Variations in carboxylase activity in marine phytoplankton cultures. β-carboxylation in carbon flux studies

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ABSTRACT: Inorganic carbon may be assimilated through the Calvin-Benson cycle via the enzyme ribulose-1,5-bisphosphate carboxylase (Rubisco) and/or by β-carboxylation [via the enzymes phosphoenolpyruvate carboxylase (PEPC), phosphoenolpyruvate carboxykinase (PEPCK) or pyruvate carboxylase]. Here, carboxylase activity measurements for marine phytoplankton are described. Two indices measuring carboxylase activity in marine phytoplankton were used. The first measures Rubisco activity per unit chlorophyll [R/Chl; nmol CO_2 (μg chl $a+b+c$)$^{-1}$ h^{-1}] while the second is the ratio of β-carboxylase activity to Rubisco activity, expressed as % (βC/R), which reflects the proportion of inorganic carbon fixed by these 2 groups of carboxylases. These ratios were studied in (1) different algal species in culture, (2) during the different growth phases of a culture, and (3) after a light-dark transition to measure the time response of carboxylase activities. These indices were different from one species to another at the same stage of growth. In autotrophic cells, β-carboxylation remained low (βC/R < 40). The βC/R ratio increased significantly when R/Chl began to decrease at the end of the growth phase of a culture of *Skeletonema costatum*. The heterotrophic dinoflagellate *Crypthecodinium cohnii*, grown on an organic medium, incorporated inorganic carbon in the dark through PEPCK activity. The wide range in βC/R ratio observed among the species confirm that in phytoplankton there may exist a continuum between autotrophy and heterotrophy. From a carbon budget point of view the 2 mechanisms are not equivalent. Rubisco fixation uses light as an energy source and results in gross production; β-carboxylation also fixes inorganic carbon but as energy source uses metabolites synthesized by other pathways in the cell or from the external medium.

INTRODUCTION

Carbon flux studies, carried out in the aquatic environment with the ultimate goal of modeling the carbon cycle, require a good knowledge of primary production including temporal and spatial scales of variation (Gieskes & Kraay 1984, Peinert et al. 1989, Williams et al. 1989). Phytoplankton production is principally studied through the radiocarbon (^{14}C) method (Steemann Nielsen 1952). Interpretation of the processes involved during incubation, especially in the dark bottle, continues to be difficult (Peterson 1980, Dring & Jewson 1982, Harris et al. 1989). These difficulties are due to the fact that incubation of the samples does not permit the study of rapid variations in carbon assimilation. Since physico-chemical environmental factors change rapidly and these changes induce metabolic

modifications, characteristic biological parameters for measurement should be chosen from those which have the shortest response time. Examination of the enzymatic pool involved in a metabolic pathway permits the maximal rate of a particular physiological function to be determined. Enzymatic activity can be measured instantaneously and may be used to determine the time scale required for the adaptation of a biological system to a change in environmental conditions.

Autotrophic phytoplankton cells mainly assimilate inorganic carbon through the Calvin-Benson cycle via the enzyme ribulose-1,5-bisphosphate carboxylase (Rubisco), but fixation by β-carboxylation [phosphoenolpyruvate carboxylase (PEPC), phosphoenolpyruvate carboxykinase (PEPCK) or pyruvate carboxylase] may be superimposed on this cycle. In diatoms and cyanobacteria, the substrate for β-carboxylation (phos-

phoenolpyruvate) originates from 3-phosphoglycerate in light (intermediate of Calvin-Benson cycle), and from a polysaccharide in darkness (Kremer & Berks 1978, Appleby et al. 1980, Morris 1980, Mortain-Bertrand et al. 1987a, b, 1988a, Owttrim & Colman 1988).

The aim of this study was to investigate variations in carboxylase activity in phytoplankton species representing different systematic groups maintained in culture and to determine the time scale of variation of these activities. Two indices measuring carboxylase activity in marine phytoplankton were used. The first measures Rubisco activity per unit chlorophyll [nmol CO_2 (μg chl $a+b+c$)$^{-1}$ h^{-1}], while the second is the ratio of β-carboxylase activity (βC) to Rubisco activity (R), expressed as %(βC/R), which indicates the proportion of inorganic carbon fixed by these 2 groups of carboxylase.

