Photosynthetic Carbon Assimilation in the Coccolithophorid Emiliania huxleyi (Haptophyta): Evidence for the Predominant Operation of the C₃ Cycle and the Contribution of β-Carboxylases to the Active Anaplerotic Reaction

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The coccolithophorid Emiliania huxleyi (Haptophyta) is a representative and unique marine phytoplankton species that fixes inorganic carbon by photosynthesis and calcification. We examined the initial process of photosynthetic carbon assimilation by analyses of metabolites, enzymes and genes. When the cells were incubated with a radioactive substrate (2.3 mM NaH¹⁴CO₃) for 10 s under illumination, 70% of the ¹⁴C was incorporated into the 80% methanol-soluble fraction. Eighty-five and 15% of ¹⁴C in the soluble fraction was incorporated into phosphate esters (P-esters), including the C₃ cycle intermediates and a C₄ compound, aspartate, respectively. A pulse–chase experiment showed that ¹⁴C in P-esters was mainly transferred into lipids, while [¹⁴C]aspartate, [¹⁴C]alanine and [¹⁴C]glutamate levels remained almost constant. These results indicate that the C₃ cycle functions as the initial pathway of carbon assimilation and that β-carboxylation contributes to the production of amino acids in subsequent metabolism. Transcriptional analysis of β-carboxylases such as pyruvate carboxylase (PYC), phosphoenolpyruvate carboxylase (PEPC) and phosphoenolpyruvate carboxykinase (PEPCK) revealed that PYC and PEPC transcripts were greatly increased under illumination, whereas the PEPCK transcript decreased remarkably. PEPC activity was higher in light-grown cells than in dark-adapted cells. PYC activity was detected in isolated chloroplasts of light-grown cells. According to analysis of their deduced N-terminal sequence, PYC and PEPC are predicted to be located in the chloroplasts and mitochondria, respectively. These results suggest that E. huxleyi possesses unique carbon assimilation mechanisms in which β-carboxylation by both PYC and PEPC plays important roles in different organelles.

Keywords: β-Carboxylation • Coccolithophorid • Emiliania huxleyi (Haptophyta) • Phosphoenolpyruvate carboxylase • Photosynthetic carbon fixation • Pyruvate carboxylase.

Abbreviations: BSA, bovine serum albumin; CA, carbonic anhydrase; CCM, carbon-concentrating mechanism; DIC, dissolved inorganic carbons (CO₂, HCO₃⁻ and CO₃²⁻); EST, expressed sequence tag; MA-ESM, Marine Art SF enriched with Erd–Schreiber medium; MDH, malate dehydrogenase; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; P-esters, phosphate esters as primary metabolites; 3-PGA, 3-phosphoglycerate; PYC, pyruvate carboxylase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; TLC, thin-layer chromatography.

The nucleotide sequences reported in this paper have been submitted to DDBJ/GenBank/EMBL databanks under accession numbers AB461362 (EhPEPC) and AB461363 (EhPYC).

Introduction

Coccolithophorids of the Haptophyta are unicellular calcifying algae that are widely distributed in the world’s oceans. The most abundant coccolithophorid, Emiliania huxleyi, frequently forms huge blooms that cover > 100,000 km² of the ocean surface. During such blooms, the alga produces massive organic and inorganic products by photosynthesis and calcification, and parts of such products sink to the bottom of the ocean. Thus, E. huxleyi plays an important role as a biological pump to transport carbon from the surface into marine sediment (Riebesell et al. 2000, Buitenhuis et al. 2001).
The dominant form of dissolved inorganic carbon (DIC) in the ocean is bicarbonate, and the concentration of CO$_2$, a substrate for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), is very low (approximately 10 µM). To avoid CO$_2$ limitation in photosynthesis, most marine algae induce active DIC uptake systems and/or carbonic anhydrase (CA), which facilitate DIC utilization by algal cells. Using the CO$_2$-concentrating mechanism (CCM), algal cells exhibit high affinity for DIC to stimulate CO$_2$ fixation under CO$_2$-limiting conditions (for reviews, see Badger et al. 1998, Kaplan and Reinhold 1999, Miyachi et al. 2003). According to previous studies, the photosynthetic affinity of E. huxleyi cells for DIC is lower than that of other microalgae such as Chlorella and Chlamydomonas, suggesting low or no activity of CCM in E. huxleyi (Sekino and Shiraiwa 1994, Sekino et al. 1996, Shiraiwa et al. 2004). Recent analysis of γ- and δ-type CAs in E. huxleyi revealed that the expression of these transcripts showed no relationship to DIC availability of the cells (Soto et al. 2006). This is consistent with previous results showing that general CA inhibitors did not affect photosynthetic DIC utilization and fixation (Sekino and Shiraiwa 1994). Therefore, unlike other microalgae, we conclude that CA does not play a significant role in carbon acquisition by E. huxleyi.

