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Translational Regulation of the Large and Small Subunits of Ribulose Bisphosphate Carboxylase/Oxygenase during Induction of the CO_2-Concentrating Mechanism in *Chlamydomonas reinhardtii*[1]

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ABSTRACT

In conditions of limiting external inorganic carbon, the unicellular alga *Chlamydomonas reinhardtii* induces a mechanism to actively transport and accumulate inorganic carbon within the cell. A high internal inorganic carbon concentration enables the cell to photosynthesize efficiently with little oxygen inhibition, even in conditions of limiting external inorganic carbon. A correlation between limiting inorganic carbon-induced induction of the CO_2-concentrating mechanism and decreased synthesis of the large and small subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase has been observed. Cells that had been transferred from elevated CO_2 to limiting CO_2 exhibit transient declines of label incorporation into both subunit polypeptides. We have found that this decrease in synthesis of large and small subunits results from specific and coordinated down-regulation of translation of both subunits possibly resulting, at least in part, from modification of large and small subunit transcripts.

Rubisco (EC 4.1.1.39) catalyzes the first steps of the photosynthetic carbon reduction cycle or the photorespiratory pathway when it reacts with CO_2 or O_2, respectively. Rubisco consists of eight catalytic LSU[3] and eight SSU of undetermined function. Precursor SSU are encoded by a multigene family (*rbc*S) on the nuclear genome and are posttranslationally transported to the chloroplast, where they are processed to mature form by proteolytic removal of an amino terminal transit peptide (reviewed in ref. 20). The large subunit is encoded by the single copy *rbc*L gene of the chloroplast genome and is translated by plastid ribosomes. Assembly of the Rubisco holoenzyme complex occurs in the chloroplast and is aided by Rubisco subunit binding protein (reviewed in ref. 18). Because pools of unassembled subunits do not accumulate in the chloroplast, successful assembly of holoenzyme requires that synthesis of subunits be regulated to ensure that each is present at stoichiometric levels.

Coordinated accumulation of Rubisco subunits may be achieved by regulatory controls at transcription (reviewed in ref. 22), posttranscriptional mRNA stability (9), translation (3), and posttranslational turnover of unassembled polypeptides (19). Within a species, control over synthesis of either subunit may be exerted at more than one level in a hierarchical manner (4, 29), and between species entirely different regulatory controls may act. Control mechanisms used by plants and algae to cause coordinated accumulation of Rubisco subunits in the event of shifts in illumination (3, 4, and reviewed in ref. 28), developmental programs (7, 15, 16), nonsense mutations of the *rbc*L gene (12, 25) or in the event of interference with chloroplast or cytoplasmic protein synthesis (17, 19, 29) have been elucidated.

Because changes in illumination have been shown to result in specific regulation of Rubisco biosynthesis, it should not be surprising that other environmental factors have similar effects. One environmental factor of central importance to the activity of Rubisco is CO_2 concentration, and one well studied system to cope with limiting CO_2 is the CO_2-concentrating mechanism of unicellular algae. *Chlamydomonas reinhardtii* and other algae possess an inducible system that functions to raise intracellular C_i concentrations in conditions of low external C_i (reviewed in ref. 1). High internal C_i concentrations effectively increase the supply of substrate CO_2 for Rubisco and diminish Rubisco oxygenase activity, thereby reducing photorespiration. The system is absent from cells cultured under elevated CO_2 (1–5% CO_2 in air) but is fully operational within 8 h in limiting external CO_2 (5, 23). Capacity for active C_i transport (24, 27) and induction of carbonic anhydrase (2) coincide with increased affinity for C_i in photosynthesis.

