Plant Physiol. (1984) 76, 472–477
0032-0889/84/76/0472/06/$01.00/0

Identification of Extracellular Carbonic Anhydrase of *Chlamydomonas reinhardtii*[1]

Received for publication May 4, 1984

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ABSTRACT

We have examined the induction of carbonic anhydrase activity in *Chlamydomonas reinhardtii* and have identified the polypeptide responsible for this activity. This polypeptide was not synthesized when the alga was grown photoautotrophically on 5% CO_2, but its synthesis was induced under low concentrations of CO_2 (air levels of CO_2). In *CW-15*, a mutant of *C. reinhardtii* which lacks a cell wall, between 80 and 90% of the carbonic anhydrase activity of air-adapted cells was present in the growth medium. Furthermore, between 80 and 90% of the carbonic anhydrase is released if wild type cells are treated with autolysin, a hydrolytic enzyme responsible for cell wall degradation during mating of *C. reinhardtii*. These data extend the work of Kimpel, Togasaki, Miyachi (1983 Plant Cell Physiol 24: 255–259) and indicate that the bulk of the carbonic anhydrase is located either in the periplasmic space or is loosely bound to the algal cell wall. The polypeptide associated with carbonic anhydrase activity has a molecular weight of approximately 37,000. Several lines of evidence indicate that this polypeptide is responsible for carbonic anhydrase activity: (a) it appears following the transfer of *C. reinhardtii* from growth on 5% CO_2 to growth on air levels of CO_2, (b) it is located in the periplasmic space or associated with the cell wall, like the bulk of the carbonic anhydrase activity, (c) it binds dansylamide, an inhibitor of the enzyme which fluoresces upon illumination with ultraviolet light, (d) antibodies which inhibit carbonic anhydrase activity only cross-react with this 37,000 dalton species.

The photosynthetic characteristics of the green alga *Chlamydomonas reinhardtii* are dependent upon the CO_2 concentration experienced by the alga during growth (1, 2). Cells grown at air levels of CO_2 (0.03%) can utilize low CO_2 concentrations much more efficiently than cells grown at high levels of CO_2 (3–5%). Since the CO_2 concentration during growth has no effect on either the mechanism of photosynthetic CO_2 fixation or the K_m (CO_2) of the principal CO_2 fixing enzyme, RuBP[4] carboxylase (2), air-grown algae must employ another method for increasing the efficiency of CO_2 utilization. Recent work has demonstrated that the appearance of both CA activity and a mechanism for the active accumulation of C_i enable air-grown algae to photosynthesize efficiently at low levels of C_i (1, 12). Growth at high levels of CO_2 suppresses these activities. Most of the induced CA is located in the periplasmic space of *C. reinhardtii* (9). Together, CA and C_i transport function to maintain a high intracellular concentration of C_i which provides sufficient substrate to saturate RuBP carboxylase and eliminate oxygen inhibition of photosynthesis (1–3). Recently, Spalding *et al.* (14, 15) selected and characterized mutants of *C. reinhardtii* which were deficient in CA activity and C_i pumping, confirming that both of these activities are essential for the efficient utilization of C_i. Some progress has also been made toward establishing the intracellular factors necessary for adaptation of algae to low CO_2 (11, 13, 16). While CA and the C_i transport system increase following the transfer of *C. reinhardtii* from high to low levels of CO_2 and appear to be required for the maintenance of CO_2 fixation in an environment deficient in C_i, the exact manner in which these activities coordinate C_i accumulation is not fully known.

In this study, we have examined the adaptation of *C. reinhardtii* to air levels of CO_2. The photosynthetic capacity of the organism at low CO_2 concentrations rapidly increases after a shift from growth on high to low CO_2 and, 5 h following the transfer, the alga displays photosynthetic characteristics similar to those observed in air-grown cultures. We have compared the kinetic parameters of CO_2 fixation during adaptation with the induction of CA and have localized and identified the major species responsible for CA activity using both biochemical and immunological procedures.

MATERIALS AND METHODS

Chlamydomonas reinhardtii 2137 mt+ (obtained from Dr. M. Spalding, Michigan State University) and the cell wall-less mutant, *CW-15*, were cultured axenically in the minimal medium described by Spalding *et al.* (13) at 28°C and a light intensity of 300 $\mu E\ m^{-2}\ s^{-1}$ (400–700 nm). Cultures were vigorously shaken and bubbled with either 5% CO_2 in air or with air alone. All experiments were performed with cells in early to midphase exponential growth.