From many studies on primary photosynthetic carbon metabolism, it is believed that the operation of the Calvin–Benson cycle (C$_3$ cycle) is predominant in algae. However, recent papers have reported evidence for the operation of C$_4$ photosynthesis as an alternative CCM in a unicellular marine diatom (Reinfelder et al. 2000, Reinfelder et al. 2004, Robert et al. 2007, McGinn and Morel 2008). In the model of carbon acquisition in diatoms, the initial carboxylation reaction is catalyzed by cytosolic phosphoenolpyruvate carboxylase (PEPC), and the products transferred into the chloroplast are subsequently decarboxylated by chloroplastic phosphoenolpyruvate carboxykinase (PEPCK; Reinfelder et al. 2004, McGinn and Morel 2008). In E. huxleyi, based on the presence of multiple numbers of PEPCK transcripts in the expressed sequence tag (EST) library, a possible contribution of PEPCK to the carbon acquisition process was suggested (Wahlund et al. 2004). However, contrary to the extensive kinetic analysis of DIC utilization (for reviews, see Paasche 2001, Shiraiwa 2003), few studies have investigated the primary mechanism of carbon assimilation and metabolism in E. huxleyi. Thus, the actual pathway and enzymes involved in photosynthetic carbon assimilation are currently unknown in E. huxleyi.

The analysis of carbon assimilation in E. huxleyi is important not only to understand physiological properties of the alga, but also to establish the wide diversity of carbon metabolism, since haptophyte algae are derived from secondary endosymbiosis (Bhattacharya et al. 2004). Considering their phylogenetic background, it is expected that coccolithophorids have distinct characteristics in primary carbon metabolism compared with primary symbiotic algae and higher plants.

In the present study, we analyzed the initial products of photosynthesis using $^{14}$C tracer techniques. Based on experimental evidence on metabolic products, transcriptional regulation and changes in enzymatic activity of β-carboxylation enzymes, we present a model of primary carbon metabolism in a coccolithophorid, E. huxleyi. Finally, we clearly show that the C$_3$ cycle is the dominant pathway in photosynthesis and that the active anaplerotic β-carboxylation reaction to produce C$_4$ compounds is also concomitantly operating in E. huxleyi.

Results
Identification of initial products of photosynthesis

We first compared the rates of carbon fixation in the light and in the dark. The rate of photosynthetic carbon fixation in the presence of DIC (final concentration, 2.3 mM) under light-saturated photosynthesis was 100 times higher than that of dark carbon fixation (Fig. 1). Although significant activity of dark carbon fixation was reported in some algae (Akagawa et al. 1972, Kremer and Küppers 1977, Kremer 1981), dark carbon fixation is negligibly low in E. huxleyi (Fig. 1).

During continuous $^{14}$C labeling of photosynthetic products, where NaH$_2$CO$_3$ was added as a carbon source, $^{14}$C was linearly incorporated into both 80% methanol-soluble and -insoluble fractions over 120 s of photosynthesis, and 65–70%
of total radioactivity was incorporated into the soluble fraction throughout the experiment (Fig. 2A). 14C in every compound in the soluble fraction increased with time immediately or after a short lag phase (Fig. 2B). The major 14C products in the 80% methanol-soluble fraction after 10 s of photosynthesis were phosphate esters (P-esters, 85%), including 3-phosphoglycerate (3-PGA) and other intermediates of the C3 cycle, and aspartate (15%). The percentage of P-esters decreased with time, from 85 to 45% over 120 s, while that of other compounds increased or was almost constant, suggesting that P-esters are the primary products (Fig. 2C).

To confirm the results from the continuous labeling experiments, we performed a pulse–chase experiment under the same conditions. After a 30 s pulse labeling with 14C during photosynthesis in the presence of 2.3 mM NaH14CO3, a subsequent chase period was started by the addition of a 50 times higher concentration of non-radioactive bicarbonate that was added in order to dilute the specific radioactivity of the radioactive substrate. Although a slight increase in total 14C fixation was observed in the first 20 s of the chase period, the total 14C in the cells remained unchanged in the subsequent chase period (Fig. 3A). As shown in Fig. 3B and C, 14C in P-esters was subsequently transferred into C4 compounds such as aspartate, other amino acids and lipids. This pattern was coincident with that of the continuous 14C labeling experiments shown in Fig. 2. These 14C labeling patterns clearly indicate that the C3 cycle is the initial path of carbon in photosynthesis of E. huxleyi. The C4 compound (aspartate) was produced subsequently from the C3 cycle by β-carboxylation reactions, but not as the initial product that can be observed in C4 photosynthesis.

Effect of light on gene expression of β-carboxylases

Although the intermediates of the C3 cycle were dominantly labeled with 14C as primary products of photosynthesis, a significant portion (approximately 15% of the soluble metabolites) of 14C was incorporated into aspartate via β-carboxylation (Fig. 2). In order to identify the β-carboxylases involved in the active anaplerosis, the effect of light on the expression of three β-carboxylase genes was investigated. Specific probes corresponding to PYC (pyruvate carboxylase), PEPC and PECK genes for Northern blotting analysis were generated based on EST sequences (see Materials and Methods). Levels of the transcripts were determined in 24 h dark-adapted cells and the subsequently illuminated cells. In the dark-adapted cells (time 0 in Fig. 4), PYC transcripts were not detectable while PEPC and PECK transcripts were clearly observed. When the dark-adapted cells were transferred to illuminated conditions, both PYC and PEPC transcripts obviously accumulated for the first 1 h (Fig. 4A, B), although accumulation was more apparent in PYC transcripts than in PEPC transcripts. In contrast to the increase in levels of PYC and PEPC transcripts in the light, the abundance of the PECK transcript was greatly decreased by illumination (Fig. 4C). Such expression patterns of PYC and PEPC genes strongly suggest the active contribution of the enzymes PYC and PEPC to the active anaplerotic β-carboxylation reactions during photosynthesis.

