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Biosynthesis of carbonic anhydrase in *Chlamydomonas reinhardtii* during adaptation to low CO_2

(glycosylation/tunicamycin/precursor/regulation)

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ABSTRACT **The unicellular green alga *Chlamydomonas reinhardtii* synthesizes carbonic anhydrase in response to low levels of CO_2 (i.e., air levels of CO_2). This enzyme, localized predominantly in the periplasmic space of the alga (or associated with the cell wall), is an important component of the machinery required for the active accumulation of inorganic carbon by *C. reinhardtii* and the saturation of ribulose-1,5-bisphosphate carboxylase at low extracellular carbon concentrations. We have begun to examine the synthesis and compartmentalization of carbonic anhydrase in *C. reinhardtii*. The monomeric species associated with carbonic anhydrase activity is synthesized as a precursor on 80S cytoplasmic ribosomes. This precursor can be detected immunologically in the profiles of translation products when a reticulocyte lysate, cell-free system is primed with poly(A)-RNA from either air-grown *C. reinhardtii* or cells shifted from growth on 5% CO_2 to air for 12 hr. It is not synthesized when the *in vitro* system is primed with poly(A)-RNA from CO_2-grown algae. Since translatable RNA for the polypeptide responsible for carbonic anhydrase activity was only present in cells that experienced low levels of CO_2, the adaptation process either involves the regulation of transcription of the carbonic anhydrase gene (and perhaps other genes involved in adaptation) or the post-transcriptional processing of the messenger RNA. Furthermore, the appearance of the mature polypeptide associated with carbonic anhydrase activity in the periplasmic space of *C. reinhardtii* is inhibited by tunicamycin, an antibiotic that prevents core glycosylation of polypeptides on the endoplasmic reticulum. Together, these results suggest that the biosynthesis of this extracellular algal enzyme involves the translation of mRNA for the carbonic anhydrase monomer on ribosomes bound to the endoplasmic reticulum, the cleavage of a signal sequence during transport of the nascent polypeptide into the lumen of the endoplasmic reticulum, and subsequent glycosylation events prior to export across the plasmalemma.**

The transfer of *Chlamydomonas reinhardtii* from growth on 5% CO_2 to air levels of CO_2 results in a dramatic change in the photosynthetic characteristics of the organism (1). Algae adapted to air levels of CO_2 exhibit both carbonic anhydrase activity and the capacity to actively transport inorganic carbon (C_i). In contrast, algae grown on 5% CO_2 have very low carbonic anhydrase activity with a limited capacity to transport inorganic carbon. The concomitant expression of carbonic anhydrase and C_i transport activities enable air-grown algae to photosynthesize much more efficiently at low CO_2 concentrations than algae grown on 5% CO_2.

The majority of carbonic anhydrase in air-grown cells of *C. reinhardtii* is located in the periplasmic space (2). In cultures of the cell wall-less mutant of *C. reinhardtii*, *CW-15*, between 80% and 90% of the carbonic anhydrase activity is released into the growth medium (3). After separation of the cells from the medium, we were able to concentrate carbonic anhydrase (by concentrating the culture medium) and identify a polypeptide with an M_r of ≈37,000 that is associated with this activity (3). In addition to carbonic anhydrase, the synthesis of other polypeptides is regulated during the adaptation of *C. reinhardtii* to air levels of CO_2 (unpublished data), although the function of many of these polypeptides remains unknown.

Carbonic anhydrase is, thus far, the most well-characterized protein synthesized by *C. reinhardtii* in response to low CO_2 levels. The biosynthesis of this extracellular protein may provide a model system for examining both the events required for the synthesis and secretion of polypeptides by eukaryotic algae and the regulatory mechanisms involved in their adaptation to a low CO_2 atmosphere.

MATERIALS AND METHODS

All chemicals were reagent grade. The $^{35}SO_4^{2-}$ was purchased from Amersham (SJS.1); Freund's adjuvant was purchased from GIBCO. All other reagents were from Sigma. Carbonic anhydrase activity in cell pressates was determined electrometrically as described (2, 4).

Culture Conditions. *C. reinhardtii* 2137 mt^+ (obtained from M. Spalding, Michigan State University) and the cell wall-less mutant of *Chlamydomonas*, *CW-15* (obtained from R. K. Togasaki, Indiana University), were cultured axenically in the medium described by Spalding and Ogren (5). The growth temperature was 28°C and the light intensity was 300 microeinsteins·m^{-2}·sec^{-1}. Cultures were shaken vigorously and bubbled either with 5% CO_2 in air or with air alone. All experiments were performed with cells in early to midlogarithmic phase growth.