For the determination of $K_{1/2}$ (CO_2) and the maximal rate of photosynthesis, P_{max}, algae grown under the appropriate conditions were harvested by centrifugation (4,000*g*), resuspended in CO_2-free, 20 mM 3-(*N*-morpholino)propanesulfonic acid buffer (pH 7.2), and the rates of O_2 evolution at varying HCO_3^- concentrations were measured at saturating light intensity and 25°C with a Clark type O_2 electrode. CA activity in cell pressates, prepared as described below, was determined electrometrically and expressed in Wilbur-Anderson units (WA) (9, 18).

For studying the induction and purification of CA we used cultures of the *C. reinhardtii* mutant, *CW-15*. This mutant lacks a cell wall, and most of the CA synthesized by *CW-15* accumulates in the culture medium. The mutant was grown at high CO_2 and then transferred to air levels of CO_2 for 24 h. Cells were

[1] This research was funded by the Carnegie Institution of Washington, Publication No. 841 and National Science Foundation Grant PCM81-17958.

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[4] Abbreviations: RuBP, ribulose-1,5-bisphosphate; CA, carbonic anhydrase; C_i, inorganic carbon.

labeled *in vivo* using $^{35}SO_4^{2-}$ during growth on high CO_2 or after transferring the cultures from growth on high CO_2 to growth on air levels of CO_2. The cells were washed and resuspended in sulfate-free culture medium prior to the addition of the label (0.5 mCi/200 ml culture medium). To isolate the proteins secreted into the medium by *CW-15*, the cells were pelleted by low speed centrifugation (4,000*g*), and protease inhibitors (1 mM benzamidine-HCl, 5 mM ε-amino-*n*-caproic acid) were added to the supernatant prior to stirring with DEAE cellulose overnight at 4°C. The cellulose was then poured into a column, washed with 1 mM Tris-HCl (pH 8.0), and the proteins eluted from the column either with 0.5 M NaCl or a gradient from 0 to 0.5 M NaCl in 1 mM Tris-HCl (pH 8.0) (containing protease inhibitors). Fractions from the column were analyzed for CA activity and the peak fractions were concentrated by precipitations with 80% $(NH_4)_2SO_4$. To try and separate CA from other periplasmic proteins the $(NH_4)_2SO_4$ precipitate was passed over a Bio-Gel A-1.5m column.

CA was also identified by reacting an aliquot of the $(NH_4)_2SO_4$ concentrated growth medium (from the DEAE cellulose column) with dansylamide (5-dimethylaminapthalene-1-sulfonamide) which forms a specific and highly fluorescent complex with the enzyme (8). This sample was applied to a native polyacrylamide gel (Laemmli system [10], 6–15% gradient) and electrophoresed at 4°C. A single fluorescent band was located by exposure of the gel to UV irradiation. This band was excised from the gel, electroeluted and electrophoresed on a SDS polyacrylamide gradient (12–18% with 8 M urea) gel.

Fractions enriched in CA from wild type cells were prepared as previously described (7). Briefly, $^{35}SO_4^{2-}$ was used to label the proteins of wild type *C. reinhardtii*. The cells were broken at 6,250 p.s.i. in a chilled French Pressure Cell in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl plus protease inhibitors. After centrifugation at 31,000*g* for 10 min to eliminate most of the thylakoid membranes (which contain essentially no CA activity), the supernatant was subjected to a high speed centrifugation (135,000*g*) for 3 h. The majority of CA activity was always associated with the pellet (high speed pellet), although a substantial amount was still present in the supernatant (high speed supernatant).

For the enzymic degradation of the *C. reinhardtii* cell wall we used the hydrolytic enzyme preparation, autolysin. This enzyme, synthesized by *Chlamydomonas* gametes prior to their fusion, was isolated according to Tamaki *et al.* (17).

The initial experiments suggested that a protein with a mol wt of 37,000 was responsible for CA activity. Antibodies were prepared against this polypeptide. The culture medium from *CW-15* cells grown on air levels of CO_2 was fractionated by DEAE cellulose chromatography (as above). Protein was eluted from the DEAE column with 0.5 M NaCl (which eluted essentially all of the protein), concentrated by $(NH_4)_2SO_4$ precipitation (80%) and electrophoresced on a 7.5 to 15% polyacrylamide gel. The 37,000-D polypeptide was excised from the gel, electroeluted, combined with Freund's adjuvant, and injected into rabbits following an immunization schedule previously described (5).

Immunoglobulin was purified from rabbit serum (5) and the effect of the antibodies raised to the 37,000-D polypeptides on CA activity was examined by incubating the purified immunoglobulin fraction with concentrated growth medium from *CW-15* cultures grown on air levels of CO_2. The incubation was for 24 h at 4°C. The samples were kept agitated on a rotary shaker and the protease inhibitors Trasylol (0.5 units/ml) and benzamidine-HCl (1 mM) were added prior to the incubation. Following the incubation, the samples were centrifuged for 15 min in an Eppendorf microfuge and the supernatant was assayed electrometrically (18). The ratio of immunoglobulin to the protein present in the sample containing CA activity was varied to ensure a proper ratio of antibody to antigen. Crossed immunoelectrophoresis was performed according to the method of Chua and Blomberg (5).