Fig. 2 Changes in 14C labeling of metabolites during 120 s of photosynthesis. The experimental conditions were as described in Fig. 1. (A) 14C incorporation into 80% methanol-soluble (filled squares) and -insoluble (filled triangles) fractions. Total fixation (filled circles) represents the sum of that in the soluble and insoluble fractions. (B) Time courses of 14C incorporation into major products in the soluble fraction. (C) Percentage distribution of 14C among soluble compounds. P-esters (open triangles), aspartate (open circles), alanine (open squares), glutamate (open diamonds) and lipids (crosses). The values are the mean of duplicate experiments in the same culture. The same pattern was confirmed in another independent culture.
Fig. 3 Changes in $^{14}$C incorporation into initial products of photosynthesis in a pulse–chase experiment. After $^{14}$C pulse labeling for 30 s, the chase phase was initiated by addition of 100 mM of non-radioactive sodium bicarbonate to dilute the specific activity of the substrate (NaH$^{14}$CO$_3$). (A) Incorporation of $^{14}$C into 80% methanol-soluble (filled squares) and -insoluble (filled triangles) fractions during the chase period. Total (filled circles) is the sum of the soluble and insoluble fractions. (B) Incorporation of $^{14}$C into major products in the soluble fraction.

Fig. 4 Effect of light and dark on levels of PYC (A), PEPC (B) and PEPCK (C) transcripts revealed by Northern blot analysis. Cells dark adapted for 24 h were transferred into light or darkness at 0 h, and aliquots of cells were taken for RNA extraction at arbitrary intervals.

Comparison of β-carboxylase activities in light-grown and dark-adapted cells

PEPC catalyzes β-carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate (OAA) in the presence of HCO$_3^-$ and Mg$^{2+}$ (or Mn$^{2+}$). When MgCl$_2$ was omitted from the reaction mixture, the activity decreased, but significant activity still remained due to the contamination with Mg$^{2+}$ (final concentration, 0.5 mM) which was carried over from the extraction buffer. Therefore, in the PEPC assay, a reaction without PEP was used as a blank. When MgCl$_2$ was replaced with MnCl$_2$, the activity was decreased to 39% of that of the complete reaction mixture (Table 1). PEPCK catalyzes β-carboxylation of PEP to OAA in the presence of Mn$^{2+}$ and CO$_2$. When MnCl$_2$ and PEP were individually omitted, the activities were

(C) Percentage distribution of $^{14}$C among soluble compounds. P-esters (open triangles), aspartate (open circles), alanine (open squares), glutamate (open diamonds) and lipids (crosses). The values are means of duplicate experiments in the same culture. The same pattern was confirmed in another independent culture.
decreased to 11 and 7% of that in the complete mixture, respectively (Table 1). However, 30% of the activity was detected even in the absence of ADP (Table 1). Since the composition of ADP-omitted PEPCK assay mixture was the same as that of Mn-containing PEPC assay mixture, the remaining activity was considered to be due to Mn-dependent PEPC activity. Therefore, to calculate the actual PEPCK activity, Mn-dependent PEPC activity that was simultaneously carried out as a blank assay was subtracted from the total PEPCK activity. PYC catalyzes $\beta$-carboxylation of pyruvate to OAA in the presence of CO$_2$, Mg$^{2+}$, ATP and pyruvate. When we used crude cell extracts for the PYC assay, its activity was not detected (data not shown). However, the activity of pyruvate-dependent carbon fixation was definitely detected in the extracts of roughly purified chloroplasts although the activity was still low for unknown reasons (Tables 1 and 2).

Although we did not examine the effect of ATP and Mg$^{2+}$, the pyruvate-dependent carbon fixation activity was completely inhibited by the addition of avidin which is known as an inhibitor of biotin enzymes such as PYC, indicating that the activity is due to PYC.

The effect of light on the activity of $\beta$-carboxylase was examined by comparing the activities in light-grown cells and 24 h dark-adapted cells (Table 2). When crude extracts were used for the enzyme assay, PEPC activity in light-grown cells was five times higher than that in 24 h dark-adapted cells. On the other hand, PEPCK activity in light-grown cells was 60% of that in dark-adapted cells (Table 2).

**Prediction of the subcellular localization of PYC and PEPC**

Since PYC and PEPC transcripts increased under illumination, both enzymes were considered to be responsible for active anaplerosis during photosynthesis in *E. huxleyi*. In order to speculate on the subcellular localization of PYC and PEPC, we isolated the full-length cDNAs of PYC and PEPC, and analyzed their deduced N-terminal sequences. In higher plants, relatively similar targeting sequences are used to target into chloroplasts or mitochondria, making it somewhat difficult to predict the correct localization. However, the chloroplast targeting sequences of haptophyte algae are distinct from those in higher plants since they have evolutionarily acquired their chloroplast via secondary endosymbiosis. As a consequence of such evolutionary development, the chloroplasts of haptophyte algae are surrounded by four membranes, of which the outermost membrane is composed of the endoplasmic reticulum. Therefore, nuclear-encoded chloroplast proteins contain bipartite targeting sequences consisting of a signal peptide followed by a transit peptide-like domain (Ishida 2005). Thus, for prediction of the chloroplast targeting sequence, SignalP (Bendtsen et al. 2004) can be used as a standard method. The transit peptide-like domain subsequent to the signal peptide can be predicted by ChloroP (Emanuelsson et al. 1999) with low sensitivity. However, the mitochondrial pre-sequences of haptophyte algae are similar to those in other eukaryotes. Thus TargetP