C. reinhardtii cells adapting to limiting CO_2 induce carbonic anhydrase and several other polypeptides (23) and, interestingly, specifically depress synthesis and/or accumulation of Rubisco LSU and SSU (6 and this study). Declines in synthesis of LSU and SSU polypeptides coincidental with establishment of the CO_2-concentrating mechanism suggest that *C. reinhardtii* cells somehow down-regulate biosynthesis of Rubisco subunits in a coordinated manner in response to limiting CO_2 concentrations. We have investigated this observation further and determined that these cells control accumulation of both subunits by specifically limiting their translation. This is in marked contrast to the posttranslational control mechanism used in *C. reinhardtii* cells deficient in

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[3] Abbreviations: LSU, large subunit; SSU, small subunit; C_i, inorganic carbon.

chloroplast protein synthesis (19) or with *rbc*L nonsense mutations (25), and demonstrates a second means by which *C. reinhardtii* cells can exert control over Rubisco biosynthesis to achieve coordinated accumulation of its subunits.

MATERIALS AND METHODS

Strains and Culture Conditions

Chlamydomonas reinhardtii wild-type strain 2137 (26) and cell wall-deficient mutant CW-15 (obtained from Dr. R. Togasaki, Indiana University) were cultured in minimal salts medium as previously reported (10). Cells were cultured on an orbital shaker under aeration with elevated CO_2 (5% CO_2 in air). Cultures to be switched from elevated CO_2 to limiting CO_2 (0.03% CO_2 in air) for induction experiments were centrifuged from the medium and resuspended in minimal salts medium buffered for low CO_2 aeration.

In Vivo Labeling Experiments

Cells cultured with elevated CO_2 or with limiting CO_2 for 4, 8, or 24 h were collected and washed with minimal salts medium lacking sulfate and resuspended in an original culture volume of minus-sulfate medium 4 h before harvesting. The 4-h sample was resuspended in sulfate-free medium at the same time that it was placed on limiting CO_2. Label (100 μCi [^{35}S]sulfuric acid) was added 30 min before harvesting and cells were harvested and disrupted as previously described (10). Soluble proteins were separated from membranes by centrifugation (48,000*g*, 20 min). For pulse-chase experiments, cells cultured with elevated CO_2 or with elevated CO_2 followed by 4 h of limiting CO_2 were washed and resuspended in minus-sulfur medium exactly as in the 30-min labeling experiments. The pulse-chase protocol developed by Schmidt and Mishkind (19) was followed. Cells were given a 5-min pulse of 1 mCi/mL [^{35}S]sulfuric acid (carrier-free; 43 Ci/mg S at 100% isotopic enrichment) followed by up to 60 min chase with 10 mM Na_2SO_4.

Protein Gels and Autoradiography

All protein samples were concentrated and unincorporated label eliminated from the samples using Amicon Centricon 10 concentrators. SDS polyacrylamide gels (10–18%) were run using equal amounts of radioactive protein per lane. Protein was transferred to nitrocellulose sheets (10) and exposed to Kodak XAR5 film.

Northern Analysis and *in Vitro* Translation

Total RNA was prepared from cells cultured with elevated CO_2 or induced with limiting CO_2 for 1, 2, 4, 8, or 24 h as previously described (10). For northern blots, RNA (10 μg/lane) was separated in formaldehyde-agarose gels and transferred to nitrocellulose sheets (8). RNA gel blots were probed using standard methods. Rubisco small subunit mRNAs were detected with the CS2.1 *rbc*S2 cDNA clone (kindly provided by Dr. M. Goldschmidt-Clermont), which is 98.5% homologous over 90% of its length to the *rbc*S1 sequence and therefore hybridizes nearly equally as well to both mRNAs (11). Rubisco large subunit mRNA was detected with the R15.4 subfragment of the *C. reinhardtii* chloroplast R15 clone (kindly provided by Dr. R. Spreitzer). Quantitation of *rbc*S1 and *rbc*S2 mRNA abundance from northern blots was performed by liquid scintillation spectroscopy of bands excised from the blot.