***In Vivo* Labeling and Gel Electrophoresis.** *In vivo* labeling was used to examine polypeptides synthesized during the adaptation of *C. reinhardtii* to low levels of C_i. Cultures were labeled with $^{35}SO_4^{2-}$ during growth on 5% CO_2 or 0.03% CO_2 and following a transfer from 5% to 0.03% CO_2. In some experiments cycloheximide (1.0 μg/ml), chloramphenicol (200 μg/ml), or tunicamycin (2.0 μg/ml) was included in cell cultures during the labeling. Generally, these inhibitors were added in the light 10 min prior to the addition of $^{35}SO_4^{2-}$. After 5 hr of labeling in the light, the cells were broken in a prechilled French pressure cell (12,500 psi; 1 psi = 6.89 kPa) and centrifuged for 3 min at 2000 × *g* to remove unbroken algae and cellular debris, and the supernatant was centrifuged at 43,000 × *g* for 30 min. Protease inhibitors (1 mM benzamidine·HCl and 5 mM ε-amino-*n*-caproic acid) were included in the buffers during the cell fractionation. The resulting pellet contained the majority of the thylakoid membranes. The supernatant was subjected to high-speed centrifugation (135,000 × *g*) for 3 hr to remove the remaining membranous material and the majority of the holoenzyme of ribulose-1,5-bisphosphate carboxylase (high-speed pellet).

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Table 1. Effect of inhibitors of protein synthesis and glycosylation on the induction of carbonic anhydrase activity

Growth condition	Carbonic anhydrase activity, WA units·mg of chlorophyll^{-1}
High CO_2	26.1
Air	1295.7
5 hr on air	610.3
+ Chloramphenicol	583.3
+ Cycloheximide	31.1
+ Tunicamycin	117.4

WA, Wilbur–Anderson.

This fraction also contained most of the carbonic anhydrase activity (3). The high-speed pellet was washed with cold 90% acetone and resuspended by sonication in 0.1 M Na_2CO_3/0.1 M dithiothreitol. The suspension was made 1.7% $NaDodSO_4$/10% sucrose, boiled, and then electrophoresed on a denaturing polyacrylamide gel (12–18% linear gradient containing 8 M urea) (6) using Laemmli buffers (7). Following electrophoresis, the gel was stained with Coomassie brilliant blue G-250, and the newly synthesized polypeptides were visualized by autoradiography.

Preparation and Electrophoretic Analysis of Extracellular Polypeptides. For the preparation of extracellular proteins from the growth medium of the cell wall-less mutant, we removed intact cells by low-speed centrifugation (4000 × *g*) and fractionated the culture medium by column chromatography. The medium was first incubated with DEAE-cellulose, at 4°C overnight. The cellulose was then poured into a column and washed with 1 mM Tris·HCl (pH 8.0), and the proteins were eluted from the column with 0.5 M NaCl in 1 mM Tris·HCl (pH 8.0) and concentrated by precipitation with 80% ammonium sulfate. The precipitated protein was resuspended in 50 mM Hepes/NaOH, pH 8.0, and precipitated with 10% trichloroacetic acid, and the pelleted protein was washed with 90% acetone. Preparation of the sample for electrophoresis involved resuspension of the pellet in 0.1 M dithiothreitol/0.1 M Na_2CO_3 by sonication and solubilization with $NaDodSO_4$ (to 1.7%). Staining for glycoproteins on polyacrylamide gels (7.5–15%, gradient gel electrophoresed at 4°C) was performed according to Zacharius *et al.* (8).