MATERIALS AND METHODS

Twenty-four marine phytoplanktonic species representing different taxonomic groups were screened. These included 9 species belonging to Diatomophyceae, 4 to the Prymnesiophyceae, 2 to the Cryptophyceae, 1 to the Chlorophyceae, 1 to the Cyanophyceae, 5 to the Dinophyceae, 1 to the Prasinophyceae and 1 to the Rhaphidophyceae. Among the Diatomophyceae, 4 species were isolated from Antarctic ocean communities.

Culture experiments. Temperate species of algae were cultivated in continuous light on f/2 medium (Guillard & Ryther 1962) at 18 °C and 40 μE m^{-2} s^{-1}, with 2 exceptions: *Pavlova lutheri* which was cultivated on S50 medium (Droop 1958) and *Cryptheco-dinium cohnii* which was grown in the dark on MLH medium (Tuttle & Loeblich 1975). Antarctic species were cultivated at 40 μE m^{-2} s^{-1} on f-medium with a salinity of 34 ‰ at 3 °C (Fiala & Oriol 1990). All cultures were axenic. The various species were studied during the exponential growth phase which was carefully checked before each experiment. All experiments were carried out in quadruplicate.

Enzyme assays. Microalgae were filtered on GF/F Whatman glass fiber filters. Filtered volume depended on the cellular density of the samples. Carboxylase measurements entail the incorporation of radioactive bicarbonate into stable products. All carboxylase assays were made on the same extract. Extraction was carried out at 0 °C. The filter was ground in a glass Potter with 50 mM tricine buffer (pH 8.0) containing 5 mM dithiothreitol (DTT), 1 mM EDTA, and Triton X-100, 10 % (Descolas-Gros & De Billy 1987). The protocol employed has previously

been described for Rubisco, PEP carboxylase and PEP carboxykinase assays (Descolas-Gros & Fontugne 1985, Descolas-Gros & De Billy 1987). For the pyruvate carboxylase assay (final assay volume of 500 μl), the reaction mixture contained 50 mM Pipes buffer (Sigma) pH 6.8; 5 mM MgCl$_2$; 5 mM ATP; 20 mM NaH^{14}CO$_3$ and 100 μl of the crude extract. Reaction was initiated after a pre-incubation period of 10 min by adding 5 mM pyruvate. For all carboxylase assays, reactions were stopped by the addition of 6N HCl. Controls were carried out with and without addition of 2-4 DNPH for β-carboxylases assays and no significant differences were observed. Liquid scintillation countings were performed with a Beckmann LS 5000CE. Each carboxylase assay was carried out in triplicate and blanks were obtained without the addition of the various substrates (RuBP, PEP, pyruvate). Rubisco activity, expressed in nmol CO_2 l^{-1} h^{-1}, was normalized to unit chlorophyll (R/Chl). Chlorophyll a, b, c determinations were carried out using the spectrofluorimetric method developed by Neveux & Panouse (1987). β-carboxylase activity was expressed as percent of Rubisco activity (βC/R).

RESULTS

Variations between species

Under standard conditions, in the exponential growth phase of cultures, it appears that Rubisco activity was present in all species including the heterotrophic dinoflagellate *Crypthecodinium cohnii* which grew in the dark (Table 1). Using our methods, 4 different β-carboxylase activities appear to be differentiated: PEPC activity, PEPC activity inhibited by Mn^{++}, PEPCK and pyruvate carboxylase activity. The identity of these carboxylases would need to be verified by purification. Pyruvate carboxylase was found in only 3 of the dinoflagellate species while all the diatoms showed PEP carboxykinase activity (Table 1).

