Membrane-Associated Polypeptides Induced in Chlamydomonas by Limiting CO₂ Concentrations

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ABSTRACT

Chlamydomonas reinhardtii and other unicellular green algae have a high apparent affinity for CO₂, little O₂ inhibition of photosynthesis, and reduced photorespiration. These characteristics result from operation of a CO₂-concentrating system. The CO₂-concentrating system involves active inorganic carbon transport and is under environmental control. Cells grown at limiting CO₂ concentrations have inorganic carbon transport activity, but cells grown at 5% CO₂ do not. Four membrane-associated polypeptides (M, 18, 21, 35, and 36 kilodaltons) have been identified which either appear or increase in abundance during adaptation to limiting CO₂ concentrations. The appearance of two of the polypeptides occurs over roughly the same time course as the appearance of the CO₂-concentrating system activity in response to CO₂ limitation.

By comparison with higher plants which use the C₃ photosynthetic pathway, Chlamydomonas reinhardtii and other unicellular green algae exhibit unusual photosynthetic characteristics. These characteristics include a very high apparent affinity for CO₂, little inhibition of photosynthesis by 21% O₂, and much reduced photorespiration (5). These characteristics apparently result from the operation of a CO₂-concentrating system that increases the intracellular CO₂ concentration to a level which suppresses photorespiration by competitive inhibition of RuBP oxygenase (1). The mechanism responsible for this effect requires energy, accumulates intracellular inorganic carbon against a very large concentration gradient, and includes carbonic anhydrase (EC 4.2.1.1) as an essential component of the system (1, 4, 17, 20, 22). A saturable component in the kinetics of inorganic carbon uptake has been demonstrated for Chlamydomonas (19), and an electrogenic process has been implicated in the inorganic carbon uptake of Chlorella (4). These points strongly suggest that the CO₂-concentrating system of unicellular algae involves active transport of inorganic carbon, probably across either the plasma membrane or the chloroplast envelope. Evidence has been presented to support either location of the transporter (3, 11–13, 16), so the actual location is still controversial.

A mutant of Chlamydomonas has been isolated and characterized which is apparently unable to accumulate inorganic carbon to any substantial extent (18). Although this mutant can be considered to be a putative transport mutant, so supports the inference that inorganic carbon transport is involved, the evidence is not conclusive. Direct physical evidence for a protein or proteins involved in transport of inorganic carbon is lacking.

Activity of the CO₂-concentrating system is under environmental control. Cells grown at limiting CO₂ concentrations (air-adapted) show inorganic carbon transport activity but cells grown at elevated CO₂ concentrations (CO₂-enriched) do not. We have identified four membrane-associated polypeptides which appear or increase in abundance during adaptation to limiting CO₂ concentrations. The appearance of two of the polypeptides coincides with induction of the CO₂-concentrating system following exposure of the cells to limiting CO₂ concentrations. One or more of these polypeptides may be candidates for direct involvement in inorganic carbon transport.

MATERIALS AND METHODS

Algal Strains and Culture Conditions

Chlamydomonas reinhardtii cell wall-less strain CW-15 mt+ (obtained from Dr. R. Togasaki, Indiana University) was cultured axenically in the minimal salts medium described by Winder and Spalding (21) with the exception that the pH of the MOPS-Tris buffer was 7.6 for CO₂-enriched cells and 7.1 for air-adapted cells. Both cell types were grown in liquid medium on a gyratory shaker (175 rpm). In addition, the CO₂-enriched cells were constantly aerated with 5% CO₂ in air. The pH of both algal cultures during growth was 7.0 to 7.1. All experiments were performed with cells in midphase exponential growth.

Induction Experiments

For induction time course experiments all cells, except air-adapted controls, were initially grown with CO₂ enrichment. The cells were collected by centrifugation, suspended in sulfate-free minimal salts medium with the appropriate pH buffer, and grown either with 5% CO₂ aeration (CO₂-enriched controls) or without aeration (air induction and air-adapted controls). To avoid any exposure to limiting CO₂, the medium for resuspension of the CO₂-enriched controls was pre aerated with 5% CO₂. Carrier-free [³⁵S]sulfate (1 mCi/L) was added to the cultures at time intervals and cells harvested 30 min after introduction of the label.

