPC, PEPC, and PEPCK during batch growth and marennine accumulation were measured in this unusual diatom.

Materials and methods

Algae culture: An axenic strain of *H. ostrearia* was isolated from oyster-ponds of the Bouin district (Vendée-France). For its morphological characteristics see Robert (1983). The clone used has a modal length of cells of 73±2 μm. Cultures were grown under axenic conditions in the liquid medium of Provasoli ES 1/3 modified by Robert (1983). The salinity of the medium was 28. The cells used for assays were taken from cultures in the exponential phase but without marennine accumulation (cultivated in semi-continuous mode under low irradiance). In all cases, 250 cm^3 of medium was transferred in 500 cm^3 glass Erlenmeyer flasks; the algae were inoculated to obtain an initial cell density of 2 000 cells per cm^3. The flasks were placed in a culture room at 16 °C under an irradiance of 100 μmol(quantum) m^{-2} s^{-1} with a 14/10 h light/dark cycle. The cultures were neither stirred, nor supplied with air. Population densities were estimated with a haemocytometer (*Nageotte*).

Photosynthetic carbon fixation: Samples were collected after the 5 h light period and concentrated to an average cell concentration of 50 000 cells per cm^3. In assays, three aliquots (1 cm^3) of the concentrated cultures were incubated in a 20 cm^3 scintillation vial at a temperature of 16±1 °C for 20 min under saturating irradiance of 500 μmol(quantum) m^{-2} s^{-1} provided by a 500 W lamp (*Mixopal*) (Tremblin and Robert 1996). Labelled ^{14}C was produced using $NaH^{14}CO_3$ (*Amersham*) at 185 GBq m^{-3} (specific activity 3.7 GBq mol^{-1}). At the end of incubation, the non-incorporated inorganic carbon was released by acidification with 6 M HCl and the entire mixture evaporated with infrared lamps. The content was re-suspended in 1 cm^3 of distilled water, and the scintillation cocktail was added. The fixed ^{14}C was measured by liquid scintillation counting (*Betamatic I, Kontron*); efficiencies were determined using the external standard channel ratio method. The photosynthetic capacity was expressed as μmol(fixed $^{14}CO_2$) per cell or per kg(Chl *a*).

Carboxylase assays: For methods used for the extraction and measurement of RuBPC, PEPC, and PEPCK in diatoms see Descolas-Gros and Fontugne (1985) and Descolas-Gros and De Billy (1987), respectively. Algae were collected by filtration (*GF/C Whatman* glass fibre filters) and frozen immediately in liquid nitrogen. Cells were re-suspended in the extraction buffer (50 mM Tricine buffer, pH 8.0 containing 5 mM dithiothreitol, 1 mM EDTA, and 10 % *Triton X-100*), and then ground in a cooled mortar (0 °C) in the presence of quartz sand. The homogenates were then centrifuged at 4 °C at 20 000×*g* for 15 min. The resulting supernatant contains the crude enzyme extract. It was stored (<30 min) at 0 °C before the enzyme test. Total (activated) capacities of RuBPC, PEPC, and PEPCK (activities measured on crude extract) were measured by incorporation of ^{14}C into acid stable products. Oxaloacetic acid (OAA) issued from β-carboxylation of phosphoenolpyruvate (PEP) by PEPCK or PEPC was fixed as the OAA-phenylhydrazone complex. The linearity of *in vitro* ^{14}C fixation (by both RuBPC and PEPCK mechanisms) with time using crude enzyme extracts from *H. ostrearia* was verified for at least 30 min; therefore, experiments involving the observation of *in vitro* $^{14}CO_2$ fixation were stopped after 10 min.

Enzymatic crude extracts for *in vitro* marennine inhibition assays on carboxylase activities were obtained from non-blue pigmented cells and a similar protocol for carboxylation enzyme measurement was used but with addition of different concentrations of partially purified blue pigment.

The fixed ^{14}C was measured by liquid scintillation counting after chemical (for RuBPC) or colour (for PEPC and PEPCK) quenching corrections. All tests were run in triplicate at a constant temperature of 30 °C. Blanks were run in the absence of RuBP or PEP, respectively.

Chl *a* and marennine contents: Chl *a* was determined by the method of Lorenzen (1967). An aliquot of the concentrated culture (20 cm^3) was filtered through glass-fibre (*Whatman GF/C*) moistened with a drop of saturated magnesium carbonate solution. The cells were homogenised by grinding with a mortar and pestle at 0 °C in acetone and the broken suspension was centrifuged. The absorption of the supernatant was measured spectrophotometrically at 665 nm before and after adding a drop of 1 M HCl. The concentration of Chl *a* was calculated

using the equation of Lorenzen (1967).