RESULTS

As shown in Table I, the transfer of *C. reinhardtii* from growth at high CO_2 concentrations (5%) to air levels of CO_2 results in a substantial increase in the affinity of whole cells for CO_2. After a 5-h exposure to low levels of CO_2, the C_i concentration required for half-saturation of photosynthesis ($K_{1/2}[CO_2]$) was 20-fold lower than the initial affinity of the cells maintained on high CO_2 concentrations. With continued exposure to air there was some additional decrease in the $K_{1/2}(CO_2)$ with the alga reaching full adaptation to low concentrations of CO_2 16 to 24 h after transfer. During adaptation, a 20% decline in the CO_2-saturated rate of O_2 evolution (P_{max}), on a Chl basis, was also noted.

CA activity was measured in *C. reinhardtii* at various times following the transfer of algal cultures from growth on high to low concentrations of CO_2 (Fig. 1). Essentially no CA was present in 5% CO_2-grown cultures but after a 5-h induction period, the level of activity was 50% of the maximal activity reached after 24 h of exposure to air. Nearly all of the CA activity is eliminated if the cells are grown on 5% CO_2; however, only partial inhibition of activity is achieved at lower (2%) CO_2 concentrations (9, 16). Little or no CA activity appeared in the culture medium of

Table I. *Changes in the $K_{1/2}$ (CO_2) during the Adaptation of C. Reinhardtii to Air Levels of CO_2*

Time[a]	$K_{1/2}$ (CO_2)	P_{max}
h	*μM*	μmol $O_2 \cdot mg^{-1}$ Chl·h
0.00	56.7	226.4
0.75	39.5	221.3
1.50	17.0	208.9
2.25	8.1	190.8
3.00	3.6	202.5
4.00	2.8	179.0
5.00	2.6	185.1
24.00	2.2	183.7

[a] Time after the transfer of wild type *C. reinhardtii* from growth on 5% CO_2 to growth on air levels (0.03%) of CO_2.

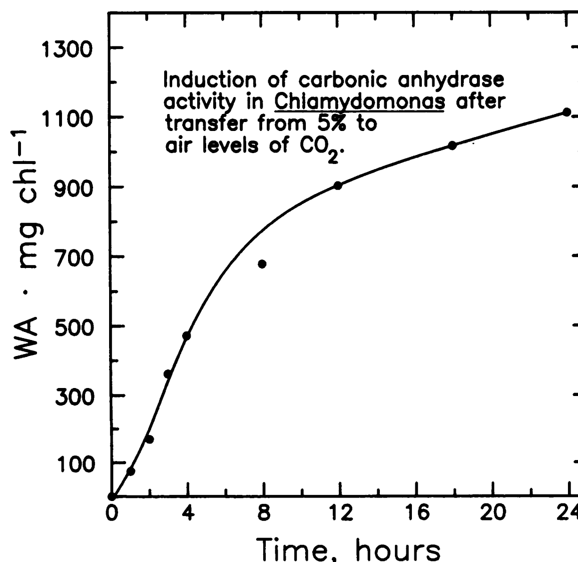

FIG. 1. Induction of CA activity in *C. reinhardtii* following transfer of cultures from 5% CO_2 to air levels of CO_2. CA activity was measured electrometrically and expressed in Wilbur-Anderson (WA) units (18).