RNA Isolation and *in Vitro* Translation. RNA was isolated by using the buffer system of Cashmore *et al.* (9). *C. reinhardtii* grown on 5% CO_2 or air levels of CO_2 or transferred from 5% to air levels of CO_2 for 12 hr was pelleted, and the pellet was resuspended in 50 mM Tris·HCl, pH 9.0/15 mM EDTA/1.0% $NaDodSO_4$/5 mM dithiothreitol. The cells became lysed under these conditions. The lysate was extracted with phenol, phenol/chloroform (1:1), and then chloroform alone, and the high molecular weight RNA was sequentially precipitated once with 0.8 vol of isopropanol, 2 M LiCl and then twice with 2 vol of ethanol. Poly(U)-derivatized Sepharose 4B was used to select for poly(A)-RNA (10), which was precipitated once with 1 vol of isopropanol and then two times with ethanol. After the final precipitation the RNA was dissolved in sterile, distilled H_2O and stored at −80°C. Poly(A)-RNA was translated *in vitro*, according to the manufacturer's instructions, in a reticulocyte lysate system purchased from Bethesda Research Laboratories. Translation mixtures were made 3% $NaDodSO_4$/15% sucrose/75 mM dithiothreitol/2 mM EDTA prior to electrophoresis on a 12–18% polyacrylamide gel containing 8 M urea (6).

Preparation of Antibodies and Immunoprecipitations. Antibodies to carbonic anhydrase were prepared against the M_r 37,000 polypeptide previously identified as the carbonic anhydrase monomer (3). This polypeptide was purified from the culture medium of *CW-15*. Proteins in the medium were concentrated by DEAE-cellulose chromatography and ammonium sulfate precipitation (80%) and then electrophoresed on a 7.5–15% polyacrylamide gel at 4°C. The M_r 37,000 polypeptide was excised from the gel, electroeluted, combined with Freund's adjuvant, and injected into rabbits following an immunization schedule previously described (11). Antibodies were also prepared against the small subunit of ribulose-1,5-bisphosphate carboxylase in a similar manner. Immunoprecipitation of the cell-free translation products was performed according to Schmidt *et al.* (12), except that protein A-Sepharose was used to precipitate the antibody instead of formalin-fixed cells of *Staphylococcus aureus*.

RESULTS

Growth of *C. reinhardtii* on air levels of CO_2 initiates the synthesis of carbonic anhydrase. As presented in Table 1, essentially no activity could be measured in cultures grown on 5% CO_2, whereas high levels of activity were observed in air-grown cells. Carbonic anhydrase activity was at an intermediate level in cultures transferred from growth on 5% CO_2 to growth on air for 5 hr. The data presented in Table 1 also demonstrate the effect of inhibitors of translation and polypeptide glycosylation on the appearance of carbonic anhydrase activity. Chloramphenicol, an inhibitor of translation on 70S organelle ribosomes, does not inhibit the synthesis of carbonic anhydrase. However, the level of carbonic anhydrase activity in the presence of cycloheximide, an inhibitor of translation on the 80S ribosomes of the cytoplasm, was similar to the level measured in cells grown on 5% CO_2. Tunicamycin also blocked the appearance of the majority of

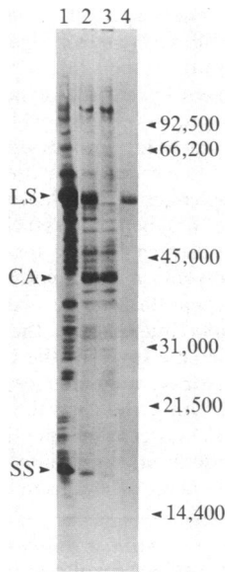

FIG. 1. Effect of chloramphenicol and cycloheximide on the synthesis of carbonic anhydrase in *C. reinhardtii*. Cells were incubated at 28°C and labeled with $^{35}SO_4^{2-}$ for 5 hr during growth on 5% CO_2 (lane 1) and following transfer from 5% CO_2 to air levels of CO_2 (lanes 2–4). The chloramphenicol concentration used was 200 $\mu g \cdot ml^{-1}$ (lane 3); the cycloheximide concentration was 1 $\mu g \cdot ml^{-1}$ (lane 4). After *in vivo* labeling, the cells were lysed and fractionated. Samples prepared from the high-speed pellet fractions were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis (12–18% polyacrylamide gel containing 8 M urea). Each lane received an equal amount of protein as determined by Coomassie brilliant blue staining. The gel was dried and exposed to Kodak XAR-5 film to visualize the newly synthesized polypeptides. The carbonic anhydrase monomer (CA) and the large (LS) and small (SS) subunits of ribulose-1,5-bisphosphate carboxylase are indicated. Molecular weight markers are phosphorylase b (M_r 92,500), bovine serum albumin (M_r 66,200), ovalbumin (M_r 45,000), bovine carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,400).

