Short Communication

Activation and De Novo Synthesis of Hydrogenase in Chlamydomonas

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ABSTRACT

Two distinct processes are involved in the formation of active hydrogenase during anaerobic adaptation of Chlamydomonas reinhardtii cells. In the first 30 minutes of anaerobiosis, nearly all of the hydrogenase activity can be attributed to activation of a constitutive polypeptide precursor, based on the insensitivity of the process to treatment with cycloheximide (15 micrograms per milliliter). This concentration of cycloheximide inhibits protein synthesis by greater than 98%. After the initial adaptation period, de novo protein synthesis plays a critical role in the adaptation process since cycloheximide inhibits the expression of hydrogenase in maximally adapted cells by 70%. Chloramphenicol (300 micrograms per milliliter) has a much lesser effect on the adaptation process.

Incubation of cell-free extracts under anaerobic conditions in the presence of dithionite, dithiothreitol, NADH, NADP, ferredoxin, ATP, Mg++, Ca++, and iron does not lead to active hydrogenase formation. Furthermore, in vivo reactivation of oxygen-inactivated hydrogenase does not appear to take place.

The adaptation process is very sensitive to the availability of iron. Iron-deficient cultures lose the ability to form active hydrogenase before growth, photosynthesis, and respiration are significantly affected. Preincubation of iron-deficient cells with iron 2 hours prior to the adaptation period fully restores the capacity of the cells to synthesize functional hydrogenase.

Many eucaryotic algae contain hydrogenase, but the activity of this enzyme is only observed after the cells have undergone a period of anaerobic incubation. This process has been termed "adaptation" by Gaffron (3). Protein synthesis inhibitors have been shown to decrease the expression of hydrogenase activity, suggesting that the adaptation process involves de novo protein synthesis. However, the presence of hydrogenase activity very shortly after the onset of anaerobiosis has been implicated as evidence for activation of constitutive hydrogenase in a process not requiring protein synthesis. Several reports have suggested that an anaerobic adaptation period is not required for hydrogenase activity if glucose or other additions are supplied to the cells (2, 13), but these results are difficult to interpret since the cells were in fact subjected to anaerobiosis in order to measure hydrogenase activity.

Oesterheld (9) provided evidence suggesting that protein synthesis is required during adaptation of Ankistrodesmus braunii, based on the effects of puromycin, gentamycin, and actinomycin D on the expression of hydrogenase activity. Likewise, Klein and Betz (7) showed that cycloheximide and chloral hydrate inhibited H2 photoproduction in Chlamydomonas moewusii. However, actinomycin D and chloramphenicol were not effective inhibitors of hydrogenase-mediated reactions in Chlorella pyenoidosa when cells were anaerobically incubated for short periods of time (12). Unfortunately, no information was provided by these authors as to the effectiveness of the inhibitors used on total protein synthesis, and therefore it is not possible to estimate the amount of total hydrogenase contributed by de novo protein synthesis and by activation of a constitutive, inactive precursor.

Iron has also been shown to affect the adaptation process. Several reports have indicated lowered hydrogenase levels in cells grown in media containing low iron concentrations (6, 14). Also, the addition of iron chelators to cell suspensions has been shown to inhibit the adaptation process in Scenedesmus obliquus (4).

In this paper, we report on the relative contributions of hydrogenase activation and de novo protein synthesis during the anaerobic adaptation period for the green alga Chlamydomonas reinhardtii. In addition, the role of iron in the adaptation process will be discussed.

MATERIALS AND METHODS

Culture and Adaptation Conditions. Chlamydomonas reinhardtii 137C (+) was grown photoheterotrophically in shaker flasks in an acetate-containing minimal medium as described previously (10). Cell suspensions were made as described in the individual figure legends and adapted under a stream of argon (99.995% purity) at 25°C for the indicated period of time.

Preparation of Cell-Free Extracts. Anaerobically adapted cells were disrupted for hydrogenase activity measurements by dilution of cell suspensions into 50 mM Mops (pH 6.8) containing 10 mM dithionite and 0.25% Triton X-100 (final concentrations) such that the Chl concentration was 15 to 20 μg/ml.