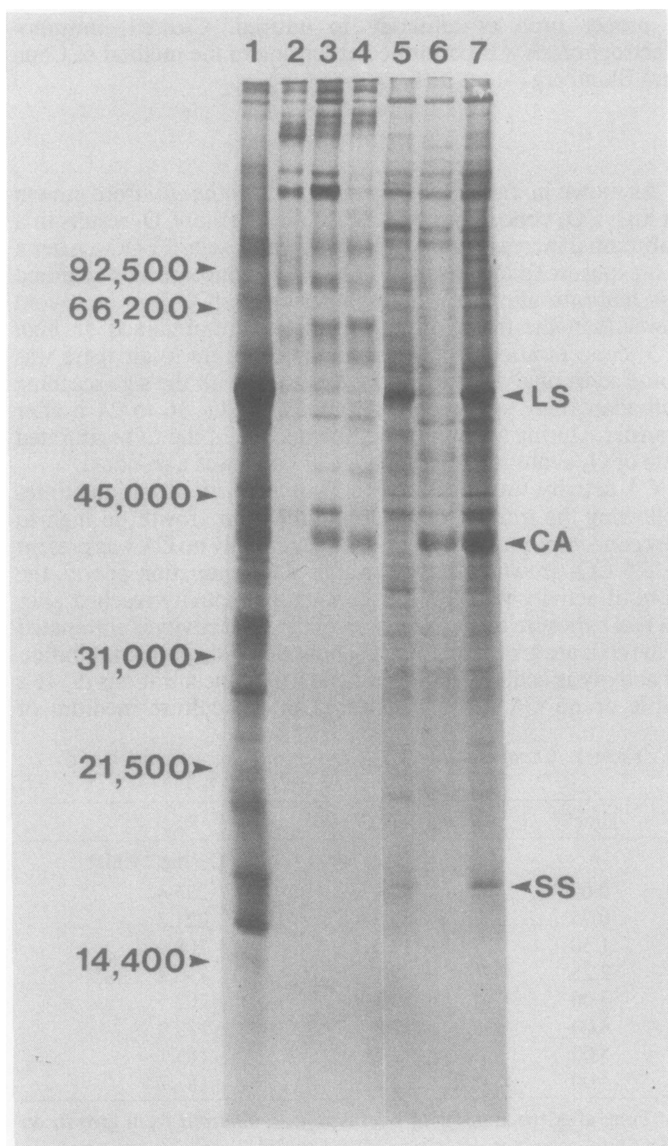

FIG. 2. Autoradiogram of newly synthesized polypeptides released into the medium by the cell wall-less mutant (*CW-15*) of *C. reinhardtii* or following autolysin treatment of wild type cells. The labeled polypeptides were concentrated by DEAE cellulose chromatography (all of the protein which bound to the DEAE cellulose was eluted with 0.5 M NaCl), and electrophoresed on polyacrylamide gels (as described in the "Materials and Methods"). The gels were dried and exposed to Kodak XAR-5 film to visualize newly synthesized polypeptides. Labeling was for 3 h at 28°C. Lane 1 shows newly synthesized polypeptides in the high speed pellet (see "Materials and Methods") from air-grown wild type cells. Lanes 2 to 4 show newly synthesized polypeptides released into the growth medium by *CW-15* grown on high CO_2 (lane 2), air (lane 4), and following transfer from high CO_2 to air (lane 3). Lanes 5 to 7 show newly synthesized polypeptides released into the growth medium after autolysin treatment of wild type cells grown on high CO_2 (lane 5), air (lane 7), or following transfer from high CO_2 to air (lane 6). The mol wt standards indicated in this figure are phosphorylase B (92,500), BSA (66,200), ovalbumin (45,000), bovine CA (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). The large subunit (LS) and small subunit (SS) of RuBP carboxylase and the presumptive CA monomer are also indicated.

normal wild type cells during this induction. However, using the cell wall-less mutant of *C. reinhardtii*, *CW-15*, which has initial kinetics of induction similar to those observed for wild type cultures (data not shown), the CA is released to the culture medium. The export of CA across the plasma membrane and into the medium suggests that a significant proportion of this enzyme is normally located, in wild type cells, within the periplasmic space (an area between the cell wall and the plasmalemma) or within the cell wall itself. In the absence of the cell wall, the enzyme is lost to the surrounding medium. Further evidence for the location of the enzyme was provided by treating air-adapted wild type cells with autolysin, a cell wall degrading enzyme synthesized by *C. reinhardtii* during mating. This treatment resulted in the release of more than 80% of the total assayable CA actvity into the medium (data not shown). Between 10 and 20% of the activity remains with the cell pellet (data not shown). CA was not being released as a result of cell lysis since only low levels of soluble proteins from the cytoplasm of *C. reinhardtii* were detectable in the medium, as shown in Figure 2, lanes 2 to 7. Both the large and the small subunits of RuBP carboxylase could be detected in the culture medium after treatment with autolysin. However, in three separate experiments the levels of those components ranged from 0.1 to 0.5% of the whole cell level (both in terms of protein and radioactivity). Furthermore, less than 0.5% of total soluble protein was found in the growth medium, again suggesting only a small amount of cell lysis. These results indicate that most of the newly synthesized enzyme (at least 80%) passes across the plasmalemma, although some CA is probably still located within the cell (possibly inside the chloroplast).

For the identification of a polypeptide associated with CA activity, we took advantage of the inducible nature of this enzyme and its appearance in the culture medium following the transfer of *CW-15* cultures from growth on high CO_2 to air. Polypeptides were labeled with $^{35}SO_4^{2-}$ for 3 h under conditions which either allowed or suppressed CA activity, and the polypeptides exported into the medium were analyzed by PAGE (Fig. 2, lanes 2 to 4). Wild type cells were used in similar experiments (Fig. 2, lanes 5 to 7) in which autolysin was employed to release periplasmic proteins into the culture medium. A polypeptide profile of a high speed pellet (see "Materials and Methods") derived from wild type cells is also presented (Fig. 2, lane 1). This fraction contains the majority of the CA activity (Table II), although a substantial amount of activity is also present in the high speed supernatant. Essentially no activity is in the thylakoid fraction.