Rubisco activity per unit chlorophyll (R/chl) ranged from 0.5 to 90 nmol CO_2 (μg chl $a+b+c$)$^{-1}$ h^{-1} except for an off-scale value recorded for the heterotrophic dinoflagellate *Crypthecodinium cohnii* (Fig. 1A). This species has a chlorophyll content near zero but incorporates large amounts of inorganic carbon through PEP carboxykinase activity (Table 1). For all the other species grown in light, the most numerous values for the R/Chl ratio were found around 40 nmol CO_2 (μg chl $a+b+c$)$^{-1}$ h^{-1} (Fig. 1B). Low values of R/Chl were encountered in *Heterosigma akashiwo*, *Pleurochrysis pseudoroscoffensis* and the autotrophic dinoflagellates. These species have low Rubisco activity per unit chlorophyll and higher β-carboxylase than Rubisco

Table 1. Marine phytoplankton species maintained in culture and their β-carboxylases. Chlorophyll $(a+b+c)$ content $(\mu g \ l^{-1})$; number of cells (ml^{-1}); Rubisco and β-carboxylase activities $(nmol \ CO_2 \ l^{-1} \ h^{-1})$

Species	β-Carboxylase	Chl $(a+b+c)$	No. cells × 1000	Rubisco	β-Carboxylase
Diatomophyceae					
Asterionella glacialis Castracane	PEP carboxykinase	541	515	20871	2409
Chaetoceros affinis Lauder	PEP carboxykinase	133	67	11262	94
• Nitzschia kerguelensis O'Meara	PEP carboxykinase	–	–	–	–
• Nitzschia turgiduloides Hasle	PEP carboxykinase	100	100	717	250
Phaeodactylum tricornutum Bohlin	PEP carboxykinase	363	1235	12489	401
Skeletonema costatum (Greville) Cleve	PEP carboxykinase	532	1897	29712	1820
• Stellarima microtrias (Ehrenberg) Hasle & Sims	PEP carboxykinase	84	8	1914	641
• Thalassiosira lentiginosa (Jan) Fryxell	PEP carboxykinase	53	4	2177	126
Thalassiosira pseudonana (Hustedt) Hasle & Heimdal	PEP carboxykinase	1321	3090	22464	2828
Prymnesiophyceae					
Emiliania huxleyi (Lohmann) Hay & Mohler	PEP carboxylase	175	1982	3314	661
Isochrysis galbana Parke	PEP carboxylase	173	492	14010	273
Pavlova lutheri (Droop) Green	PEP carboxylase	1069	2730	40650	2088
Pleurochrysis pseudoroscoffensis Gayral & Fresnel	PEP carboxylase inhibited by Mn⁺⁺	258	63	2995	9138
Cryptophyceae					
Rhinomonas fragarioides (Butcher) Hill & Wetherbee	PEP carboxylase	130	238	3023	7
Rhodomonas baltica Karsten	PEP carboxylase	288	225	109	13
Chlorophyceae					
Dunaliella marina Butcher	PEP carboxylase	221	133	8090	52
Cyanophyceae					
Synechocstis sp.	PEP carboxylase	428	–	1459	318
Dinophyceae					
Amphidinium carterae Hulburt	Pyruvate carboxylase	53	38	32	550
Amphidinium operculatum Claparede & Lachmann	Pyruvate carboxylase	93	42	33	262
Crypthecodinium cohnii (Seligo) Chatton	PEP carboxykinase	1	37	672	9878
Prorocentrum micans Ehrenberg	PEP carboxylase	120	7	519	2392
Scrippsiella trochoidea (Stein) Loeblich III	Pyruvate carboxylase	131	8	434	240
Prasinophyceae					
Pyramimonas sp.	PEP carboxylase	–	–	–	–
Raphidophyceae					
Heterosigma akashiwo (Hada) Hada	PEP carboxykinase	851	92	31	1204
*Antarctic species					

activity. Marine phytoplankton with a βC/R ratio <100 % (Fig. 1B), and particularly when less than 10 %, appeared to have the highest Rubisco activity per unit chlorophyll (>20 nmol CO_2 (μg chl $a+b+c)^{-1}$ h^{-1}); this occurred principally in the diatoms. The highest βC/R ratios among the diatoms were found in 2 Antarctic species.