### Table 1 Substrate dependency of PYC, PEPC and PEPCK

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction used for assay</th>
<th>Reaction mixture</th>
<th>Rate of carbon fixation a (nmol mg protein$^{-1}$ min$^{-1}$)</th>
<th>Relative rate of carbon fixation (% of complete reaction mixture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYC</td>
<td>Homogenates of isolated chloroplasts b</td>
<td>Complete</td>
<td>1.11 ± 0.34</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–Pyruvate</td>
<td>0.16 ± 0.09</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ Avidin c</td>
<td>0.17 ± 0.04</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–Pyruvate + Avidin c</td>
<td>0.14 ± 0.05</td>
<td>13</td>
</tr>
<tr>
<td>PEPC</td>
<td>Crude extract</td>
<td>Complete</td>
<td>8.61 ± 0.31</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–MgCl$_2$ d</td>
<td>3.36 ± 0.09</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–PEP</td>
<td>0.57 ± 0.02</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–MgCl$_2$ d , + MnCl$_2$</td>
<td>3.39 ± 0.13</td>
<td>39</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Crude extract</td>
<td>Complete</td>
<td>11.01 ± 0.34</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–MnCl$_2$</td>
<td>1.21 ± 0.01</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–ADP</td>
<td>3.39 ± 0.13</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–PEP</td>
<td>0.71 ± 0.04</td>
<td>7</td>
</tr>
</tbody>
</table>

a Enzymatic activities were determined by measuring substrate-dependent $^1$C fixation as described in Materials and Methods. Mean values ± SD of three (in PEPC and PEPCK) or six (in PYC) measurements are shown.

b PYC activity was not detected in the crude cell extracts, but was detected in homogenates of isolated chloroplasts.

c Avidin was added into the reaction mixture at the final concentration of 2 U ml$^{-1}$.

d The reaction mixture in the ‘–Mg’ assay contains 0.5 mM MgCl$_2$ which was carried over from the extraction buffer.
Emanuelsson et al. (2000) can be used for the prediction of pre-sequences. The deduced polypeptide of \textit{E. huxleyi} PYC had significant homology to PYCs from various organisms such as \textit{Saccharomyces cerevisiae} PYC1 (accession No. NP_011453, 50% identity) and \textit{Mus musculus} PYC (NP_032823, 50% identity). By analysis of the N-terminal sequence of \textit{E. huxleyi} PYC, a bipartite chloroplast targeting sequence consisting of the signal peptide and a subsequent transit peptide-like domain (TP) in EhPYC are underlined by black and gray lines, respectively. SignalP and ChloroP were used for the prediction of SP and TP, respectively. The putative mitochondrial targeting pre-sequence in \textit{M. musculus} mitochondrial PYC (MmPYC) is marked with a dashed underline. The sequences shown in this figure are \textit{Corynebacterium glutamicum} PYC (CgPYC, NP_599921), \textit{Saccharomyces cerevisiae} cytosolic PYC1 (ScPYC1, NP_011453) and \textit{M. musculus} PYC (NP_032823).

The deduced amino acid sequence of \textit{E. huxleyi} PYC had a ‘QNTG’ motif, which is characteristic of plant-type PYCs (Mamedov et al. 2005). The \textit{E. huxleyi} PEPC showed significant homology to other plant-type PEPCs, such as \textit{Arabidopsis thaliana} PEPC3 (NP_188112, 41% identity) and \textit{Chlamydomonas reinhardtii} PEPC1 (AAS01722, 41% identity). In the analysis of the N-terminal targeting sequence of PEPC, a mitochondrial targeting pre-sequence was predicted by TargetP with low reliability (reliability class 4). Another prediction program, iPSORT (Bannai et al. 2002), also supports the existence of a mitochondrial pre-sequence. The bipartite chloroplast targeting sequence was not predicted by analysis with SignalP and ChloroP; therefore, the \textit{E. huxleyi} PEPC is suggested to be located in the mitochondria.

Although we isolated full-length cDNAs based on approximately 20,000 EST sequences published by Wahlund et al. (2004), it is conceivable that other genes of the three \(\beta\)-carboxylation enzymes exist in the genome of \textit{E. huxleyi}. The genome of \textit{E. huxleyi} was estimated to be 220 Mbp (Grossman 2005), which is larger than other eukaryotic algae such as the diatom \textit{Thalassiosira pseudonana} (32 Mbp) and

### Table 2
Comparison of \(\beta\)-carboxylase activities between light-grown cells and 24 h dark-adapted cells of \textit{E. huxleyi}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction used for assay</th>
<th>Enzyme activity (nmol carbon fixation mg protein(^{-1}) min(^{-1})) in cells adapted to</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYC</td>
<td>Homogenates of isolated chloroplasts*</td>
<td>Light: 0.95 ± 0.34(^{b}) Dark: –</td>
</tr>
<tr>
<td>PEPC</td>
<td>Crude cell extracts</td>
<td>Light: 7.8 ± 0.27 Dark: 1.7 ± 0.19</td>
</tr>
<tr>
<td>PEPC</td>
<td>Crude cell extracts</td>
<td>Light: 7.9 ± 0.23 Dark: 13.1 ± 0.72</td>
</tr>
</tbody>
</table>

Enzymatic activities were determined by measuring substrate-dependent \(^{14}\)C fixation as described in Materials and Methods. Mean values ± SD of three (in PEPC and PEPK) or six (in PYC) measurements are shown. –, not determined.