Comparisons of the profiles of labeled polypeptides demonstrate that both wild type cells and *CW-15* synthesize a specific periplasmic polypeptide (mol wt 37,000) which is only present during growth on air (Fig. 2, lanes 4 and 7) or after the cultures are transferred from high CO_2 to air for 5 h (Fig. 2, lanes 3 and 6). Cells grown continuously on high CO_2 (Fig. 2, lanes 2 and 5) do not synthesize this polypeptide. In samples prepared from the autolysin-treated wild type cells, as previously noted, a small amount of cell lysis indicated by the release of RuBP carboxylase into the medium, occurs. However, the prominance of the 37,000-D protein after autolysin treatment is in complete agreement with the distribution and regulation of CA activity demonstrated in the experiments with cultures of *CW-15*. The 37,000-D protein is generally a broad band and may, on occasion, be resolved into two separate species (discussed below).

Table II. *CA Activity in Cellular Fractions from Wild Type C. reinhardtii*

The methods for preparing the different cellular fractions are described in "Materials and Methods".

Fraction	Total Activity
	%
High speed supernatant (135,000*g*)	42.2
High speed pellet (135,000*g*)	56.0
Thylakoids (31,000*g*)	1.8

To establish whether this inducible, 37,000-D species represents CA, we attempted to obtain an enriched fraction of the enzyme from the medium of *CW-15* cultures. Following a 24-h induction period, *CW-15* cells were removed from the culture medium by low speed centrifugation and the medium was fractionated by DEAE cellulose chromatography (eluted with a 0–0.5 M NaCl gradient) followed by gel filtration (described in "Materials and Methods"). The fractions showing the highest CA activity (the void volume of the gel filtration column, Bio-Gel A-1.5m, contained the peak activity) were analyzed by SDS-PAGE (Fig. 3, lanes 2–10). While many bands are observed in these profiles, the 37,000-D band is a prominent constituent. To confirm the possibility that the 37,000-D polypeptide represented the CA monomer, we used dansylamide, a fluorescent compound which complexes with CA (8). Proteins concentrated by $(NH_4)_2SO_4$ precipitation of growth medium from *CW-15* cultures were reacted with dansylamide, applied to a native polyacrylamide gel, and electrophoresed at 4°C. A single, fluorescent band was located by exposure of the gel to UV illumination (data not shown). This band was excised from the gel, electroeluted, and electrophoresed on a SDS polyacrylamide gradient gel. A diffuse band with a mol wt of approximately 37,000 D (Fig. 3, lane 1) comigrated with both the prominent constituent in the fractions containing peak CA activity from the agarose column (Fig. 3, lanes 2–10) and the polypeptide induced during CO_2 deprivation of *CW-15* and wild type cultures (Fig. 2). Denaturation and electrophoresis of greater quantities of the electroeluted CA does sometimes result in bands in addition to the 37,000-D species (an additional band at approximately 60,000 is occasionally observed). However, the appearance of these bands is variable and therefore most likely reflects contamination of the excised, native CA (from the nondenaturing gel) with other proteins. The data suggest that CA activity, induced when cultures of *Chlamydomonas* are exposed to low levels of CO_2, is associated with a polypeptide with an approximate mol wt of 37,000 D.

To confirm the identity of the CA monomer, we prepared antibodies against the 37,000-D polypeptide. The effect of this antibody on CA activity in the medium from cultures of *CW-15* is presented in Table III. The ratio of protein in the purified immunoglublin fractions from both preimmune serum and serum from rabbits injected with the 37,000-D polypeptide to protein in the CA enriched fraction was varied form 1:1 to 3:1.