Marennine was extracted in Na/K phosphate buffer (pH 8.0) after concentration of the cells by centrifugation. Pigment concentration was measured spectrophotometrically at 669 nm using the specific extinction coefficient ($E^{1\%}_{1\,cm}$ = 17.2) proposed by Robert *et al.* (2001).

Results

Algal growth, marennine accumulation, and Chl *a* content: The time courses of *H. ostrearia* grown in batch culture (Fig. 1*A*) are typical of those obtained in many experiments. The lag period of 48 h corresponds to the photoacclimation of cells. Therefore, photosynthetic rate and carboxylase activities were measured from the third day of culture. Maximum cell numbers (stationary phase) were obtained after 6 d; exponential growth ceased when the culture reached a mean population density of 50×10^9 cells per m^3. At this time the marennine content was high (Fig. 1*A*) and the blue pigment was observed in a large part (close to 50 %) of the cell structure (Robert *et al.* 1975). In contrast, during batch growth, Chl *a* content was not significantly modified with an average value of 3.8±0.2 µg(Chl *a*) per 10^6 cells (Fig. 1*B*).

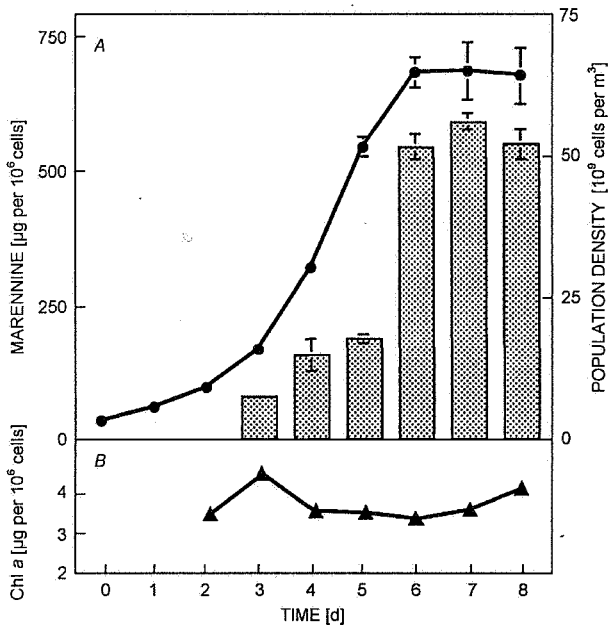

Fig. 1. Growth (increase in population density) and marennine (*A*) and chlorophyll *a* (*B*) contents during batch culture of *Haslea ostrearia*. Mean values, vertical bars = ± S.E., n = 5. Unseen error bars are too small to be discernible.

Changes in photosynthetic capacities: During algal growth, photosynthetic capacities of *H. ostrearia* cells changed markedly (Fig. 2). Carbon fixation per cell or per Chl *a* was high during the lag period, but it decreased before the end of the exponential growth phase and was stabilised at a minimum rate during the stationary phase. After 7 d, the photosynthetic capacity of the cells was less than 30 % of the maximum reached during the early stages of the exponential growth phase and about 25 % when based on Chl *a* content.

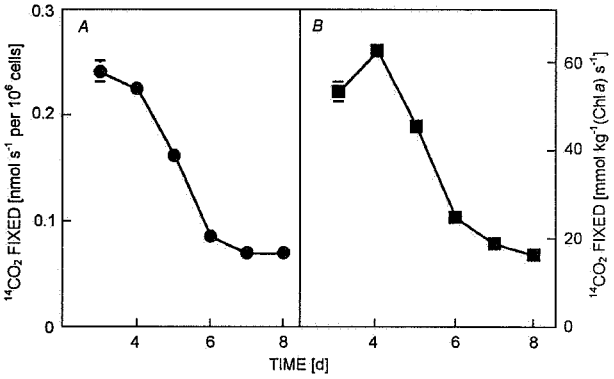

Fig. 2. Time course of $^{14}CO_2$ fixation per cells (*A*) or per chlorophyll *a* (*B*) in *Haslea ostrearia*. Mean values, vertical bars = ± S.E., n = 3. Unseen error bars are too small to be discernible.