Variations in carboxylase activities during growth

During the growth of the diatom *Skeletonema costatum*, Rubisco activity per unit chlorophyll $(a+c)$ (R/Chl) increased very rapidly (×30) and reached 60 nmol CO_2 (μg chl $a+c)^{-1}$ h^{-1}. During the same period the βC/R ratio remained very low (< 1 %) and increased only at
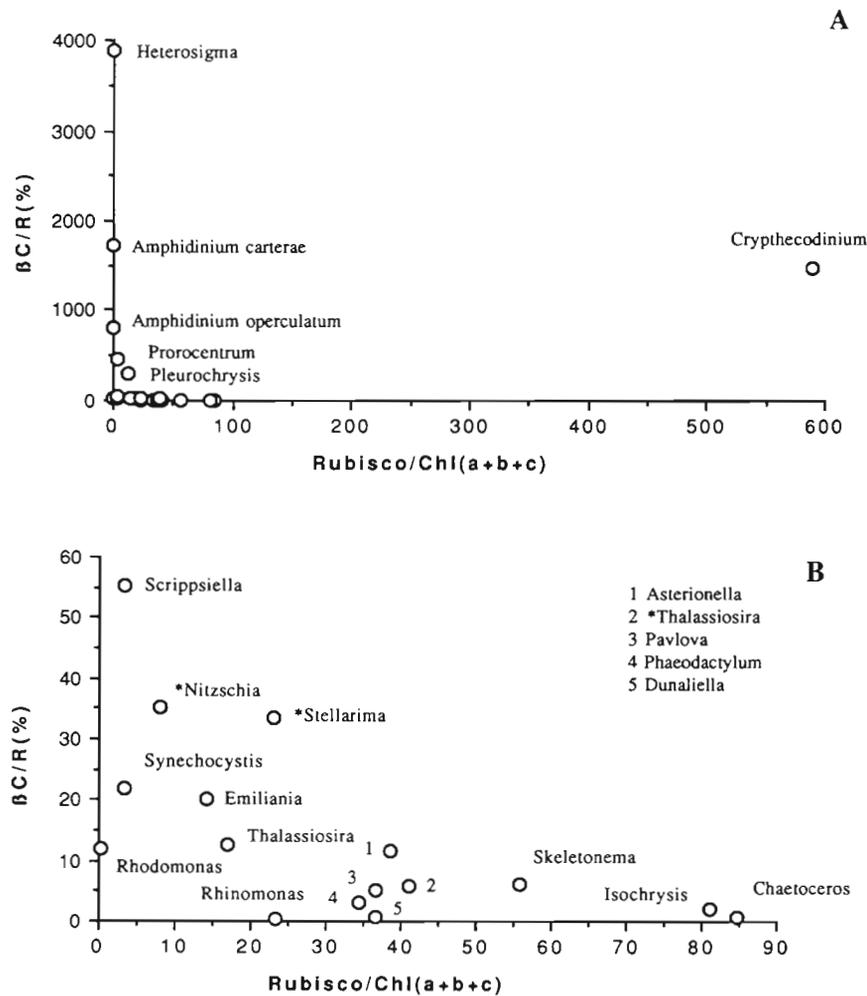
Fig. 1. Rubisco activity per unit chlorophyll [nmol CO_2 (μg chl $a+b+c$)$^{-1}$ h^{-1}] versus βC/R ratio (the % ratio of β-carboxylase to Rubisco activity ratio) for 22 species given in Table 1. (A) Full range of R/Chl values; (B) R/Chl values from 0 to 90. *Antarctic species

the end of the culture period when R/Chl decreased. The increase in R/Chl was faster than the increase of biomass expressed as cell counts (Fig. 2). During growth larger variations were recorded in R/Chl than in the βC/R ratio.

Influence of light-dark transition

To obtain information on the short-term time scale of response of carboxylase activity to physical perturbation, 2 cultures, of the diatom *Skeletonema costatum* and the prymnesiophycean *Isochrysis galbana,* at the same growth stage, were put in the dark (Fig. 3). The R/Chl and the βC/R (%) ratios were measured each hour for several hours. The number of cells and the chlorophyll ($a+c$) content was also measured in order to verify that there was no increase in biomass during the experimental time. For both species, it appeared that R/Chl did not change but a rapid increase in βC/R was observed, linked to the increase in β-carboxylase activity.

DISCUSSION

β-carboxylase activities detected in microalgae complement data previously obtained for marine phytoplankton species, summarized by Beardall (1989) and Glover (1989). The phosphoenolpyruvate carboxykinase activity detected in diatom species (Holdsworth & Bruck 1977, Kremer & Berks 1978) was confirmed for 3 of these species and found in 6 further diatom species. The pyruvate carboxylase activity detected in *Amphidinium carterae* and *Gymnodinium* sp. (Appleby et al. 1980) was confirmed in *A. carterae* and found in 2 other dinoflagellates species, *Amphidinium operculatum* and *Scrippsiella trochoidea*.