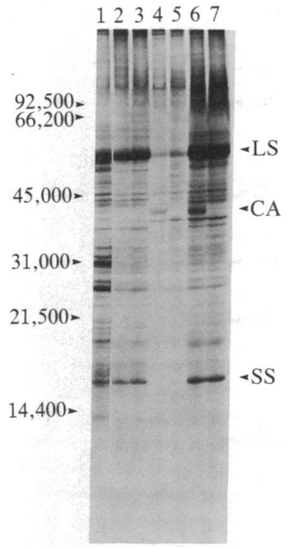

FIG. 2. Effect of tunicamycin on the synthesis of carbonic anhydrase by *C. reinhardtii*. Cells grown at 28°C and the appropriate CO_2 concentration were incubated with $^{35}SO_4^{2-}$ for 5 hr in the presence or absence of tunicamycin (2 $\mu g \cdot ml^{-1}$). The newly synthesized polypeptides in the high-speed pellet fraction were analyzed by gel electrophoresis followed by autoradiography, as described in the legend of Fig. 1. Lane 1, a profile of the stained polypeptides. Treatment with tunicamycin during growth at high or low CO_2 concentrations did not lead to a noticeable alteration in the stained polypeptide profile. Newly synthesized polypeptides from cells grown on 5% CO_2 (lanes 2 and 3) or air levels of CO_2 (lanes 6 and 7) and after transfer from 5% to air levels of CO_2 (lanes 4 and 5) are presented. Lanes 3, 5, and 7, cultures containing tunicamycin during *in vivo* labeling; lanes 2, 4, and 6, untreated cultures. The carbonic anhydrase monomer (CA) and the large (LS) and small (SS) subunits of ribulose-1,5-bisphosphate carboxylase are indicated. Molecular weight markers are as in the legend of Fig. 1.

carbonic anhydrase activity during adaptation. In most experiments we found that tunicamycin reduced the level of activity to between 10% and 15% of that measured in air-grown cells.

Fig. 1 shows the effect of inhibitors of translation on the synthesis of polypeptides in the high-speed pellet (see *Materials and Methods*), the fraction that contained the majority of the carbonic anhydrase activity. Of particular interest is the effect of these inhibitors on the synthesis of a polypeptide, M_r of ≈37,000, which is responsible for carbonic anhydrase activity (3). This polypeptide cannot be detected in cultures grown on 5% CO_2 but is synthesized during growth on air (Fig. 1, compare lanes 1 and 2). Its synthesis, like the synthesis of most polypeptides in the high-speed pellet fraction, is inhibited by cycloheximide but not by chloramphenicol (Fig. 1, compare lanes 3 and 4). The reduced synthesis of the large subunit of ribulose-1,5-bisphosphate carboxylase (Fig. 1, lane 3) during *in vivo* labeling in the presence of chloramphenicol indicates that this antibiotic is working properly. Cycloheximide inhibited the synthesis of nearly every other polypeptide, including the carbonic anhydrase monomer. Therefore, the adaptation of *C. reinhardtii* to low levels of CO_2 probably requires the *de novo* synthesis of carbonic anhydrase on 80S cytoplasmic ribosomes.

The effect of tunicamycin on the appearance of carbonic anhydrase in the high-speed pellet is presented in Fig. 2. The stained profile of polypeptides of the high-speed pellet is in lane 1, whereas the newly synthesized polypeptides, visualized following autoradiography, both in the presence and absence of tunicamycin, are presented in lanes 2–7. The synthesis of few polypeptides was affected by this antibiotic. Indeed, as demonstrated in Fig. 2, only the synthesis of the carbonic anhydrase monomer (indicated as CA in Fig. 2) in this fraction appears to be affected by inhibition of polypeptide glycosylation. Carbonic anhydrase is absent in cells grown on 5% CO_2 (Fig. 2, lanes 2 and 3) but is present in both air-grown cells (Fig. 2, lane 6) and cells adapting to air levels of CO_2 (Fig. 2, lane 4). Tunicamycin prevents the appearance of carbonic anhydrase in both adapting cells and cells grown continuously on air (Fig. 2, lanes 5 and 7). Therefore, protein glycosylation is required for either the synthesis, stabilization, export, or maturation of the monomeric species responsible for carbonic anhydrase activity.