Polarographic H2 and O2 Measurements. H2 production was measured with a Clark-type electrode at 25°C as described previously (10), using 3.3 μM Fd isolated from C. reinhardtii as the electron mediator. Photosynthetic O2 production was also measured at 25°C with a Clark-type electrode using cells suspended in growth medium equilibrated under an atmosphere of 1.5% CO2 in air. Saturating light was provided by a tungsten lamp.

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* Abbreviations: Mops, morpholinopropanesulfonic acid.
HYDROGENASE FORMATION IN CHLAMYDOMONAS

RESULTS AND DISCUSSION

The adaptation process leading to the expression of hydrogenase activity in C. reinhardtii appears to involve both de novo protein synthesis and activation of a constitutive precursor polypeptide. During the initial 30 min of anaerobiosis, cycloheximide (15 μg/ml) has no effect on the appearance of hydrogenase activity, despite a 1-h aerobic preincubation period with the antibiotic (Fig. 1). This finding indicates that during this time period nearly all of the hydrogenase activity can be attributed to activation of a preexisting polypeptide in a process that does not require protein synthesis. However, after a 5-h adaptation period (which is the time normally required for the appearance of maximal hydrogenase activity) cycloheximide inhibits the expression of hydrogenase activity by nearly 70%. Thus, complete adaptation mainly involves synthesis of hydrogenase, or some other protein required for the production of functional hydrogenase, on cytoplasmic ribosomes. If it is true that hydrogenase is synthesized in the cytoplasm, it would be necessary to transport the protein into the chloroplast where it exists in the active state (8). A similar situation exists for the small subunit of ribulose bisphosphate carboxylase/oxygenase, which is synthesized on cytoplasmic ribosomes as a higher mol wt precursor and then transported into the chloroplast and cleaved to the functional peptide (5).

In order to ascertain that protein synthesis was successfully inhibited by the concentration of cycloheximide used, an experiment was performed in which the incorporation of [14C]arginine into soluble protein was followed. Cells were aerobically incubated with [14C]arginine in the presence or absence of cycloheximide, and samples were removed after various times for analysis. Figure 2 indicates that 15 μg/ml cycloheximide does, in fact, inhibit both protein and Chl synthesis by greater than 98%. Samples of these same cells which had undergone aerobic treatment with cycloheximide for various periods of time were then placed under dark, anaerobic conditions for 3 h. Despite the apparent inability of these cells to synthesize protein, active hydrogenase could still be formed (Fig. 2). The hydrogenase activity present after the 3-h adaptation period declines as the aerobic incubation time with cycloheximide increases, however. This decrease may reflect turnover of the constitutive polypeptide precursor.

A recent report by Maione and Gibbs (8) indicated that anaerobically incubated chloroplasts isolated from nonadapted C. reinhardtii cells were capable of carrying out hydrogenase-mediated photoreduction at a rate which was approximately one-third of the rate observed in whole cells. It is possible that the activity reported by these investigators may represent the cycloheximide-insensitive hydrogenase activity that we have observed.

Fig. 1. Effects of cycloheximide and chloramphenicol on adaptation of C. reinhardtii cells. Cells were suspended in 50 mM MOPS (pH 6.8) containing 17 mM FeCl3 and 17 mM EDTA at a concentration of 40 μg Chl/ml and allowed to preincubate under normal culture conditions for 1 h in the presence of the antibiotics. Cell-free extracts were made at various times after the onset of anaerobiosis (0 h) and assayed for hydrogenase activity as described in "Materials and Methods." Additions were as follows: 15 μg/ml cycloheximide (●); 500 μg/ml chloramphenicol (▲); no additions (○).