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2 Abbreviations: RuBP, ribulose-1,5-bisphosphate; ACA,  α-amino-n-caproic acid; BAM, benzamidine; IEF, isoelectric focusing; MOPS, 3-(N-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.
Cell Fractionations and Electrophoresis

Cells from 800 mL of culture were harvested by centrifugation (2000 rpm, Sorvall GSA or GS3 rotor, 10 min), resuspended in buffer A (0.3 M sucrose, 10 mM EDTA, 10 mM ACA, 2 mM BAM, 10 mM Tris-HCl, pH 7.5), collected again by centrifugation (5000 rpm, Sorvall SS-34 rotor, 5 min), and resuspended in 5 mL of buffer A plus 0.125 mL PMSF (40 mM in isopropanol). The cells were homogenized thoroughly (750 rpm, 50–100 passes) in a Potter-Elvehjem tissue homogenizer with a motor-driven Teflon pestle. The cell homogenates were centrifuged (2000 rpm, SS-34 rotor, 5 min) to remove unbroken cells and starch grains. The supernatant was recentrifuged (5000 rpm, SS-34 rotor, 30 min) to collect a low-speed membrane pellet. The resulting supernatant was again centrifuged (19,000 rpm, SS-34 rotor, 30 min) to collect a high-speed membrane pellet and a soluble protein fraction. The above procedures were all performed at 4°C.

SDS polyacrylamide gel electrophoresis was performed using the method of Laemmli (10, 14) on 10 to 18% acrylamide gradient gels at room temperature. The gels were either silver-stained (9) for protein or impregnated with PPO (15), dried and used for fluorographic detection of labeled polypeptides. Molecular size markers used for silver-stained gels were β-galactosidase (116 kD), phosphorylase b (97 kD), BSA (68 kD), glutamate dehydrogenase (53 kD), ovalbumin (43 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), soybean trypsin inhibitor (20 kD), and Cyt c (12 kD). The 14C-labeled molecular size markers (Bethesda Research Labs) for fluorography of 35S-labeled gels were myosin (200 kD), phosphorylase b (97 kD), BSA (68 kD), ovalbumin (43 kD), α-chymotrypsinogen (26 kD), β-lactoglobulin (18 kD), and lysozyme (14 kD).

Assays of Photosynthesis and Internal Inorganic Carbon Accumulation

Inorganic carbon accumulation was determined as previously described (1, 19). Cells were exposed to NaH14CO3 (60 μM, pH 7.0) for 30 s prior to centrifugation through silicone oil. Photosynthetic rate was estimated simultaneously from the acid-stable 14C in the pellet following centrifugation of the cells through silicone oil.

RESULTS

Observation of the polypeptide profiles of total cell extracts from CO2-enriched and air-adapted Chlamydomonas cells revealed no obvious differences between the two when compared by SDS-PAGE (Fig. 1). There also were no obvious differences in the polypeptide profiles of the soluble protein fraction from the two cell types (Fig. 1). However, when low-speed and high-speed membrane fractions from the two cell types were compared, four membrane-associated polypeptides were apparent in the membrane fractions of the air-adapted cells which were either absent or present in very reduced amounts in the same fractions from CO2-enriched cells (Fig. 1). These included two polypeptides of approximately 35 and 36 kD, one polypeptide of approximately 21 kD, and one polypeptide of approximately 19 kD. Appearance of the 19 and 35 kD polypeptides was somewhat variable, although it is possible they were not always completely resolved from other polypeptides of similar size. The two polypeptides at 35 and 36 kD and the 19 kD polypeptide appeared to be more abundant in the low-speed membrane fraction, while the 21 kD polypeptide appeared more abundant in the high-speed membrane fraction.

Because of a substantial presence of thylakoid membranes in both membrane fractions, purified thylakoid membranes were prepared as described by Greer et al. (8) from CO2-enriched and air-adapted cells and their polypeptide profiles compared, by SDS-PAGE, with each other and with the crude, low-speed membrane fraction shown in Figure 1. The 19 and 21 kD polypeptides were absent from the purified thylakoids. There appeared to be some slight retention of the 35 and 36 kD polypeptides in the purified thylakoids from the air-adapted cells, but the relative abundance was much decreased compared with the crude membrane fraction (not shown). This indicates that it is unlikely any of the four air-specific polypeptides are resident in the thylakoid membranes.

Appearance of newly synthesized polypeptides following exposure of CO2-enriched cells to limiting CO2 was investigated using 35S-labeling (Fig. 2). Using this method, a 21 kD polypeptide was apparent in the whole-cell profile 2 h following transfer to limiting CO2. In addition, two new polypeptides at about 45 to 50 kD appeared to be heavily labeled in both the whole-cell and soluble-protein profiles following exposure to limiting CO2, and the large and small subunits of Rubisco exhibited reduced relative labeling in both profiles. In the low-speed membrane fraction, labeling of the 21 and the 36 kD polypeptides were apparent 2 h following transfer to limiting CO2. The other two air-specific polypeptides were not readily observed at 2 h following transfer but, because of the lower resolution afforded by fluorography, the 35 kD polypeptide might have been obscured by the larger one. A similar pattern was observed in the high-speed membrane fraction.