Changes in capacities of carboxylases (Table 1): In *H. ostrearia* similarly as in all diatom species, carboxylation *via* RuBPC is the most important carboxylation system. No ^{14}C was fixed by an enzymatic preparation if the reaction mixture only contained phosphoenolpyruvate as a carboxylation substrate and Mg^{2+} as a cofactor corresponding to PEPC. In contrast, in the presence of ADP and Mn^{2+}, significant activity of β-carboxylation was found in the crude extract, so carboxylation capacity other than CO_2-fixation *via* RuBPC was mainly due to PEPCK.

Table 1. *In vitro* ^{14}C fixation [pmol(CO_2) s^{-1} per 10^6 cells] by enzyme extracted from no blue-pigmented cells of *H. ostrearia*. Mean values ± S.E., n = 3. n.d.: not detected.

Enzyme	RuBPC	PEPC	PEPCK
Test substrate	RuBP	PEP	PEP ADP
Test co-substrate	Mg^{++}	Mg^{++}	Mn^{++}
^{14}C fixed	45.33±1.33	n.d.	37.17±2.00

The carboxylation enzyme activities measured *in vitro via* RuBPC and PEPCK were maximum in this species with an almost equivalent level in the early stages of the exponential growth phases, but declined significantly with time during the late exponential and stationary phases (Fig. 3). This rapid decrease in activity was higher for RuBPC than PEPCK but reached an

immeasurable value for the two carboxylases at the stationary phase (after 7 d of culture). An important inhibition of RuBPC and PEPCK activities was observed in relation with marennine concentration, but it was almost equivalent for the two carboxylases (Fig. 4).

Discussion

The PEPCK activity previously detected in other diatom species (Descolas-Gros and Oriol 1992) was found in *H. ostrearia* and was equivalent to RuBPC activity (under our experimental conditions) when cells did not accumulate the blue pigment. However, no PEPC activity was detected.

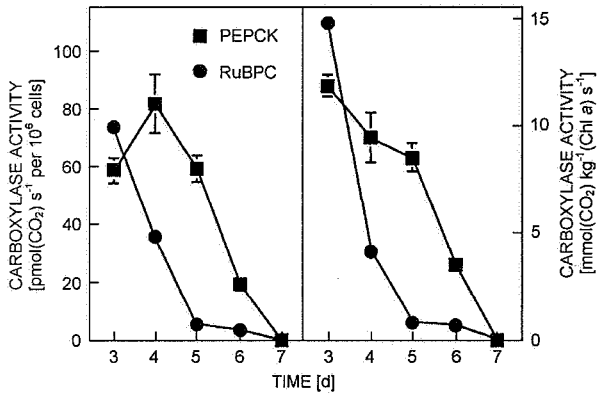

Fig. 3. Changing rates of RuBPC and PEPCK activities during batch culture of *Haslea ostrearia*. Mean values, vertical bars = ± S.E., $n = 3$. Unseen error bars are too small to be discernible.

The difference between PEPCK and PEPC involves the phosphorylation of ADP to ATP by PEPCK and a requirement of $MnCl_2$ for activation. In a manner similar to other autotrophic phytoplankton cells (Descolas-Gros and Oriol 1992), *H. ostrearia* assimilates inorganic carbon through the Calvin-Benson cycle *via* the RuBPC pathway, but β-carboxylation by PEPCK provides an efficient mechanism that may be superimposed on this cycle.

In batch cultures of other diatom species (Ebata and Fujita 1971, Griffiths 1973, Beardall *et al.* 1976), the photosynthetic capacity and related carboxylase activities of the cells significantly decrease with time along with a reduction in Chl *a* content. In *H. ostrearia* all these changes occur without major changes in the concentration of Chl *a*, but with an important marennine accumulation. During batch culture, changing photosynthetic capacities in *H. ostrearia* correlated more closely with changes in the activity of PEPCK (as previously observed by Beardall *et al.* 1976 for PEPC in other diatoms) than RuBPC, the activity of which was quickly and significantly reduced.

There was a correlation between the reduced photosynthetic rate (*in vivo* CO_2 uptake) and the changing of the carboxylase activities (*in vitro* CO_2 fixation) but after 7 d no carboxylase activity was detected *in vitro* whereas *in vivo* significant ^{14}C fixation still occurred. However, since the crude enzymatic extract contains marennine which *in vitro* inhibits carboxylase activities (Fig. 4), the *in vivo* effects of this egnimatic blue pigment on carbon fixation are not clear and thus further experiments are required.