*PYC activity was not detected in the crude cell extracts, but was detected in homogenates of isolated chloroplasts.

\(^{b}\)PYC activity in the isolated chloroplasts was completely (97%) inhibited by addition of avidin (2 U ml\(^{-1}\)), a specific inhibitor of biotin enzymes such as PYC.

### Fig. 5
Comparison of the deduced amino acid sequence of \textit{E. huxleyi} PYC and PYCs from other organisms. (A) Comparison of the pyruvate binding motif (open box) conserved in PYCs from various organisms. The alignment was constructed by the ClustalW program. Identical amino acids are shaded by gray boxes. (B) Analysis of the targeting signal on the N-terminal end of \textit{E. huxleyi} PYC. The N-terminal sequences were right-aligned to the left of the conserved amino acids (A and N). The predicted signal peptide (SP) and transit peptide-like domain (TP) in EhPYC are underlined by black and gray lines, respectively. SignalP and ChloroP were used for the prediction of SP and TP, respectively. The putative mitochondrial targeting pre-sequence in \textit{M. musculus} mitochondrial PYC (MmPYC) is marked with a dashed underline. The sequences shown in this figure are \textit{Corynebacterium glutamicum} PYC (CgPYC, NP_599921), \textit{Saccharomyces cerevisiae} cytosolic PYC1 (ScPYC1, NP_011453) and \textit{M. musculus} PYC (NP_032823).
the green alga *C. reinhardtii* (121 Mbp). Currently, the genome sequencing of *E. huxleyi* is in progress and therefore we cannot completely exclude the possibility of the existence of other genes for the enzymes we characterized in this study. The complete sequence of the *E. huxleyi* genome is essential to complete identification of the respective genes for these active enzymes.

**Discussion**

We characterized the initial process of photosynthetic carbon assimilation in the coccolithophorid *E. huxleyi* using 14C tracer experiments. In *E. huxleyi*, a relatively high percentage (approximately 30%) of 14C was incorporated into the 80% methanol-insoluble fraction in this study (Figs. 2, 3). Previously, similar results showing a high level of production of the insoluble fraction were also reported in a diatom and a cryptophyte alga (Suzuki and Ikawa 1985, Suzuki and Ikawa 1993), and the major alcohol-insoluble 14C-labeled product of the diatom was β-1,3-glucan (Suzuki and Ikawa 1993). Since *E. huxleyi* also produces similar β-polyglucans that contain β-(1→3) and β-(1→6) linkages (Vårum et al. 1986), the insoluble 14C-labeled compounds in *E. huxleyi* are considered to be β-polyglucans.

Analysis of 80% methanol-soluble metabolites revealed that the dominant initial photosynthetic products of *E. huxleyi* are P-esters, namely intermediates of the C4 cycle and C3 compounds (aspartate). In general, the production of C4 compounds is explained by the operation of C4 photosynthesis or anaplerotic β-carboxylation reactions to replenish carbon skeletons for nitrogen assimilation. The 14C labeling pattern supported the contribution of anaplerotic β-carboxylation reactions to the production of C4 compounds because: (i) 14C of P-esters, predominantly produced as an initial product, was subsequently transferred into other compounds such as amino acids and lipids; and (ii) no decrease in 14C of C4 compounds (aspartate) was observed (Figs. 2, 3). Therefore, we concluded that the C4 cycle is predominantly driven as the initial pathway of carbon assimilation, and that the C4 compound aspartate was produced by the anaplerotic β-carboxylation reaction that is simultaneously driven when the C3 cycle is also active. The production of glutamate also supports the operation of an active anaplerotic pathway since glutamate is produced from 2-oxoglutarate, an intermediate of the tricarboxylic acid cycle, by coupling with nitrogen assimilation during photosynthesis.

According to our previous study (Sekino and Shiraiwa 1994), the concentration of DIC used in this study (2.3 mM) is limiting to CO2 assimilation in this alga. Therefore, the present results on the analysis of primary photosynthetic products indicate that the C3 cycle is predominantly functioning in *E. huxleyi* even under such CO2-limiting conditions where the operation of the C4 pathway is generally preferable to exert high activity. The increase of total 14C fixation during the first 20 s of the chase period was probably caused by the addition of a high concentration of substrate, which is expected to enhance carbon assimilation under CO2-limiting condition.

Compared with other algae such as *Chlorella* and *Dunaliella* that only possess the C3 pathway (Hogetsu and Miyachi 1979, Aizawa et al. 1985), the amounts of C4 compounds produced were significantly high in *E. huxleyi*, although the C4 cycle is the dominant main carbon fixation pathway (Figs. 2, 3). The active production of C4 compounds via anaplerotic β-carboxylation was also reported in brown algae. The 14C labeling pattern of the C4 compound, aspartate, in *E. huxleyi* (Fig. 2) was similar to that in brown algae (Kremer and Kuppers 1977). In the case of brown algae, β-carboxylation contributes to both photosynthetic and dark carbon fixations, and hence the rate of dark carbon fixation reached 4–16% of photosynthetic carbon fixation (Kremer and Kuppers 1977). On the other hand, the rate of dark carbon fixation was only 1% of photosynthetic carbon fixation in *E. huxleyi* (Fig. 1). Therefore, unlike brown algae, the anaplerotic β-carboxylation reactions are mostly light-dependent processes in *E. huxleyi*. Such light-dependent metabolism was also observed in nitrate assimilation in *E. huxleyi*, whereas in other algae, such as brown algae and diatoms, a high rate of nitrate assimilation can proceed even in the dark (Needoba and Harrison 2004, Young et al. 2007).