Since all four polypeptides were present in the low-speed membrane fraction, a more detailed 35S-labeling time course was performed to further investigate the appearance of newly synthesized polypeptides in this fraction following transfer of CO2-enriched cells to limiting CO2 (Fig. 3). The labeled polypeptides at 21 and 36 kD were apparent on the fluorograph by only 1 h following transfer and were prominent thereafter. When the low-speed membrane fractions from the same time course were separated by SDS-PAGE and silver-stained, all four of the air-specific polypeptides appeared over the 8-h time course (Fig. 4). In agreement with the fluorograph in Figure 4, traces of the 21 and the 36 kD polypeptides appeared after only 2 h of limiting CO2, and the two polypeptides were quite apparent after 4 h and remained prominent thereafter. The 19 and the 35 kD polypeptides were observed after 4 h in limiting CO2 but were not present in substantial amounts even after 8 h.

For purposes of comparison with the appearance of air-specific polypeptides, a time course was performed for increase in photosynthetic rate at limiting CO2 and capacity for inorganic carbon accumulation in CO2-enriched Chlamydomonas cells exposed to limiting CO2 (Fig. 5). Both were found to increase slightly during the first hour, to increase substantially between 1 and 8 h and to reach by 8 h nearly the level observed with 8 h following transfer to limiting CO2. These
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Figure 1. Silver-stained SDS-PAGE gel of fractions from CO₂-enriched (lanes 2, 4, 6, and 8) and air adapted (lanes 3, 5, 7, and 9) Ch. reinhardtii cells. The fractions are: total protein (lanes 2 and 3), low-speed membrane fraction (lanes 4 and 5), high-speed membrane fraction (lanes 6 and 7), and soluble proteins (lanes 8 and 9). Lane one contains molecular size markers. Arrowheads indicate polypeptides apparently specific for air-adapted cell fractions.

Figure 2. Fluorograph of SDS-PAGE gel of ³⁵S-labeled fractions from CO₂-enriched (lanes 3, 6, 9, and 12), air-adapted (lanes 5, 8, 11, and 14), and CO₂-enriched Ch. reinhardtii cells exposed to limiting CO₂ for 2 h (lanes 4, 7, 10, and 13). Labeled polypeptide patterns are shown for total cell protein (lanes 3, 4, and 5), low-speed membrane fractions (lanes 6, 7, and 8), high-speed membrane fractions (lanes 9, 10, and 11) and soluble proteins (lanes 12, 13, and 14). Lanes 1, 2, and 15 contain ¹⁴C-labeled molecular size markers. Arrowheads in lanes 3 and 14 indicate the large and small subunits of Rubisco. Arrowheads in other lanes indicate polypeptides apparently induced by limiting CO₂.

increase correlated well with the appearance of the 21 and 36 kD polypeptides.

DISCUSSION

The work described in this paper has demonstrated the presence of four membrane-associated polypeptides of 19, 21, 35, and 36 kD apparent Mₜ specific to air-adapted Chlamydomonas reinhardtii cells when compared with CO₂-enriched cells. Although not demonstrating involvement in the microalgal CO₂-concentrating system, the air-specific nature of the polypeptides is consistent with some potential involvement in the system. Since the polypeptides are all associated
with membranes, it is additionally possible that one or more of them might be involved in inorganic carbon transport.

It is also not clear with which membrane(s) the polypeptides are associated, although it seems clear they are not specifically associated with the thylakoid membranes. Crude membrane fractions were purposefully used in this study, because there is some controversy over the location of the inorganic carbon transport in eukaryotic microalgae (16). Following purification of the proteins and antibody production, we hope to be able to establish unambiguously the intracellular location using immunohistochemical methods.

Coleman et al. (7) have previously demonstrated the specific occurrence of a 37 kD, soluble, periplasmic carboxylic anhydrase in air-adapted versus CO$_2$-enriched *Chlamydomonas* cells, and Coleman and Grossman (6) have identified a 20 kD soluble peptide specifically labeled with $^{35}$S$^{-}$ during adaptation of CO$_2$-enriched cells to limiting CO$_2$. In addition, Badour and Kim (2) have reported air-specific polypeptides of 42, 22, and 20 kD in detergent extracts of whole cells of *Chlamydomonas segnis*. None of these polypeptides has been demonstrated to have any association with membranes but might be involved in the CO$_2$-concentrating system in some way.

A soluble, 20 kD air-specific polypeptide was reported by Coleman and Grossman (6). We observed no obvious air-specific polypeptides of any size in our soluble fraction by either protein staining or $^{35}$S-labeling, but did occasionally observe a 21 kD soluble polypeptide by both methods to be faintly but specifically present in the soluble fraction of air-adapted cells (not shown). Ehrhardt and Grossman (6) also reported two polypeptides

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**Figure 3.** Fluorograph of SDS-PAGE gel of low-speed membrane fractions from CO$_2$-enriched (lane 2), air-adapted (lane 7), and CO$_2$-enriched cells transferred to limiting CO$_2$ for 1 h (lane 3), 2 h (lane 4), 4 h (lane 5), and 8 h (lane 6). Lanes 1 and 8 contain molecular size markers. Arrowheads indicate polypeptides apparently induced by limiting CO$_2$.