Fig. 2. Effects of cycloheximide on hydrogenase activity, protein synthesis, and Chl concentration of C. reinhardtii cultures. [14C]Arginine and cycloheximide were added to cell cultures at 0 h, followed by incubation under normal aerobic culture conditions. At the indicated times, samples were removed and analyzed for Chl content and for the amount of [14C]arginine incorporated into soluble protein. Samples were also removed and placed under anaerobic conditions for 3 h for hydrogenase activity determinations. (O, △, □), Control cells; (●, ▲, ■), cells incubated in the presence of 15 μg/ml cycloheximide. Hydrogenase activity (●, △, □); incorporation of [14C]arginine into soluble protein (▲, ■, ■); Chl concentration of cultures (▲, △).

fiber optic system. When used, dimethylbenzquinone was injected directly into the electrode chamber to a final concentration of 1 mM. Dark respiratory O2 uptake was also measured polarographically.

Measurement of Protein Synthesis. Cells from an early stationary phase culture were harvested and resuspended in fresh medium at a concentration of 25 μg Chl/ml. A portion of this suspension (100 ml) was placed into each of two 250-ml flasks. At time zero, 5 μM [14C]arginine (8.33 mCi/mmol) was added to each flask, and 15 μg/ml cycloheximide was added to one flask. The flasks were placed on a shaker under fluorescent lights (10.5 W/m2) and, at various time points, 2-ml samples were removed from each flask and immediately frozen in liquid N2 for subsequent 14C incorporation determinations. Samples were also removed at this time and placed under anaerobic conditions for 3 h in order to determine the capacity of cells for active hydrogenase formation. The frozen cells were thawed, refrozen, and thawed again, followed by removal of cellular debris by centrifugation at 800 g for 10 min. One ml of the supernatant solution was added to 1 ml of cold 10% TCA and allowed to sit on ice for 30 min. The precipitated protein was collected by positive pressure filtration through a Whatman GF/C filter on top of a Millipore 0.45 μm nitrocellulose filter. The collected precipitate was then washed with an additional 23 ml of cold 5% TCA. The filter was then placed in 5 ml of Beckman HS scintillation cocktail for counting. Corrections for quenching were made by the H# program of a Beckman LS900 scintillation counter.

Chl Determinations. Chl was quantified spectrophotometrically by the method of Arnon (1).
cycloheximide (A). Amol compounds, for (A); growth as Oxygen thesis, subsequent pended active and Fe mM), hydrogenase in vitro by a extracts cell-free cation anaerobic or transport is due to secondary effects in this study. Chloramphenicol (500 µg/ml) is much less inhibitory than cycloheximide (Fig. 1). It is not clear whether this slight inhibition is due to secondary effects of the antibiotic or to a possible requirement for peptides of chloroplastic origin in the processing or transport of hydrogenase.

Hydrogenase activity is destroyed upon exposure of anaerobically adapted cells to O2. If the cells are again placed under anaerobic conditions, the kinetics for active hydrogenase formation are identical to that of the initial adaptation period. Thus, it is unlikely that the activation process simply involves modification of O2-inactivated hydrogenase. It appears that upon inactivation by O2, the active form of hydrogenase is irreversibly damaged.

In an effort to determine the nature of cycloheximide-insensitive hydrogenase activation, we attempted to produce active hydrogenase in vitro by a variety of treatments. Various combinations of dithionite (10 mM), DTT (2 mM), Fe (10 µM), NADH (1 mM), NADP (1 mM), ATP (1 mM), Mg2+ (1 mM), Ca2+ (1 mM), and Fe (100 µM) failed to convert the hypothetical precursor into an active form of hydrogenase when included with sonicated cell-free extracts (300 µg Chl/ml) incubated under an argon or H2 atmosphere. In addition, combining equal volumes of extracts from adapted and nonadapted cells under anaerobic conditions did not lead to greater-than-expected hydrogenase activity. The nature of the activation process therefore remains unknown.