Gene expression analysis demonstrated that PYC and PEPC transcripts increased, whereas PEPC transcripts decreased under illumination (Fig. 4). These results suggest the possibility of the dominant contribution of PYC and PEPC to the light-dependent β-carboxylation in *E. huxleyi*.

The change in PEPC activity between light and dark was greater than that in the levels of PEPC transcripts (Fig. 4, Table 2). PEPC seems to be regulated at both transcriptional and post-transcriptional levels. The post-transcriptional regulation of PEPC activity by reversible phosphorylation is well characterized in higher plants (Izui et al. 2004). However, the phosphorylation motif was absent in *E. huxleyi* PEPC according to amino acid sequence analysis (data not shown). Among eukaryotic PEPCs, lack of the phosphorylation motif was also reported in diatoms (Granum et al. 2005) and *Chlamydomonas* (Mamedov et al. 2005). This evidence demonstrates that the regulation of algal PEPC should be distinct from that in higher plants.

PEPC has been well characterized in higher plants and algae. On the other hand, little information is available on PYC at both gene and protein levels despite the wide distribution of its genes among eukaryotic algae. The overall primary structure of *E. huxleyi* PYC is quite similar to previously characterized PYCs in other organisms (Supplementary Fig. S1), and three functional domains common in biotin enzymes, namely the biotin carboxylation domain, the transcarboxylation...
domain and the biotin carboxyl carrier domain, are well conserved in *E. huxleyi* PYC. Since the *E. huxleyi* PYC has a pyruvate binding motif (Fig. 5A), this enzyme is clearly distinguished from other biotin enzymes such as acetyl-CoA carboxylase. The present study is the first report showing the presence of regulation of PYC transcription by light. In addition, based on the N-terminal targeting sequence (Fig. 5B), PYC is suggested to be located in the chloroplast of *E. huxleyi*. The predicted localization of *E. huxleyi* PYC is unique since PYC is known as a cytosolic or mitochondrial protein. Therefore, experimental confirmation of its localization will be necessary in the future. Since PYC activity was not well detected in crude cell extracts, we attempted to isolate chloroplasts from *E. huxleyi* by the Percoll gradient method. The PYC activity was then definitely detected in partially purified chloroplasts, although the activity was not high (Table 1). The reasons for such low activity are still unclear, although the presence of some inhibitory factors in the extracts may suppress PYC activity.

PEPCK is well known to function in CO₂ fixation in photosynthesis as a major anaplerotic enzyme in various algae (Kremer and Küppers 1977, Cabello-Pasini and Alberte 2001, Cabello-Pasini et al. 2001). However, the transcriptional analysis and its enzymatic assay performed in this study did not support such evidence on the contribution of PEPCK to light-dependent anaplerotic β-carboxylation in *E. huxleyi* since both transcription and enzymatic activities were greatly increased in dark-adapted cells and obviously decreased in light-adapted cells (Fig. 4 and Table 2). The increase in PEPCK activity during light to dark transition has also been reported in another haptophyte alga, *Isochrysis galbana* (Descolas-Gros and Oriol 1992). Thus, unlike other algae, PEPCK in haptophyte algae probably plays an important role in the dark, but not in the light.

In conclusion, in this study we show that the C₃ cycle is the initial pathway for carbon assimilation, although a light-dependent anaplerotic β-carboxylation reaction produces C₄ compounds simultaneously. Our transcriptional analysis indicated the contribution of both PYC and PEPC to such C₄ compound production, while PEPC and/or PEPCK are currently considered as the major anaplerotic enzyme(s) in algae in general (Fig. 6).

As shown in Fig. 6, photosynthetic carbon assimilation metabolism seems to be complicated in *E. huxleyi* due to the multiplicity and uniqueness of the predicted localization of the β-carboxylases. PYC located in the chloroplasts may play an important role in the biosynthesis of proteins and nucleic acids since OAA is known as a precursor of the aspartate family amino acids, purines and pyrimidines. We have no direct evidence on the localization of aspartate aminotransferase, and OAA and malate were not detected in ¹⁴C tracer experiments. However, OAA produced by PYC and PEPC may be immediately converted into aspartate by aspartate aminotransferase. Aspartate aminotransferase may be located in both chloroplasts and mitochondria where PYC and PEPC are predicted to be located, respectively. Interestingly, gene products of three enzymes that convert 3-PGA to pyruvate, namely phosphoglycerate mutase, enolase and pyruvate kinase, are also predicted to have bipartite chloroplast targeting sequences (Supplementary Table S1). Thus, pyruvate, the substrate of PYC, seems to be produced from 3-PGA via 2-PGA and PEP in the chloroplast of *E. huxleyi* (Fig. 6). This feature is different from the case in chloroplasts of higher plants in which PEP, a precursor of pyruvate, is supplied from the cytosol into the chloroplast through the function of a PEP/phosphate translocator (Streatfield et al. 1999). On the other hand, PEPC is predicted to be localized in mitochondria; therefore, triose phosphate exported from the chloroplast is presumably converted to PEP in the cytosol, and PEP is then provided as the substrate for PEPC (Fig. 6). Hence, the localization of enzymes in carbon metabolism, especially those involved in the chloroplasts, in *E. huxleyi* seems to be different from that in higher plants. To confirm the unique primary carbon metabolism in the coccolithophorids, studies on the subcellular localization of primary carbon fixation and metabolism will be essential, as well as the biochemical characterization of enzymes that contribute to carbon assimilation.

