Light Requirement for Induction and Continuous Accumulation of an Ammonium-Inducible NADP-Specific Glutamate Dehydrogenase in Chlorella

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ABSTRACT

The ammonium-inducible NADP-specific glutamate dehydrogenase of Chlorella sorokiniana was shown to require light for both its induction by ammonia in uninduced cells, and its continuous accumulation in fully induced cells. Addition of ammonia to uninduced cells in the light resulted in a 35-minute induction lag followed by linear and coincident increases in enzyme activity and antigen. Enzyme activity was not induced in the dark; however, transfer of these cells to the light resulted in an immediate increase in enzyme activity and antigen. The absence of an induction lag suggested that mRNA sequences and/or an enzyme precursor with different antigenic properties than the active holoenzyme accumulated in cells in the dark in ammonium medium. When fully induced cells were transferred to the dark, the activity of the enzyme quickly ceased to accumulate. In contrast to the NADP-specific isozyme, the cells also contain a constitutive NAD-specific isozyme which was shown to accumulate in cells in the dark in either ammonium or nitrate medium.

Chlorella cells have been reported (11, 18, 21) to contain two GDH isozymes with different coenzyme specificities. In this laboratory the NAD-GDH and NADP-GDH isozymes have been purified from C. sorokiniana and shown to be chemically, physically, and immunologically distinct molecular species (7, 16).

The NAD- and NADP-GDH isozymes have been shown to be regulated independently (10, 11, 21). The former isozyme has been observed in cells cultured in either nitrate or ammonium medium, whereas the latter one only in cells cultured in ammonium medium. The inducibility of the NADP-GDH has been examined in cell cycle experiments in which synchronous cells growing in nitrate medium were periodically challenged to synthesize the enzyme by addition of inducer. The enzyme was shown to be inducible throughout the cell cycle (21, 22). The cell cycle regulation of this enzyme has also been studied in fully induced synchronous cells growing in the continuous presence of inducer for an entire cell cycle (10, 11). This latter experimental approach revealed the operation of a regulatory system which alters the timing between gene replication and the expression of newly replicated genes in cells growing at different rates in ammonium medium. Under these different cell cycle conditions, the activity of the NAD-GDH increased in a typical step pattern during the last 0.5 h of the cell cycle.

In studies on the cell cycle inducibility of the NADP-GDH, it was observed that the initial rate of enzyme induction (i.e., enzyme potential) was proportional to the rate of accumulation of total cellular protein (21, 22). Since the accumulation rate of cellular protein has been observed to be proportional to the effective light intensity per cell (17, 19), it seemed possible that the induction or accumulation of the NADP-GDH might be light-dependent. The experimental evidence described in the present paper is consistent with a light requirement for both the induction and continuous accumulation of the NADP-GDH in Chlorella cells in ammonium medium.

MATERIALS AND METHODS

Organism and Growth Conditions. Cells of Chlorella sorokiniana were cultured autotrophically in an inorganic salts medium with either nitrate or ammonia as the sole nitrogen source as described earlier (10, 22). The cells were synchronized in nitrate medium by three light/dark periods of 10/6 h, respectively. During cell cycle experiments, the culture turbidities were held constant by hourly dilutions with fresh pre-equilibrated (4% CO2-air, 38.5 C) culture medium.

Enzyme Induction Experiments. The enzyme induction conditions, cell harvest and washing procedures, and cell breakage procedures were performed as described previously (10, 22). The enzyme induction measurements were initiated with synchronized cells harvested from nitrate medium immediately prior to the onset of the light period and also after 3 h of synchronous growth in nitrate medium in continuous light. The cells were washed twice in nitrogen-free medium and then resuspended in culture medium containing 30 mM ammonia (unless otherwise stated). The initial culture turbidity was 6.8 (A500 nm, Beckman DB spectrophotometer; 1-cm light path; 160-170 × 106 cells/ml). During the induction period, the culture turbidity was held constant by hourly dilutions (beginning at 0.5 h) with fresh pre-equilibrated culture medium. To avoid possible problems in interpretation of enzyme induction kinetic data due to changes in gene dosage, the induction studies were initiated and terminated before the onset of the period of DNA replication (9) in these synchronized cultures.