To confirm this finding, and note the effect of tunicamycin on other polypeptides destined for the periplasmic space, we analyzed the effect of tunicamycin on the synthesis of polypeptides exported into the growth medium in cultures of *CW-15*. Electrophoretic analysis of these polypeptides (concentrated from 200 ml of medium) is presented in Fig. 3. Stained polypeptide profiles for air-grown and 5% CO_2-grown cells are presented in lanes 1–4, whereas the autoradiogram showing newly synthesized, extracellular polypeptides after a 5-hr labeling period is presented in lanes 5–8. No stainable carbonic anhydrase band is observed in the polypeptide profiles from high CO_2-grown cells (lanes 1 and 2) or cells grown on air in the presence of tunicamycin (lane 4). However, air-grown cells in the absence of tunicamycin do exhibit stainable levels of the M_r 37,000 polypeptide (lane 3). These results are more dramatically demonstrated when newly synthesized polypeptides are visualized by *in vivo* labeling. No carbonic anhydrase is synthesized by cells maintained on high CO_2 in either the presence or absence of tunicamycin (Fig. 3, lanes 5 and 6). Air-grown cells, on the other hand, export large amounts of carbonic anhydrase unless tunicamycin is included in the culture medium (Fig. 3, compare lanes 7 and 8) during the labeling period. Tunicamycin reduces the export of several polypeptides regardless of the CO_2 concentration. In the presence of tunicamycin, cultures

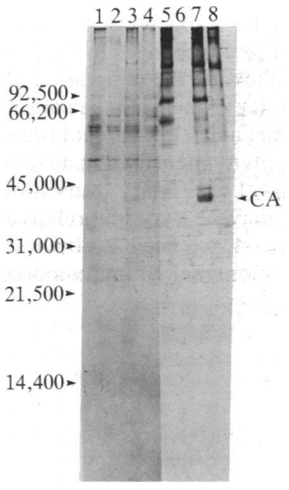

FIG. 3. Effect of tunicamycin on the export of polypeptides from *CW-15* cells. Cultures were incubated with $^{35}SO_4^{2-}$ at 28°C and the appropriate CO_2 concentration for 5 hr. The polypeptides released into the medium were concentrated on DEAE-cellulose and then subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The stained gel profile (lanes 1–4) and the autoradiogram (lanes 5–8) are presented. Lanes 1 and 2 and lanes 5 and 6, polypeptides in the medium of cells grown at 5% CO_2 in the presence (lanes 2 and 6) and absence (lanes 1 and 5) of tunicamycin (2 $\mu g \cdot ml^{-1}$). Lanes 3 and 4 and lanes 7 and 8, polypeptides in the medium of cells transferred from 5% CO_2 to air levels of CO_2 in the presence (lanes 4 and 8) and absence (lanes 3 and 7) of tunicamycin. The carbonic anhydrase monomer (CA) is indicated. Molecular weight markers are as in the legend of Fig. 1.

grown at high levels of CO_2 export almost no polypeptides (or very low levels) over the labeling period, whereas air-grown cultures clearly export polypeptides. However, some of these polypeptides do not comigrate with those from cultures labeled in the absence of tunicamycin (Fig. 3, compare lanes 7 and 8). These results suggest that even if export is occurring, the extracellular polypeptides may not be properly or completely processed. For example, is the M_r 33,000 polypeptide that is only exported by air-grown cells in the presence of tunicamycin (lane 8) related to carbonic anhydrase? Incomplete or improper glycosylation may allow for more rapid degradation of the newly synthesized polypeptide (13–15).

To confirm that the M_r 37,000 polypeptide responsible for carbonic anhydrase activity is covalently linked to sugar residues, we stained the extracellular polypeptides from the medium of *CW-15* specifically for glycoproteins. The carbonic anhydrase monomer and several other extracellular polypeptides showed a strong affinity for the glycoprotein stain (Fig. 4). The carbonic anhydrase monomer can, on occasion, be split into two bands on a 7.5–15% polyacrylamide gel. These two bands may reflect different states of glycosylation of the carbonic anhydrase monomer or partial proteolysis of the polypeptide (in spite of the fact that protease inhibitors were included in all solutions used during the isolation). Generally, only one band is observed. Furthermore, antibodies prepared against the M_r 37,000 polypeptide inhibit carbonic anhydrase activity and appear to react with only one species as determined by crossed immunoelectrophoresis (3).