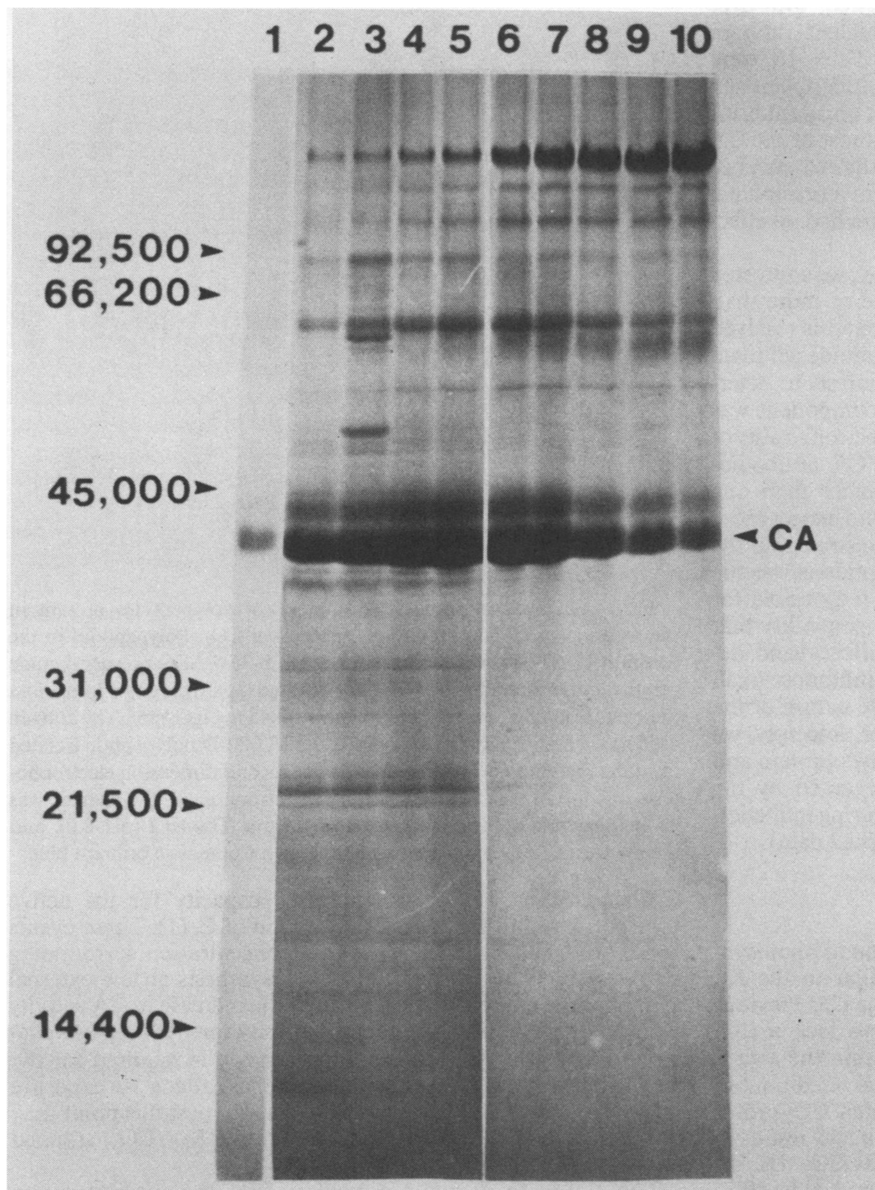

FIG. 3. Enrichment for CA by gel filtration and nondenaturing PAGE. The sample in lane 1 was prepared from a band exhibiting CA activity on a nondenaturing Laemmli gel (6–12% polyacrylamide). Activity on the gel was located by ultraviolet fluorescence of dansylamide, which associates tightly with CA (8). The nondenatured sample was excised from the gel, electroeluted, and re-electrophoresed on a denaturing polyacrylamide gel (lane 1). Lanes 2 to 10 show stained polypeptides from fractions exhibiting CA activity after a combination of DEAE cellulose (0.0–0.5 M NaCl gradient) and agarose gel column chromatography. The peak activity was found in the fractions of lanes 3 to 7. The mol wt standards indicated were as described in the legend of Figure 2. The large (LS) and small (SS) subunits of RuBP carboxylase and the band thought to be associated with CA activity are also indicated.

Table III. *Inhibition of CA Activity with Antibodies Prepared Against the 37,000-D Polypeptide from the Medium of CW-15*

Immunization of the rabbits with the 37,000-D polypeptide electroeluted from SDS polyacrylamide gels was according to the schedule of Chua and Blomberg (5). Incubation with either the immune or preimmune serum was for 24 h at 4°C. The carbonic anhydrase activity was measured after a 15-min centrifugation in an Eppendorf microfuge.

Protein in Preimmune serum to Protein in the Carbonic Anhydrase Preparation	Protein in Immune Serum to Protein in the Carbonic Anhydrase Preparation	WA Units	Inhibition of Carbonic Anhydrase
ratio			%
		16	0
1:1		17	0
2:1		16	0
3:1		17	0
	1:1	10	37
	2:1	5	69
	3:1	1	94

The CA activity is inhibited by the immunoglobulin fraction from immunized rabbits at all concentrations tested, with over 90% of the activity being suppressed at the highest ratio of antibody to antigen. The results presented in Table III were obtained after a brief centrifugation to pellet the antibody-antigen complex, although some inhibition (10–20%) was apparent prior to this centrifugation. These results suggest that most of the CA antibody is not directed against the active site of the enzyme, but is effective in complexing with the enzyme to form a precipitate. Immunoglobulin prepared from preimmune serum had no effect on the level of CA activity.