In vitro translation was performed in the presence of 50 μCi of L-[^{35}S]methionine (New England Nuclear) using rabbit reticulocyte lysate (Promega) according to the supplier's instructions. *In vitro* translations in 50 μL were primed with 1.5 μg poly(A^+) RNA or 15 μg poly(A^-) RNA isolated from either 4-h limiting CO_2-induced cells or cells cultured with elevated CO_2 (10). *In vitro* translation products were analyzed by SDS-PAGE and fluorography as described (10).

RESULTS AND DISCUSSION

We have confirmed an earlier observation (6, 23) that when *C. reinhardtii* cells adapt from a CO_2-enriched environment to a CO_2-limiting environment, synthesis of Rubisco LSU and SSU polypeptides temporarily decline but then return to the levels in high CO_2-grown cells. *C. reinhardtii* cells cultured with elevated CO_2 were exposed to limiting CO_2 for 4, 8, or 24 h and *in vivo* labeled at each time point to monitor accumulation of newly synthesized polypeptides. Autoradiograms of SDS-PAGE-separated soluble protein from experiments using either wild-type (2137) or a cell wall-deficient mutant (CW-15) are shown in Figure 1. Both *C. reinhardtii* strains had diminished synthesis and/or accumulation of LSU and SSU between 4 and 8 h of limiting CO_2 growth, but between 8 and 24 h, newly synthesized LSU and SSU again accumulated as in high CO_2-grown cells. Silver-stained gels of proteins were not quantitative enough to determine whether a decrease in the amount of LSU and SSU occurred.

Decreased subunit synthesis and/or accumulation in 4- and

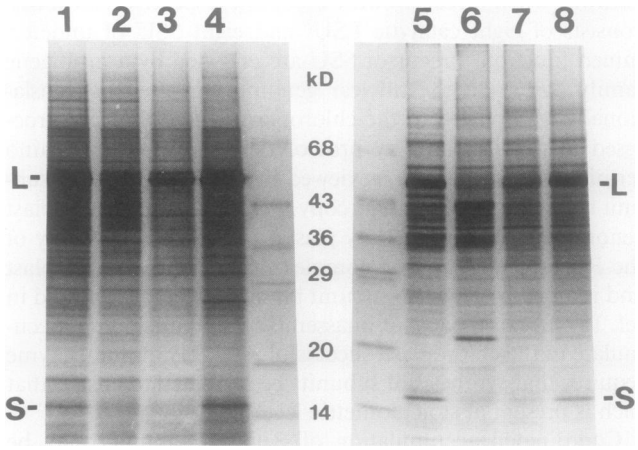

Figure 1. *In vivo* labeled polypeptides in the soluble fraction of *C. reinhardtii* strains CW-15 (lanes 1–4) and 2137 (lanes 5–8) cultured with elevated CO_2 (lanes 1 and 5) or with limiting CO_2 for 4 h (lanes 2 and 6), 8 h (lanes 3 and 7), and 24 h (lanes 4 and 8). Polypeptides were labeled for 30 min prior to harvesting. Radioactive polypeptides were transferred to nitrocellulose and autoradiographed. Positions of Rubisco LSU (L) and SSU (S) polypeptides are indicated.