Since carbonic anhydrase is a cytoplasmic enzyme that is exported into the periplasmic space of the cell, we decided to further characterize the biosynthetic events involved in the maturation of this protein. Translation of poly(A)-RNA from cells grown on 5% and air levels of CO_2, as well as from cells transferred from 5% CO_2 to air for 12 hr, was performed in a reticulocyte lysate system. Profiles of primary translation products are presented in Fig. 5, lanes 2–4. Lane 1 is the control in which no exogenous RNA was added to the translation system. Few changes in the profile of translation products occur following the adaptation of *C. reinhardtii* to low levels of CO_2 (lanes 2–4). Since carbonic anhydrase is a very active enzyme, and a low level of translatable RNA may account for the activity measured in air-grown cultures of *C. reinhardtii* (the level of primary translation product for carbonic anhydrase may be very low relative to other primary translation products), we used antibodies raised to the carbonic anhydrase monomer to immunoprecipitate a primary translation product for this enzyme. An immunoprecipitation from translation mixtures primed with poly(A)-RNA derived from *C. reinhardtii* grown on 5% CO_2 is presented in Fig. 5, lane 8. This profile is similar to the profile observed when preimmune serum was used for immunoprecipitations from translation products from RNA isolated from 5% CO_2-grown cells or cells grown in air (data not shown). No specific products were immunoprecipitated, although some major primary translation products did contaminate the precipitates that we obtained. At least one of these major species represents the precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase (Fig. 5, lane 5). Immunoprecipitations from the primary translation products of either air-grown cells or cells that had been transferred from 5% CO_2 to air for 12 hr (Fig. 5, lanes 6 and 7) enrich for a specific band that is a minor species in the total population of translation products and is not apparent in profiles of primary translation products prior to immunological enrichment. This species has an M_r of ≈44,000, an apparent M_r of between 6000 and 7000 more than the mature carbonic anhydrase monomer.

Together, the results presented here are consistent with the events reported for the biosynthesis of secretory proteins from a number of organisms. This system does, however, offer the unique possibility for the analysis of molecular events required for the maturation and export of periplasmic proteins by a unicellular alga and the induction of polypep-

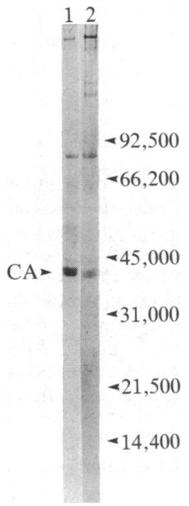

FIG. 4. Glycoprotein staining of polypeptides released into the medium by air-adapted *CW-15*. Polypeptides in the medium of *CW-15* cells adapted to air levels of CO_2 were concentrated on DEAE-cellulose and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. Lane 1, the profile of stained polypeptides released into the medium; lane 2, the identical profile treated with reagents that specifically stain glycoproteins (8). The carbonic anhydrase monomer (CA) is indicated. Molecular weight markers are as in the legend of Fig. 1.

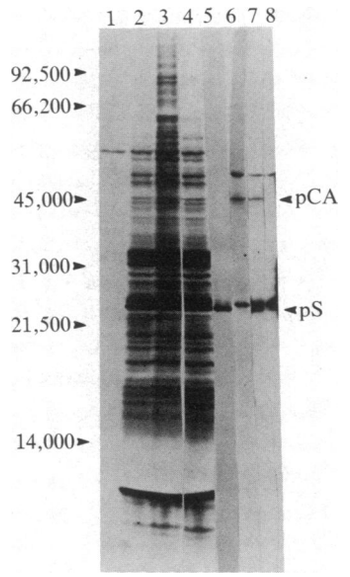

FIG. 5. Immunoprecipitation of carbonic anhydrase from primary translation products of poly(A)-RNA of *C. reinhardtii*. Poly(A)-RNA was translated *in vitro* in a reticulocyte lysate system purchased from Bethesda Research Laboratories. Translation was either in the absence of RNA (lane 1) or in the presence of RNA isolated from algae grown on 5% CO_2 (lane 2) or air levels of CO_2 (lane 4) or after transfer from growth on 5% CO_2 to growth on air levels of CO_2 for 12 hr (lane 3). Immunoprecipitates from primary translation products of algae grown on 5% CO_2 (lane 8) or air levels of CO_2 (lane 6) or transferred from growth on 5% CO_2 to air levels of CO_2 for 12 hr (lane 7), by using antibodies raised against the extracellular carbonic anhydrase of *C. reinhardtii*, are also presented. Lane 5, an immunoprecipitation from primary translation products of high CO_2-grown cells by using antibodies raised to the small subunit of ribulose-1,5-bisphosphate carboxylase. The primary translation product precipitated by using the carbonic anhydrase antibody is indicated as pCA (precursor to carbonic anhydrase), whereas the product precipitated with the antibody to the small subunit of ribulose-1,5-bisphosphate carboxylase is indicated as pS (precursor to the small subunit). Molecular weight markers are as in the legend of Fig. 1.

tides specific to the periplasmic space under diverse environmental conditions.