**Materials and Methods**

**Organism and culture conditions**

The organism used in this study was the coccolithophorid *E. huxleyi* (NIES 837), sometimes called *E. huxleyi* EH 2 (Iwamoto and Shiraiwa 2003). Cells were grown in artificial seawater Marine Art SF (produced by Tomita Seiyaku Co., Ltd.,
Tokushima, Japan, and distributed by Osaka Yakken Co. Ltd., Osaka) enriched with Erd–Schreiber medium (MA-ESM), in which soil extract was replaced with 10 mM sodium selenite according to Danbara and Shiraiwa (1999). Cells used for experiments were grown under constant illumination (100 µmol m⁻² s⁻¹) at 20°C.

Radioisotope labeling of metabolites

Cells in the logarithmic growth phase grown in ordinary air were harvested by centrifugation at 650×g for 10 min and then resuspended in MA-ESM containing 20 mM HEPES-NaOH (pH 8.2), in which DIC had been equilibrated to air before use and therefore contained 2 mM DIC. The cell suspension was kept under illumination (100 µmol m⁻² s⁻¹) at 20°C until use. A commercial glass filtration apparatus (Millipore, Bedford, MA, USA) with a glass filter (GF/F, 25 mm diameter, Whatman, Springfield Mill, UK) was used as a reaction chamber to avoid disturbing the development on thin-layer chromatography (TLC) by contamination from salts in the medium. A 1 ml aliquot of cell suspension was placed on the glass filter and pre-incubated for 1 min under illumination of 120 µmol m⁻² s⁻¹ at 20°C. After pre-incubation, ¹⁴C labeling was started by the addition of an aliquot of NaH¹⁴CO₃ (680 kBq, 2 GBq mmol⁻¹; final concentration, 0.3 mM) to the air-equilibrated MA-ESM already containing about 2 mM cold DIC. Hence, during ¹⁴C labeling the cells were exposed to 2.3 mM DIC. The reaction was terminated by collection of cells on the glass filter through quick removal of medium by suction and subsequent rapid washing of cells with 150 µl of ice-cooled distilled water. The filter was immediately dipped into 5 ml of ice-cooled 80% (v/v) methanol to kill the cells. The time taken for this sampling process was within 5 s. In the pulse–chase experiment, after 30 s of pulselabeling with ¹⁴C, the following chase period was begun by the addition of 100 mM non-radioactive bicarbonate to dilute the radioactivity of the ¹⁴C-labeled substrate.

Analysis of metabolites by two-dimensional TLC

Following extraction in cold 80% (v/v) methanol, cells on the filter were further extracted twice with 5 ml of 80% (v/v) methanol at 70°C for 5 min for complete extraction. Radioactivities in the 80% methanol and in the glass filter were obtained as 80% (v/v) methanol-soluble and -insoluble fractions, respectively. The radioactivity of the fractions was determined using a liquid scintillation counter (LS5000TD, Beckman, Fullerton, CA, USA). The 80% (v/v) methanol-soluble fractions were concentrated by evaporation, and the concentrated sample was resuspended in a small volume of 50% (v/v) ethanol and then applied to the silica gel 70 plate (Wako, Osaka, Japan). Phenol:acetic acid:EDTA:water (47:84:5.5:1.14, by vol.) was used for the first development. A mixture (1:1, v/v) of 1-butanol:water (74:5, v/v) and propionic acid:water (9:11, v/v) was used for the second development. After development, the TLC plate was dried and then put in contact with the imaging plate (Fuji Film, Tokyo, Japan). The radioactive spots detected were quantified by BAS 1800 and Image gauge software (Fuji Film, Tokyo, Japan). The ¹⁴C-labeled metabolites were identified by co-chromatography with authentic compounds. There were no obvious radioactive spots corresponding to malate and OAA on TLC.

Preparation of crude extracts for enzymatic assay

Cells from 3 liters of culture harvested at the logarithmic growth phase were washed with 100 ml of 100 mM EDTA (adjusted to pH 8.0) for decalcification. The cells were further washed with 20 ml of extraction buffer containing 100 mM HEPES-NaOH (pH 7.5), 10 mM MgCl₂, 1 mM Na₂ EDTA, 100 mM KCl, 20% (v/v) glycerol and 2 mM dithiothreitol. The washed cells were resuspended in 4 ml of extraction buffer and subjected to disruption by passing them through a French pressure cell. The crude homogenates were centrifuged at 31,000×g for 20 min. The resulting supernatant was applied onto a PD10 desalting column (GE Healthcare BioSciences, Tokyo, Japan) equilibrated with the extraction buffer and then used for the enzymatic assay as crude extracts. Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay kit, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard.