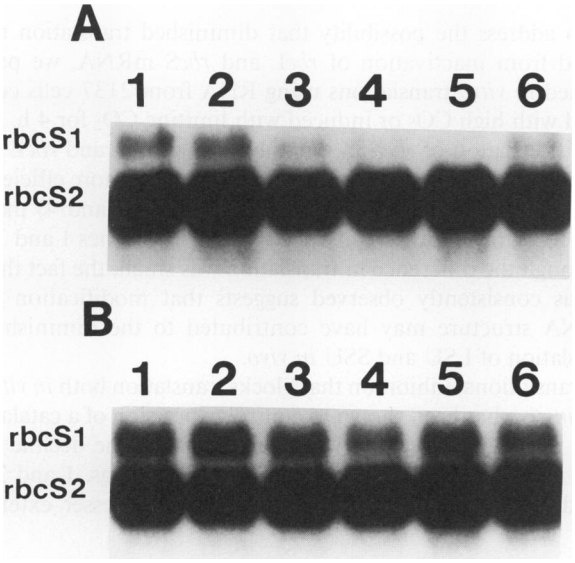

Figure 2. *rbc*S mRNA accumulation in *C. reinhardtii* strains CW-15 (A) and 2137 (B) cultured with elevated CO_2 (lanes 1) or with limiting CO_2 for 1 h (lanes 2), 2 h (lanes 3), 4 h (lanes 4), 8 h (lanes 5), and 24 h (lanes 6). Cellular RNA was separated in formaldehyde-agarose gels, transferred to nitrocellulose, and probed with a cDNA for *rbc*S2, which hybridizes to both mRNAs.

8-h cells as measured by diminished *in vivo* labeling could result from: (a) decreased abundance of translatable mRNA for one or both of the subunits, (b) inhibition of translation elongation or initiation, or (c) rapid degradation of one or both of the newly made subunits. We have conducted experiments aimed at determining which stage of Rubisco subunit biosynthesis is regulated to cause the depression in subunit labeling in limiting CO_2-adapting *C. reinhardtii* cells.

Comparisons between the wild-type and CW-15 strains were used throughout this study because a difference in *rbc*S mRNA abundance had been observed during adaptation to limiting CO_2 in the two strains. Adjustment of *rbc*L and *rbc*S mRNA pool sizes is the principal way plants and algae coordinate Rubisco protein levels during developmentally induced changes (reviewed in ref. 28). To determine if *C. reinhardtii* cells undergoing adaptation to limiting CO_2 use this level of control to regulate Rubisco levels, accumulation of hybridizable *rbc*S and *rbc*L mRNAs was monitored for strains 2137 and CW-15 adapted to limiting CO_2 for 1, 2, 4, 8, and 24 h, as well as for high CO_2-grown cells. The more abundant transcript (*rbc*S2) of the two-gene *rbc*S family remained essentially unchanged in abundance in both strains over the time course (Fig. 2), but a more significant change in abundance was observed for *rbc*S1 mRNA in CW-15 cells (but not in 2137 cells) at 2, 4, and 8 h of limiting CO_2 growth.

We do not suspect involvement of *rbc*S1 mRNA regulation in control over SSU polypeptide levels in our study because we found constitutive expression of *rbc*S1 mRNA in 2137 cells and because *rbc*S2 mRNA in both strains remains essentially unchanged in abundance and contributes most of the mRNA to the *rbc*S mRNA pool. We conclude, therefore, that the decline of *rbc*S1 mRNA in CW-15 is a strain-specific effect: if it played a significant role in the down-regulation of SSU synthesis, we would expect the decline to be seen in 2137 cells as well. Nevertheless, in view of evidence (11, 13) that under other conditions *rbc*S1 is regulated at the level of mRNA abundance, it remains intriguing that the patterns of *rbc*S1 mRNA abundance and SSU accumulation appeared to be exactly coordinated.

We saw no evidence for control of *rbc*L mRNA pool size in limiting CO_2-adapting *C. reinhardtii* cells because *rbc*L mRNA abundance did not vary during the time course of adaptation to limiting CO_2 (Fig. 3). Abundance of *rbc*L mRNA does not vary at any time of the cell cycle in synchronous *C. reinhardtii* cells (14), suggesting that regulation of LSU synthesis by mRNA abundance is not common in *C. reinhardtii*.