DISCUSSION

In this study we have defined some of the events that occur during the biosynthesis of the extracellular carbonic anhydrase of *C. reinhardtii*. This enzyme is synthesized when *C. reinhardtii* is grown at low CO_2 concentrations and is important for the adaptation of this alga to air levels of CO_2 (1, 16–18). The enzyme is translated on cytoplasmic, 80S ribosomes as a higher molecular weight precursor. Since no translatable mRNA is detected in cells grown on high CO_2, the induction of this species either involves activation of the gene(s) encoding carbonic anhydrase or a post-transcriptional modification that converts translationally inactive messenger to active messenger. Although the difference in apparent molecular weight between the precursor and mature product is 6000–7000, the exact difference is difficult to assess since processing of this polypeptide involves the addition of sugar residues, which alters the apparent molecular weight of the polypeptide.

Generally, the glycosylation of polypeptides exported into the periplasmic space occurs prior to transport across the plasmalemma. Addition of sugar residues to the monomer associated with carbonic anhydrase activity was indicated in experiments that employed tunicamycin, an antibiotic that inhibits the appearance of carbonic anhydrase in the periplasmic space during adaptation, and was confirmed with a stain specific for glycoproteins. Decreased levels of carbonic anhydrase in the presence of tunicamycin are probably due to inhibition of core glycosylation on the endoplasmic reticulum (19, 20), although the exact mechanism by which this results in the absence of the band that corresponds to carbonic anhydrase is still unclear. The addition of carbohydrate residues may be required for the export process itself, or, it may confer resistance to proteolytic degradation (either within the cell or in the periplasmic space) on the newly synthesized polypeptide. The low level of carbonic anhydrase measured following the transfer of *C. reinhardtii* cultures from growth on 5% CO_2 to growth on air in the presence of tunicamycin may be due to another form of the enzyme that does not require glycosylation (perhaps localized within the chloroplast), the activity of a nascent polypeptide that remains within the cell, or an extracellular unglycosylated species (a species that has not undergone complete maturation and does not migrate to the same position on a polyacrylamide gel).

In addition to carbonic anhydrase, other enzymes synthesized in response to nutrient deprivation are located in the periplasmic space of *C. reinhardtii*. When this alga is deprived of phosphate, the acquisition of this nutrient is dependent upon the activity of periplasmic phosphatases (21–23). Some of these phosphatases are derepressed during phosphate starvation (23). The identities of other polypeptides present in the periplasmic space or associated with the cell wall of *C. reinhardtii* remain to be established.

The regulation of extracellular polypeptides is being analyzed in a number of organisms (23–25). Recently, extensive information has been obtained on the synthesis of invertase in *Saccharomyces cerevisiae* and the regulation of the invertase genes. Extracellular invertase synthesis in *S. cerevisiae* is regulated by glucose repression, whereas the intracellular form of this enzyme is synthesized constituitively (26). The invertase gene, *SUC2*, encodes two mRNAs that differ in their 5′ ends and are differentially regulated. Translation of the larger mRNA results in the secreted, glycosylated form of the enzyme, whereas translation of the smaller messenger yields the intracellular nonglycosylated form (27, 28). The larger mRNA contains a sequence for a hydrophobic signal peptide, required for the secretion of the polypeptide into the lumen of the endoplasmic reticulum, whereas the smaller mRNA does not include the complete coding sequence for the signal peptide. Preliminary data suggest that an intracellular form of carbonic anhydrase is present in air-grown cultures of *C. reinhardtii*. Yet to be established are the location of this intracellular enzyme and its role in the adaptation of *C. reinhardtii* to low levels of CO_2, the relationship of the intracellular carbonic anhydrase to its extracellular counterpart, and the regulatory events involved in the synthesis of both forms.

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