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3 Abbreviations: GDH, glutamate dehydrogenase; NAD-GDH, nicotinamide adenine dinucleotide-specific glutamate dehydrogenase; NADP-GDH, nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase; IgG, immunoglobulin G.
Enzyme Assays. The aminating and deaminating activities of the NAD- and NADP-GDH isozymes, respectively, were measured by a spectrophotometric assay described by Turner et al. (22).

Total Cellular Protein and Cell Number Measurements. Total cellular protein was extracted from cells with 1 M NaOH for 12 h at 22 C (9, 22) and then was assayed by the method of Lowry et al. (14). Cell number per ml culture was determined with a hemacytometer (American Optical) or with a model B Coulter Counter.

Ammonium Uptake Studies. To measure accurately the uptake of ammonia from the culture medium in certain experiments, the initial concentration of ammonia in the medium was decreased from 30 mM to either 2 or 3 mM. The rate of accumulation of the ammonium-inducible NADP-GDH was previously shown to be maximal at ammonia concentrations of 1 mM or higher in the culture medium. During uptake measurements, the culture medium was rapidly separated from the cells by rapid filtration through a Millipore filter (0.8 μm). The concentration of ammonia in 100- to 200-μl samples was determined by the method of Harwood and Kuhn (8). The reagent and sample volumes were decreased proportionally so that the volume of the final reaction mixture was 5 ml. Ammonium sulfate dissolved in nitrogen-free culture medium was used to establish a standard curve for ammonia.

Rocket Immunoelectrophoresis. Rocket immunoelectrophoresis (7, 12) was performed in 1% agarose prepared by boiling 0.15 g of agarose in 15 ml of Bio-Rad Swendsen buffer III for 5 min. This solution was allowed to cool to approximately 55 C then 7.5 μl rabbit anti-NADP-GDH IgG (0.25 mg/ml) was added. The anti-NADP-GDH IgG was purified from rabbit antisera by use of antigen-affinity chromatography as described by Yeung et al. (23). The agarose-antibody mixture was poured onto a 10 X 10 cm glass plate to give a gel 1.5 mm thick. A Bio-Rad gel-puncher and punching template was used to punch 4-mm wells in the gel 7 mm apart. Fifteen μl sample (0–20 ng NADP-GDH) from cell homogenates were pipetted into the wells, and electrophoresis was performed for 14 h at 150 v in a Bio-Rad electrophoresis cell (model 1400) at 4 C. Since the enzyme was previously shown (3, 23) to remain active after immunoprecipitation by anti-NADP-GDH, the "rockets" were visualized by a modified tetrazolium activity stain. The stain was composed of 10 ml of the deaminating enzyme-assay mixture, 10 ml H2O, 0.25 mg phenazine methosulfate solution (49.8 mg/25 ml H2O), 0.5 ml nitro-blue tetrazolium solution (126.5 mg/25 ml H2O); pH 8.3; incubated at 38.5 C for 6 h.

RESULTS

Coinduction of NADP-GDH Activity and Antigen in the Light. As an initial step in the elucidation of the mechanism of the induction of Chlorella NADP-GDH activity by ammonia, it was essential to establish whether the induced accumulation of catalytic activity was due to activation of pre-existing enzyme antigen or to de novo synthesis of new enzyme molecules. Rocket immunoelectrophoresis (12) was selected as the technique to examine the relationship between the changes in level of enzyme activity and antigen during an induction period. It was shown earlier in this laboratory that "rocket" heights are directly proportional to the amount of NADP-GDH antigen (7). In addition, the NADP-GDH was shown to remain active after immunoprecipitation with rabbit anti-NADP-GDH IgG (23). The immunoprecipitation bands, resulting from reaction of the enzyme with its antibody in agarose, were shown to be readily detectable by a very sensitive enzyme activity stain (3).

In the present study, rocket immunoelectrophoresis was used, in conjunction with the enzyme activity stain, to show that the levels of NADP-GDH activity and antigen increased in a linear and coincident manner upon transfer of uninduced synchronous daughter cells to ammonium medium in continuous light (Fig. 1). This accumulation of enzyme-antigen during induction is consistent with an earlier observation that the protein synthesis inhibitor, cycloheximide, blocks the induction of activity of this enzyme (10, 11, 21).