Because accumulation of newly synthesized Rubisco subunits in limiting CO_2-adapting *C. reinhardtii* cells was not correlated with their corresponding mRNAs levels, translational or posttranslational controls must act in these cells to cause the observed decline in Rubisco subunit synthesis. Depression of synthesis of LSU and SSU could be coordinated if one subunit was regulated translationally and the other was rapidly turned over posttranslationally. Alternatively, both subunit polypeptides could be synthesized at normal rates but rapidly degraded so that synthesis appears to be decreased. In higher plants, degradation of excess mature SSU polypeptide seems to allow for the most rapid equilibration of subunit concentrations (29), and this strategy is utilized in algae when LSU polypeptide does not accumulate (12, 17, 19, 25).

Pulse-chase experiments with either high CO_2-grown or 4 h limiting CO_2-adapted cells would indicate if one (or both) of the subunit polypeptides was being synthesized but rapidly degraded. High CO_2-grown cells pulsed for 5 min synthesized LSU and SSU that were stable throughout the 60-min chase period (Fig. 4, lanes 1–5), but when cells were cultured in limiting CO_2 for 4 h prior to the pulse (Fig. 4, lanes 6–10),

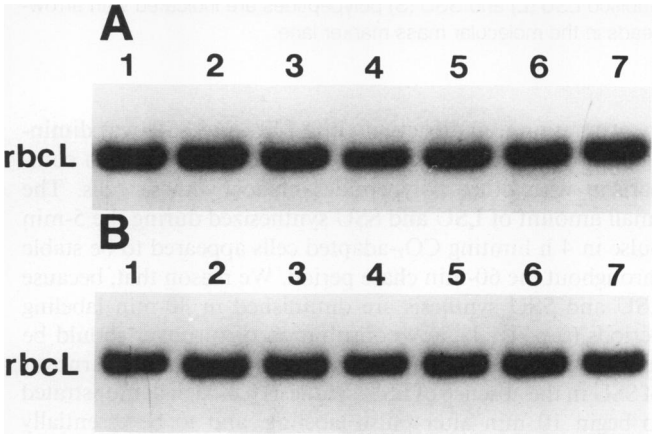

Figure 3. *rbc*L mRNA accumulation in *C. reinhardtii* strains CW-15 (A) and 2137 (B) cultured with elevated CO_2 (lanes 1) or with limiting CO_2 for 1 h (lanes 2), 2 h (lanes 3), 4 h (lanes 4), 8 h (lanes 5), 12 h (lanes 6), and 24 h (lanes 7).