At the stationary phase, cells accumulated a large amount of marennine (Fig. 1). So, *in vitro* CO_2 fixation by carboxylases was entirely inhibited on the 7th d (Fig. 3). In cells, marennine is principally accumulated in a cytoplasmic vesicular complex (Nassiri *et al.* 1998) meaning that perhaps, *in vivo*, this blue pigment cannot directly inhibit enzymatic reactions of carboxylation. Unfortunately, both the marennine biosynthesis pathway and the cell compartment origin are still unknown. If chloroplasts are involved in this synthesis, marennine can act directly as an inhibitor of carbon fixation by RuBPC in the chloroplast stroma. If PEPCK is involved in cytosol primary fixation of CO_2, marennine can act as an inhibitor before its accumulation in vesicles (Nassiri *et al.* 1998).

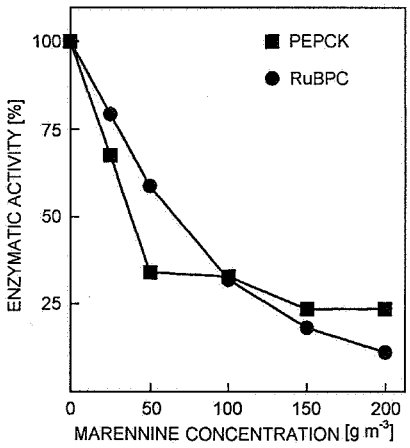

Fig. 4. *In vitro* inhibition of RuPBC and PEPCK activities by marennine extract. Both enzyme activities calculated on a per cell basis and expressed relative to the maximum value (see Table 1). The S.E. for each set of values (not plotted) was 5 % or less the magnitude of the mean.

The *in vitro* RuBPC activity in *H. ostrearia* is lower than the *in vivo* photosynthetic inorganic carbon fixation but is similar to that described previously for other diatoms (Zimba *et al.* 1990, MacIntyre and Geider 1996, MacIntyre *et al.* 1996, 1997). MacIntyre *et al.* (1997) proposed that some of the differences between RuBPC capacity and carbon fixation may have been due to proteolysis during extraction, incomplete extraction of

RuBPC in the crude extract, or to inhibition of the enzyme by the presence of metabolites such as marennine in the reaction mixture (see above). However, the rate of carboxylation by RuBPC measured *in vitro* could be low relative to photosynthesis if there were a high rate of β-carboxylation in intact cells. The higher PEPCK than RuBPC capacities measured along the batch culture in *H. ostrearia* support this hypothesis.

The importance of C_4-like metabolism with significant fixation of carbon by PEPCK (or other equivalent carboxylases) amongst marine microalgae and more precisely in diatoms is the subject of some debate (Morris 1980, Beardall 1989, Glover 1989, Zimba *et al.* 1990, Descolas-Gros and Oriol 1992, Raven 1997). Descolas-Gros and Oriol (1992) found an important variability between species. Our results suggest an important variability with the physiological stage, with an increase in the β-carboxylase activity to RuBPC activity at the end of the stationary phases of the culture as previously shown in the ubiquitous diatom *Skeletonema costatum* (Descolas-Gros and Oriol 1992). In *H. ostrearia*, similar as in other diatoms, a continuum between strict autotrophy (RuBPC fixation using photons as an energy source) and mixotrophy (PEPCK fixation using metabolites previously synthesised) for carbon (Descolas-Gros and Oriol 1992) and nitrogen (Maestrini and Robert 1984) assimilation may exist during the growth cycle (characterised by marennine accumulation).

Whatever the relative importance of these two pathways, the fixation of inorganic carbon still remains controversial. However, Reinfelder *et al.* (2000) recently presented evidence that C_4 photosynthesis (confined to the cytoplasm) occurring simultaneously with the RuBPC process (which occurs in the chloroplast) supports carbon assimilation in the marine diatom *Thalassiosira weissflogii*. Therefore, in *H. ostrearia*, short-term and long-term pathways of ^{14}C fixation must be quantitatively investigated to define precisely the relative importance of the radio-labelled products directly due to β-carboxylation in the carbon budget of this diatom.

Finally, in oyster-ponds and during the benthic stage, the cells synthesise biofilms that may be responsible for shading. In addition, the cells accumulate a large amount of marennine (Robert 1983). Since this blue pigment has an *in vivo* maximal absorption spectrum maximum at 663 nm (Robert and Hallet 1981), photosynthetic activity *via* long wavelength absorption by Chl may be reduced. As a result of this shielding it is possible that cell metabolism could be partially oriented to a heterotrophic pathway. Therefore, at the benthic stage, the real physiological importance of the negative effect observed here on the level of carboxylases might be overestimated.

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