Since the CA monomer is a diffuse band on a gel, we addressed the question of whether this band contained one or more than one antigenic species. Sometimes the 37,000-D band is resolved into two components on a 7.5 to 15% polyacrylamide gel (data not shown). We used crossed immunoelectrophoresis to determine whether one or more than one antigenic component was present in the diffuse 37,000-D band (Fig. 4). Electrophoresis of this band into an agarose bed containing the CA antibodies results in one broad peak of precipitation. If more than one antigenic species were present in this peak we would expect more than one arc of precipitation. These results demonstrate that the antibody prepared against the 37,000-D polypeptide is specific for one antigenic component (the polypeptide responsible for CA activity). Although we cannot eliminate the possibility that some proteolysis may be responsible for the diffuse band described here (in spite of the addition of protease inhibitors to all solutions used in the isolation of CA), the diffuse nature of the species may reflect glycosylation of the monomer. Recently, we have shown that the 37,000 mol wt protein is a glycoprotein and its appearance in the extracellular space is prevented by the inclusion of tunicamycin in the growth medium during induction (6: J. R. Coleman and A. R. Grossman, unpublished data).

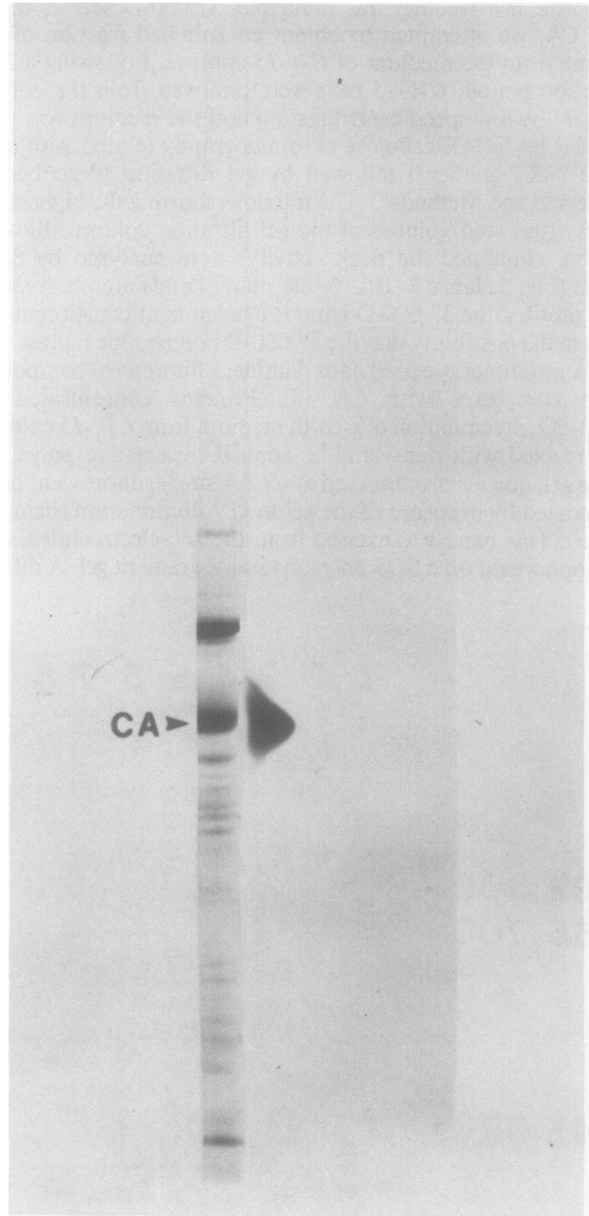

FIG. 4. Crossed immunoelectrophoresis of polypeptides present in the medium of *CW-15* grown on air levels of CO_2. Polypeptides in the medium of *CW-15* were separated on a 7.5–15% SDS polyacrylamide gel (first dimension) and then electrophoresed (at a right angle to the first dimension) into an agarose bed containing 0.45 mg IgG/cm^2. The antigen used to elicit antibody formation was the 37,000-D polypeptide isolated from the medium of *CW-15* cultures. The second dimension electrophoresis was for 16 h at 100 v. Following electrophoresis, the agarose was washed extensively with 0.15 M NaCl, 50 mM Tris-HCl (pH 8.0), and then with distilled H_2O prior to staining with Coomassie brilliant blue.