Enzymatic assay of PYC, PEPC and PEPCK

The activities of PYC, PEPC and PEPCK were determined by measuring substrate-dependent ¹⁴CO₂ (H¹⁴CO₃⁻) fixation. To assay PYC, a reaction mixture containing 100 mM Tris–HCl (pH 8.0), 5 mM MgCl₂, 2 mM ATP, 2 mM dithiothreitol, 0.25 mM NADH, 5 µl⁻¹ malate dehydrogenase (MDH), 100 mM KCl, 0.1 mM acetyl-CoA, 5 mM pyruvate and 0.2 mg protein ml⁻¹ of crude extract was pre-incubated at 25°C for 3 min. The reaction was started by adding 10 mM NaH¹⁴CO₃ (6.8 MBq mmol⁻¹) and terminated by adding acetic acid [final concentration, 10% (v/v)]. A blank assay was performed without pyruvate. After the removal of acid-labile ¹⁴C compounds under an air stream, the ¹⁴C in acid-stable compounds was determined by liquid scintillation counting.

In the PEPC assay, the reaction mixture contained 100 mM HEPES-NaOH (pH 7.0), 5 mM MgCl₂, 0.25 mM NADH, 5 U ml⁻¹ MDH, 2 mM ADP, 5 mM PEP and 0.2 mg protein ml⁻¹ of crude extract, pre-incubated at 25°C for 3 min. The reaction was started by adding 20 mM NaH¹⁴CO₃ (6.8 MBq mmol⁻¹) and terminated by adding acetic acid [final concentration, 10% (v/v)]. To assay PEPC, the reaction mixture was the same as that for PEPC, but MnCl₂ was replaced with MgCl₂, and ADP was omitted. A blank assay was carried out without ADP and PEP for PEPC and PEPC, respectively.
Identification of cDNAs encoding β-carboxylases from the EST database

To obtain specific probes for Northern blotting, we searched for β-carboxylase genes in the EST database. Saccharomyces cerevisiae PYC1 (GenBank accession No. CAA96765), A. thaliana PEPC3 (NP_188112) and A. thaliana PEPC (Q9T074) sequences were used as query sequences for the tBLASTn search against ESTs of E. huxleyi. As a result of the search, we obtained three, one and 10 ESTs that showed significant homology (e-value < 10^-4) with PYC, PEPC and PEPC, respectively (Supplementary Table S2).

A comparison of three PYC-homologous ESTs revealed that two overlapping ESTs (CX778169 and CX771508) correspond to the C-terminal region of S. cerevisiae PYC, while another non-overlapping EST (CV068798) corresponds to the middle of the S. cerevisiae PYC. Using PCR amplification with the primer set pyc2F and pyc2R, we obtained a fragment containing both middle and C-terminal regions. Thus, the three ESTs represent a single mRNA species. We also examined redundancy of PEPC-homologous ESTs by sequence comparison and PCR amplification, and confirmed that all PEPC-homologous ESTs are also derived from a single mRNA. Consequently, we obtained three mRNA sequences corresponding to PYC, PEPC and PEPC. These mRNA sequences were subjected to BLASTx analysis against non-redundant proteins and confirmed to have significant homology (e-value < 10^-4) to the corresponding proteins. Specific probes corresponding to PYC, PEPC and PEPC were then generated by PCR with the primer sets pycF1 and pycR1, ppfF1 and ppfR2, and pckF and pckR, respectively.

Northern blot analysis

Total RNA was extracted from cells harvested in the logarithmic growth phase using a Total RNA Isolation system (Promega, Madison, WI, USA). Samples containing 10μg of total RNA were subjected to electrophoresis on a 1.4% agarose–formaldehyde gel. After electrophoresis, the total RNA in the gel was transferred to a Hybond-N + membrane (GE Healthcare Bio-Sciences, Tokyo, Japan) and fixed on the membrane using a UV cross-linker (FS-800, Funakoshi, Tokyo, Japan). Hybridization was performed at 42°C according to Church and Gilbert (1984). The probes were 32P labeled using the BcaBest labeling kit (TAKARA BIO INC., Otsu, Japan).

Cloning of full-length cDNA encoding PYC and PEPC

Total RNA was extracted from cells harvested in the logarithmic growth phase using a Total RNA Isolation system (Promega). mRNA was isolated using a PolyATtract mRNA Isolation system (Promega). A CapFishing full-length cDNA kit (Seegene, Seoul, Korea) or a SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) was used to obtain full-length cDNAs of PYC and PEPC. Primers used for cloning are described in Supplementary Table S3. For PCR amplification, LA Taq with GC buffer or PrimeSTAR GXL (TAKARA BIO INC.) were used. Amplified PCR fragments were cloned into pGEM T-easy vector (Promega) and sequenced with a DNA sequencer model 3130 with BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA, USA).

Isolation of chloroplasts from E. huxleyi

Emiliania huxleyi cells harvested at the late logarithmic growth phase by centrifugation at 360×g were washed and resuspended in an ice-cold isolation buffer containing 600 mM sorbitol, 1 mM MgCl2, 10 mM KCl, 0.1% BSA and 50 mM HEPES-NaOH, pH 8.0. Cells were then disrupted in a Yeda pressure cell under nitrogen at 3.9 MPa. After filtration of the homogenates through Miracloth, the filtrates were layered onto a discontinuous gradient of 30, 20 and 10% Percoll, and then centrifuged at 2,500×g for 20 min. The chloroplasts obtained at the boundary between the 20 and 30% Percoll fractions were used to assay PYC.

Supplementary data

Supplementary data are available at PCP online.

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