The first index used, Rubisco per unit chlorophyll, when expressed in mgC (mg chl $a+b+c$)$^{-1}$ h^{-1}, showed large variations, between 0.1 and 1.1, for algae at the same exponential growth phase. These values are similar to those reported in previous studies (Kelly 1989). The wide range of values recorded indicate that there are interspecific and/or physiological differences
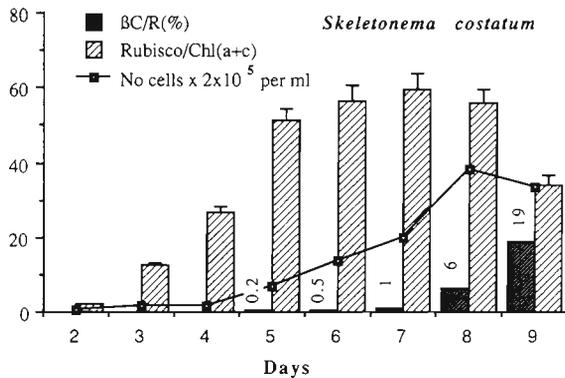
Fig. 2. *Skeletonema costatum*. Variations in Rubisco activity per unit chlorophyll($a+c$) (R/Chl) in nmol CO_2 (µg chl $a+c$)$^{-1}$ h^{-1} and βC/R ratio during growth of a culture. Each point is the mean value from 12 measurements. Experimental error was ± 7 % for R/Chl

operating during inorganic carbon autotrophic fixation. Inorganic carbon assimilation is also closely related to the light regime experienced by the culture. A 2-fold increase of the R/Chl ratio was noted when cultures of the diatom *Skeletonema costatum* were
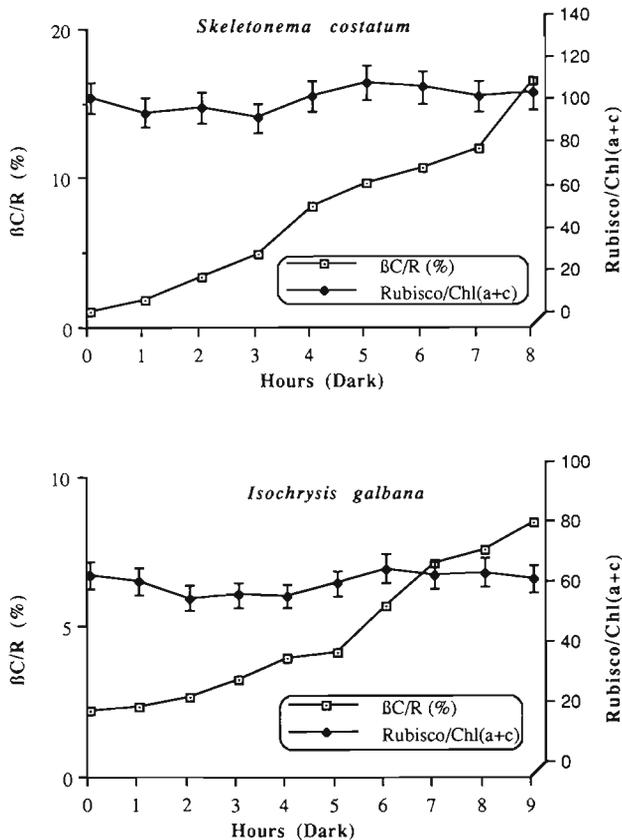

Fig. 3. *Skeletonema costatum* and *Isochrysis galbana*. Rubisco activity per unit chlorophyll ($a+c$) [nmol CO_2 (µg chl $a+c$)$^{-1}$ h^{-1}] and % βC/R ratio following a light dark transition

acclimatized at 40 and 120 µE m^{-2} s^{-1} (Mortain-Bertrand et al. 1988b). Rivkin (1990) has shown a correlation between the Rubisco content of cells and photosynthetic capacity (P_{max}); Orellana et al. (1988) found a similar correlation between photosynthetic rate and Rubisco carboxylase content of cells. On the other hand, Sukenik et al. (1987), working with *Dunaliella tertiolecta* showed that Rubisco/PSII was the most important ratio to consider.