DISCUSSION

The C_i concentration required for half saturation of photosynthesis by high CO_2-grown *C. reinhardtii* is similar to the K_m (CO_2) of RuBP carboxylase isolated from the alga (2). Previous studies (1) suggest that the high CO_2-grown cells lack a CO_2 concentrating mechanism and the level of C_i within the alga is approximately the same as that of the external medium. In contrast, the $K_{1/2}$ (CO_2) of photosynthesis for high CO_2-grown cells transferred to air decreases rapdily with time and results in a substantial increase in the whole cell affinity for CO_2. During this adaptation period, *C. reinhardtii* exhibits increased levels of CA activity, as well as an increased capacity for the active transport and intracellular accumulation of C_i (1). These events appear to result in an elevated CO_2 concentration surrounding RuBP carboxylase and efficient photosynthesis at low external CO_2 concentrations. A comparison of the increase in CA activity with the increase in the whole cell affinity for CO_2 suggests that only a portion of the total induced activity is required for the large initial decline in the $K_{1/2}$ (CO_2) observed after a 5-h exposure to air. The continued increase of CA activity past this point may be required for the maximal reduction in the $K_{1/2}$ (CO_2) attained after extended periods of air exposure.

The ability to release CA activity into the medium upon

treatment of wild type *C. reinhardtii* with autolysin, together with the observation that this enzyme is exported into the culture medium by the cell wall-less mutant, *CW-15*, indicates that the majority of CA is localized in the periplasmic space or cell wall. This finding is in agreement with previous observations (9). Since CA would act to maintain a rapid equilibrium between HCO_3^- and dissolved CO_2, the location of this enzyme within the periplasmic space would be particularly useful if the velocity of active transport of C_i (whether HCO_3^- or CO_2 were the transported species) into the cell exceeds the uncatalyzed equilibration. CA in the periplasmic space would also act as a 'trap' for CO_2 which is passively diffusing out of the algal cell. By rapidly hydrating this CO_2, it would be immediately available for active transport back into the alga (if HCO_3^- were the transported species). This could help to establish a low CO_2 compensation point. The location of CA in the periplasmic space or cell wall would also be advantageous to *C. reinhardtii* when the aqueous environment is limited to a thin layer surrounding the organism, as would be the case for soil organisms (*C. reinhardtii* does grow in such environments). Rapid equilibration between CO_2 and HCO_3^- would allow for efficient utilization of C_i by the transport system (no matter which species was transported).

In vivo labeling of polypeptides using SO_4^{2-} during the adaptation of *Chlamydomonas* to air levels of CO_2 indicates that carbonic anhydrase activity appears as the result of the synthesis of a 37,000-D polypeptide. Its increased level probably reflects control at the transcriptional level (or posttranscriptional processing of the RNA) since we have not found translatable RNA for the CA monomer in cultures of *C. reinhardtii* grown on 5% CO_2. On the other hand, the translation of poly A RNA from both adapting cells and cells grown continuously on low levels of CO_2 yields a primary translation product which can be immunoprecipitated with antibodies raised against the 37,000-D polypeptide (J. R. Coleman and A. R. Grossman, unpublished data).

The polypeptide which we have associated with CA activity forms a broad band on SDS polyacrylamide gels which can sometimes be resolved into two diffuse bands. We have shown that the broad band contains one antigenic component and that antibodies prepared against this component inhibit CA activity. The morphology of the 37,000-D polypeptide and its resolution into two species may reflect different glycosylation states of the CA monomer, but may also result from proteolysis (in spite of the precautions taken).

In contrast to our finding, an earlier report has described CA of *Chlamydomonas* as a hexameric enzyme containing monomérs of 27,000-D (4). However, since only 80 to 90% of the CA is measured in the medium of cultures of *CW-15* (or after autolysin treatment of wild type cells), an intracellular form of the enzyme may also exist.

In sum, several lines of evidence have helped us establish the identity of the extracellular form of the *C. reinhardtii* carbonic anhydrase. The monomeric species for this enzyme is a diffuse band at 37,000 D which appears following the transfer of high CO_2 grown cells to air levels of CO_2. Like the majority of the CA activity, it is excreted into the culture medium by the cell wall-less mutant, *CW-15*. It is the major component of a nondenatured protein which binds dansylamide, a specific inhibitor of CA. And finally, antibodies prepared against the 37,000-D polypeptide inhibit CA activity, and although two species sometimes can be discerned from this diffuse band on a gel, the antibody reacts with only one antigenic component.

By clearly establishing the identity of the polypeptide responsible for CA activity and preparing an antibody to this constituent, we have set the foundation for analyzing other aspects of the response of *C. reinhardtii* to low levels of CO_2. For example, we have recently defined some of the events involved in the biosynthesis of CA and have determined that the monomer is a glycoprotein which is synthesized in the cytoplasm of the cell as a higher mol wt precursor (J. R. Coleman and A. R. Grossman, unpublished data). Using the antibody, we may be able to detect an internal species of CA and determine its subcellular location. Such an analysis would help us model a system which would more clearly explain the mechanism by which *C. reinhardtii* accumulates and utilizes C_i.

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