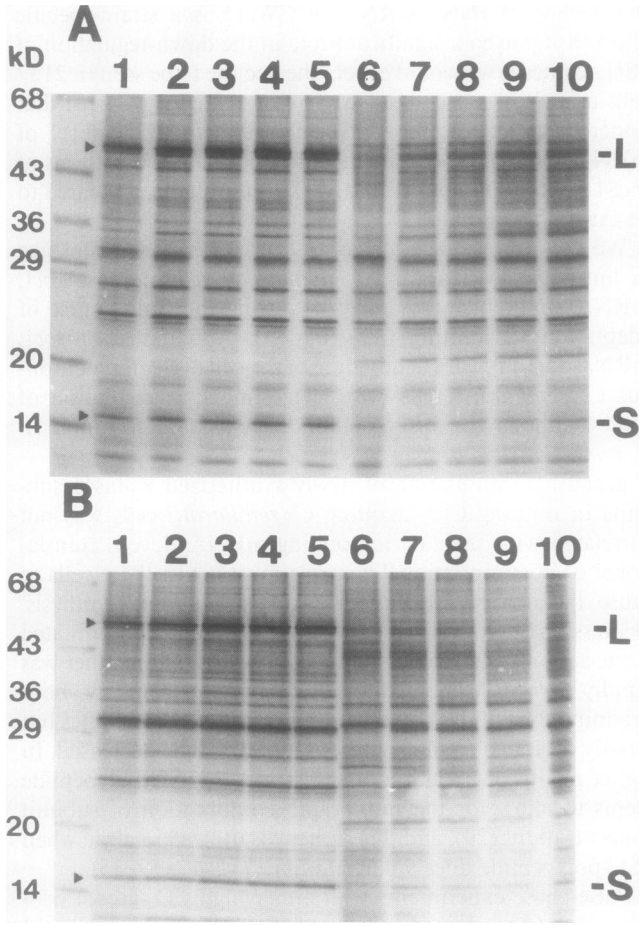

Figure 4. Pulse-chase labeled polypeptides found in the acetone insoluble fraction of *C. reinhardtii* strains CW-15 (A) and 2137 (B). Pulse-chase labelings of cells cultured with elevated CO_2 (lanes 1–5) and cells switched from elevated CO_2 to limiting CO_2 for 4 h (lanes 6–10) are shown. Polypeptides from cells pulse-labeled for 5 min (lanes 1 and 6) and from cells chased for 10 min (lanes 2 and 7), 20 min (lanes 3 and 8), 30 min (lanes 4 and 9), and 60 min (lanes 5 and 10) were analyzed by SDS-PAGE and autoradiography. Positions of Rubisco LSU (L) and SSU (S) polypeptides are indicated with arrowheads in the molecular mass marker lane.

incorporation of radioactivity into LSU and SSU was diminished in comparison with high CO_2-grown cells and in comparison with other polypeptides made by these cells. The small amount of LSU and SSU synthesized during the 5-min pulse in 4 h limiting CO_2-adapted cells appeared to be stable throughout the 60-min chase period. We reason that, because LSU and SSU synthesis are diminished in 30-min labeling periods (see Fig. 1), any contribution of turnover should be seen in the 60-min chase. In fact, posttranslational turnover of SSU in the absence of LSU synthesis has been demonstrated to begin 10 min after pulse labeling, and to be essentially complete after 60 min (19, 25). The results presented in Figure 3 clearly indicate that synthesis of SSU followed by rapid degradation in the absence of LSU is not occurring. Rather, it appears that translation of both subunits is diminished, and that the level of control for SSU is the same as that for LSU.

To address the possibility that diminished translation resulted from inactivation of *rbc*L and *rbc*S mRNA, we performed *in vitro* translations using RNA from 2137 cells cultured with high CO_2 or induced with limiting CO_2 for 4 h. *In vitro* translation of *rbc*S in the poly(A^+) fraction and *rbc*L in the poly(A^-) fraction apparently was somewhat more efficient with RNA from high CO_2 cells (Fig. 5, lanes 2 and 4) than with RNA from low CO_2-induced cells (Fig. 5, lanes 1 and 3). Although the difference in translation was small, the fact that it was consistently observed suggests that modification of mRNA structure may have contributed to the diminished translation of LSU and SSU *in vivo*.

Translational inhibition that blocks translation both *in vitro* and *in vivo* has been shown to control expression of a catalase gene in maize (21). However, compared with the decline in LSU and SSU synthesis observed *in vivo* (see Figs. 1 and 3), the decline seen *in vitro* appears to be of a lesser extent.