In a previous publication (11), we provided evidence that algal hydrogenase is an iron-containing protein. Therefore, it is not unusual that past investigations have suggested a role for iron in the adaptation process. Yamanagi and Sasa (14) reported an apparent correlation between the hydrogenase activity of synchronously dividing Scenedesmus cells and the iron content of the growth medium, although cell cycle effects may have been a more important determinant of hydrogenase activity in these studies. Iron chelators have also been shown to inhibit the formation of active hydrogenase in Scenedesmus (4). It was therefore of interest to determine the effect of iron deficiency on the adaptation of C. reinhardtii cells. Figure 3 shows values for growth (measured as the increase in culture Chl concentration), photosynthesis, respiration, and hydrogenase activity measured at various times after an inoculum of iron-replete cells was added to an iron-deficient medium. Growth is exponential for the first 20 to 25 h after inoculation. Likewise, a substantial decrease in photosynthesis does not occur until more than 19 h after the time of inoculation. From this point on, photosynthetic O2 production with both CO2 and dimethylbenzoquinone as the electron acceptor follows the same time course of inactivation, suggesting that iron deficiency has a deleterious effect on the functioning of PSI1, and that the availability of the iron-sulfur protein FeFd is not rate-limiting under these conditions. A 2-h aerobic incubation with added iron (followed by up to 3 h of anaerobic incubation) does not lead to a significant recovery of the photosynthetic capacity of these cells. Dark respiration is constant throughout the duration of the experiment.

Expression of hydrogenase activity during anaerobic incubation is affected at an earlier stage of iron deficiency than growth and photosynthesis. Hydrogenase activity was determined after a 3-h adaptation period for cells aerobically preincubated for 2 h with either no additions (control) or with added iron (17 µM) in the presence or absence of cycloheximide (15 µg/ml). Preincubation of cells collected at the time of inoculation with iron has little effect on the amount of active hydrogenase produced. Cycloheximide inhibits hydrogenase expression by approximately 62% at this point. Twelve h later, the control rate remains unchanged, although approximately 80% of cycloheximide-insensitive hydrogenase activity was lost, suggesting that the hydrogenase precursor is either unstable or produced in lesser quantities when the iron supply of the cells is reduced to low levels. During the next 7 h, the control rate of hydrogen production decreases to about 20% of the initial level, although the addition of iron 2 h prior to the adaptation period fully restores the capacity for hydrogenase formation. In fact, the hydrogenase activity of cells resupplied with iron is about twice as high as the initial control rates. The reason for this enhanced activity is not known, although it is possible that iron-limited C. reinhardtii cells produce siderophores which allow higher rates of uptake when iron is again made available. The rapid resumption in hydrogenase production capacity after the addition of iron to starved cultures suggests that the primary effect of iron deficiency involves the synthesis of the iron-sulfur group believed to be an integral part of the hydrogenase polypeptide.

The results of this investigation suggest that the formation of active hydrogenase during anaerobic incubation of C. reinhardtii cells is due in part to the activation of a constitutive polypeptide precursor (accounting for approximately one-third of the total activity) and to cytoplasmic de novo synthesis of hydrogenase, or some other protein required for the expression of hydrogenase activity. The molecular events occurring during the activation process are unknown at the present time. It is clear, however, that cells must have an adequate iron supply for maximal hy-

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**Fig. 3.** Effects of iron limitation on hydrogenase activity, photosynthesis, respiration, and growth of C. reinhardtii. Normal growth medium lacking added iron was inoculated with iron-replete cells at 0 h. At the indicated times, cells were harvested from culture samples and resuspended in the same medium at a Chl concentration of 20 µg/ml for subsequent measurements. A. Photosynthesis, respiration, and growth. Oxygen flux (in µmol·mg⁻¹·Chl·h⁻¹) was determined polarographically as described in "Materials and Methods." Gross photosynthetic O2 production with CO2 as the oxidant (●); photosynthetic O2 production with dimethylbenzoquinone (1 mM) as the oxidant (●); dark respiration (●); growth (— —). B. Hydrogenase activity. Hydrogen production (in µmol·mg⁻¹·Chl·h⁻¹) was measured as described in "Materials and Methods." Cell suspensions in acid-washed test tubes were allowed to preincubate for 2 h under normal culture conditions in the presence of added compounds, and then placed under anaerobic conditions for 3 h prior to hydrogenase activity measurements. No additions (●); +17 µM FeCl3, and 17 µM EDTA (●); +17 µM FeCl3, 17 µM EDTA, and 15 µg/ml cycloheximide (●).
hydrogenase activity to be expressed. This finding provides additional evidence that algal hydrogenase is an iron-sulfur protein.

**LITERATURE CITED**

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