The second index, the βC/R(%) ratio, also shows a wide range of values between the different species. In autotrophic cells, β-carboxylation remained low (βC/R(%) < 40). The βC/R(%) ratio increased significantly when R/Chl began to decrease at the end of the growth of a culture of *Skeletonema costatum*. This ratio increased when cells were not in optimal growth conditions and/or in a poor physiological state. In such a case an inverse relationship was observed between R/Chl and βC/R(%). β-carboxylation may be a compensatory mechanism. High rates of β-carboxylation have previously been observed in *S. costatum* with suboptimal conditions of light and temperature (Mortain-Bertrand et al. 1988b) and by Guy et al. (1989) during NH_4^+ assimilation by N-limited green algae. For autotrophic cells, such as diatoms and cyanobacteria grown in light, β-carboxylation occurs using a substrate (phosphoenolpyruvate) derived from the Calvin-Benson cycle; in the dark, phosphoenolpyruvate is obtained from the dissimilation of reserve carbohydrate as is also the case in brown macroalgae (Kremer 1981, Mortain-Bertrand et al. 1988a).

The heterotrophic dinoflagellate *Crypthecodinium cohnii*, grown on an organic medium, incorporates inorganic carbon in the dark through PEPCK activity. The β-carboxylase activity is of the same order of magnitude as Rubisco activity for autotrophic cells in our experimental assays conditions (Table 1). We verified (unpubl. data) that inorganic carbon assimilation by intact cells took place in the dark. Strict heterotrophic species may incorporate inorganic carbon (Levedahl 1968, Raven 1974). In heterotrophically grown *Euglena gracilis*, inorganic carbon assimilation occurred through PEPCK activity (Briand et al. 1981, Peak & Peak 1981, Pönsgen-Schmidt et al. 1988). However, for heterotrophic cells, information is lacking about the origin of phosphoenolpyruvate. It is probably derived from reserve carbohydrate or supplied by the incorporation of external organic substances. These organic substances are presumably also used to fulfill the energy and reductive requirements of the alga (Barton et al. 1991, Lewitus & Caron 1991a).

For the other species of dinoflagellates and *Pleurochrysis pseudoroscoffensis*, inorganic carbon assimilation by β-carboxylation was greater than through Rubisco activity. These species were cultivated in light

on inorganic medium but their βC/R(%) ratio is similar to that of the heterotrophic species and thus they probably also have heterotrophic potential (Richardson & Fogg 1982, Rivkin et al. 1984, Amblard 1991). The wide range in the observed βC/R(%) ratio confirms that in phytoplankton there may exist a continuum between autotrophy and heterotrophy, corroborating results recently obtained using other methods by Lewitus & Caron (1991a, b).

Primary production measured using the radiocarbon (^{14}C) method give a global measure of inorganic carbon fixation without differentiating between the relative contributions of the 2 metabolic pathways concerned, i.e. β-carboxylation and the Calvin-Benson cycle. This is important because the 2 pathways are not equivalent when considered from a carbon budget point of view. Rubisco fixation uses light as an energy source and is responsible for gross production; on the other hand, β-carboxylation also fixed inorganic carbon but uses metabolites synthesized by other pathways in the cell or from the external medium as an energy source. Carboxylase activity measurements give the percentage of carbon assimilated by the 2 pathways (autotrophic Rubisco activity pathway and β-carboxylation), especially inorganic carbon fixation by β-carboxylation in heterotrophs. This produces a clearer picture of the processes associated with inorganic carbon assimilation *in situ*. This method also removes the need to carry out incubations. Our results show that rapid changes in environmental conditions such as light-dark transitions can be detected by following their impact on the carbon metabolism of the cells. This technique can be used easily both in coastal areas (Descolas-Gros & Fontugne 1990, Collos et al. 1992) and in open ocean, even in oligotrophic situations (Descolas-Gros & Fontugne 1988, Fontugne et al. 1991).

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Erratum

● On p. 165, Table 1:
Read <u>PEP carboxykinase</u> instead of PEP carboxylase for the
following three species of Prymnesiophyceae: *Emiliana huxleyi,
Isochrysis galbana*, and *Pavlova lutheri*