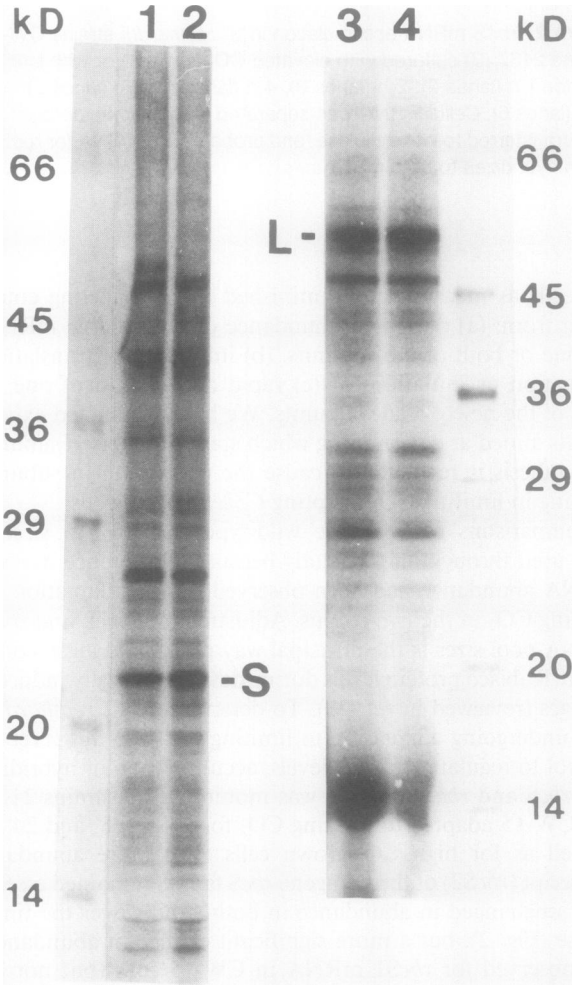

Figure 5. *In vitro* translation products of poly(A^+) RNA (lanes 1 and 2) and poly(A^-) RNA (lanes 3 and 4) analyzed by SDS-PAGE and fluorography. RNA was from 4 h limiting CO_2-induced cells (lanes 1 and 3) or cells cultured with elevated CO_2 (lanes 2 and 4). Molecular mass markers are identified by size in kD. Positions of the Rubisco SSU (S) and LSU (L) are indicated.

Therefore, it is unclear whether inactivation of subunit mRNA (as measured by *in vitro* translation) is sufficient enough to account for the observed decline in label incorporation into LSU and SSU polypeptides *in vivo*.

If translational factors other than transcript modification are involved in reduced *in vivo* Rubisco subunit translation, we do not know whether these involve inhibition of elongation or initiation or both, as in the case of amaranth seedlings (3). However, we have ruled out rapid degradation of newly synthesized subunit polypeptides as well as control of mRNA levels as mechanisms used to coordinately down-regulate synthesis of LSU and SSU polypeptides during adaptation to limiting external C_i, so regulation clearly occurs at the level of transcript modification or translation or both.

When accumulation of LSU was blocked in algae, either by antibiotic treatment (17, 19) or by genetic defects (12, 19, 25), SSU synthesis proceeded but unassembled SSU was unstable, with a half-life of only 7.5 min (19). This type of control over biosynthesis of Rubisco has been regarded (29) as a final adjustment mechanism to attain stoichiometric levels of LSU and SSU. Control by translational regulation is in marked contrast with this mechanism and appears to be like that found in higher plants shifted from light to dark (3). Translational regulation of LSU and SSU by limiting CO_2-adapting cells might be regarded as a coarse control, functioning in response to changes in growth conditions that induce major shifts in metabolism of the cell. Therefore, whether stoichiometric accumulation of LSU and SSU is attained by transcriptional, posttranscriptional, translational, or post-translational mechanisms may be dependent not only upon genetic determinants but also upon the nature of the stimulus affecting the altered Rubisco levels.

Induction of the CO_2-concentrating mechanism of *C. reinhardtii* involves changes in gene transcription (2), protein synthesis (2, 6), and energy-requiring C_i transport (27). As a consequence of these demands for energy and components to build macromolecules placed upon the cell by this growth condition, production of expendable macromolecules might be temporarily interrupted in a specific or a general manner. Although Rubisco activity is not dispensable to the cells in our experimental conditions, the stability of the holoenzyme may make production of new subunits expendable, particularly if cell growth and/or division is arrested. Evidence from our lab (L. Marek, unpublished observations) suggests that cell division possibly is interrupted for 4 to 6 h immediately after transfer of cells from elevated CO_2 to limiting CO_2.

Although we can only speculate on the causes for diminished demand for new Rubisco subunits in limiting CO_2-adapting cells, this study clearly shows that diminished Rubisco subunit synthesis results from a specific translational control that similarly affects both subunits.

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