**Figure 4.** Silver-stained SDS-PAGE gel of low-speed membrane fractions from CO$_2$-enriched (lane 2), air-adapted (lane 7), and CO$_2$-enriched *Ch. reinhardtii* cells transferred to limiting CO$_2$ for 1 h (lane 3), 2 h (lane 4), 4 h (lane 5), and 8 h (lane 6). Lanes 1 and 8 contain molecular size markers. Arrowheads indicate polypeptides apparently induced by limiting CO$_2$.

**Figure 5.** Time course of increase in photosynthetic $^{14}$CO$_2$ fixation at limiting CO$_2$ (○) and internal inorganic carbon accumulation (●) following transfer of CO$_2$-enriched *Ch. reinhardtii* to limiting CO$_2$. Assays for both parameters were performed simultaneously 30 s after introduction of $^{14}$CO$_2$ (60 $\mu$M NaHCO$_3$, pH 7.0).

The apparent discrepancy with the work of Coleman and Grossman, but our soluble fraction was a 40,000g supernatant while theirs was a 135,000g supernatant. It is possible that the 20 kD polypeptide would be enriched, and therefore more apparent, in a 135,000g supernatant. Since we were specifically interested in membrane-associated polypeptides, this was not investigated further.
between 45 and 50 kD which were heavily $^{35}$S-labeled early in a time course of adaptation of CO$_2$-enriched *Chlamydomonas* cells to limiting CO$_2$. They observed a simultaneous decrease in the relative labeling of the Rubisco large and small subunits. The results described in this paper confirm those observations (Fig. 2).

Although it cannot be ruled out at this point, it seems unlikely that either the 35 or 36 kD polypeptide described in this paper corresponds to the 37 kD periplasmic carbonic anhydrase described by Coleman et al. (7). In the cell-wallless *Ch. reinhardtii* strain (CW-15) used here the periplasmic carbonic anhydrase is released into the growth medium (7) so would probably not be present in the cell extracts described in this paper. In the work described here, no evidence of a 37 kD air-specific polypeptide was seen by either protein staining or $^{35}$S-labeling in either the whole-cell or soluble protein fractions. It is still possible that the 36 or 35 kD (or both) polypeptide reported here represents a membrane-associated form of the periplasmic carbonic anhydrase.

It is not clear whether any of the air-specific polypeptides reported by Badour and Kim (2) correspond to any of those reported in this paper. Since Badour and Kim extracted whole cells with a detergent mixture (2% Nonidet P40 and 0.04% SDS), it was not established whether the polypeptides reported by them were soluble or membrane-associated. Badour and Kim identified the air-specific polypeptides following two-dimensional gel electrophoresis (IEF and SDS-PAGE). Because of the difficulties inherent in IEF of membrane proteins, it is not clear that membrane proteins would have been readily observed in their protocol. A large smear of protein was apparent on the alkaline edge of their two-dimensional gels which may represent many of the membrane proteins. It is possible, however, that the 22 and 20 kD air-specific polypeptides reported by Badour and Kim (2) correspond to the 21 and 19 kD air-specific polypeptides described in this paper.

In addition to demonstrating four membrane-associated, air-specific polypeptides, a comparison of the kinetics of appearance of the four polypeptides with the appearance of the functional activity of the CO$_2$-concentrating system revealed that two of the polypeptides (M, 21 and 36 kD) appeared with roughly the same kinetics as the CO$_2$-concentrating system. This correlation is consistent with a possible role in the CO$_2$-concentrating system, though it does not prove any involvement. The other two air-specific polypeptides (M, 19 and 35 kD) were somewhat variable in their appearance and had a delayed appearance relative to the increase in the activity of the CO$_2$-concentrating system, so would appear to be less likely to be directly involved in the system. However, some involvement of these two polypeptides cannot be ruled out.

In conclusion, four membrane-associated polypeptides have been demonstrated to be specifically present in air-adapted but not CO$_2$-enriched *Chlamydomonas* cells. The appearance of two of these polypeptides (M, 21 and 36 kD) during induction of the CO$_2$-concentrating system correlate well with increase in the activity of the system. Although the results reported here have the possibility that one or more of these membrane-associated, air-specific polypeptides might be directly or indirectly involved in the microalgal CO$_2$-concentrating system, no involvement has actually been demonstrated.

**LITERATURE CITED**