As discussed in detail by Bascomb et al. (3), this rocket immunoelectrophoresis/activity stain procedure will not detect inactive enzyme-antigen for which the anti-NADP-GDH IgG has a much different affinity than for the active enzyme. Such an inactive antigen would form a rocket in a different position than the active enzyme and would go undetected by the activity stain. By this procedure, one cannot eliminate the possibility that uninduced cells contain an enzyme precursor with different antigenic properties than the active enzyme. The above experiment establishes unequivocally that the increase in NADP-GDH activity is not due to activation of an enzyme precursor with the same antigenicity as the native enzyme.

Light-dependent Induction of NADP-GDH Activity. In contrast to the rather short lag time (i.e. approximately 0.5 h) for the induction of the NADP-GDH observed in continuous light (Fig. 1), the addition of ammonia to uninduced synchronous daughter cells in the dark did not induce the accumulation of NADP-GDH activity (Fig. 2). However, after a 3-h dark period in ammonium medium, the cells were transferred to the light and an immediate increase in NADP-GDH activity was observed (Fig. 2). Although the cells absorbed ammonia from the culture medium for 2 h during dark period, this amount of ammonia alone was not sufficient to induce the enzyme or to support the accumulation of total cellular protein. Furthermore when this same amount of ammonia was absorbed during the first 20 min of the subsequent light period, the activity of the enzyme was induced (Fig. 2).

Light Requirement for Continuous Accumulation of NADP-GDH Activity in Induced Cells. In the previous experiment, light was shown to be required for the initial induction of NADP-GDH activity in uninduced cells (Fig. 2). To determine whether light is also required for the continuous accumulation of NADP-GDH activity in induced cells, synchronous daughter cells (previously uninduced) were induced in ammonium medium for 3 h and then transferred to darkness. The NADP-GDH activity and total cel-

![Fig. 1. Comparison of catalytic activity and antigen of Chlorella NADP-GDH during induction in synchronous daughter cells in ammonium medium (30 mM) in continuous light. Zero time was the beginning of the cell cycle and the time of addition of ammonia. NADP-GDH antigen was measured by a rocket immunoelectrophoresis/activity stain procedure. Pure NADP-GDH was used to establish a standard curve of "rocket" heights versus enzyme concentration. Catalytic activity (●●●●), enzyme antigen (O—O).](https://www.plantphysiol.org/figure/1/1/1.png)
were placed in the dark at the time of the anticipated step-increase in activity by this enzyme. The NAD-GDH isozyme showed the same 4-fold step increase in activity in cells in dark as in cells in a parallel synchronous culture in continuous light (Fig. 4). In fact, although the cell number patterns were coincident in the dark and lighted synchronous cultures, the NAD-GDH activity increased somewhat faster in the darkened culture. Thus, light is not required for accumulation of NAD-GDH activity in synchronous cells in nitrate medium.

A direct comparison of the light requirements for accumulation of the NAD-GDH and NADP-GDH isozymes was also made in synchronous cells. Uninduced cells were allowed to grow to the 3rd h of the cell cycle in nitrate medium. At this time, the cells were transferred to ammonium medium, and the cellular activities of the two GDH isozymes were measured before and after the cells were placed in the dark at the 6th h of the cell cycle. Because Israel et al. (10) had shown that in cells in ammonium medium the onset of the step increase in NAD-GDH activity occurred 1 to 2 h earlier in the cell cycle than in nitrate medium, the cells were placed in the dark at the 6th rather than 8th h of the cell cycle. During the 3-h light period in ammonium medium, the activity of the NAD-GDH increased very slightly, whereas the activity of the NADP-GDH increased rapidly in a linear manner (Fig. 5). At the onset of the dark period, the NAD-GDH activity began to increase in a linear pattern, and the NADP-GDH activity immediately ceased to increase. In this particular experiment, the ammonium concentration was increased to 30 mM to avoid any possible problems with low levels of ammonia resulting in the deinduction of the NADP-GDH during the light or dark periods. Therefore, the observed initial rapid loss in NADP-GDH activity observed during the dark period was unanticipated (Fig. 5).

Absence of Light Requirement for Accumulation of Activity of the NAD-GDH Isozyme. Talley et al. (21) observed that the activity of the constitutive NAD-GDH isozyme increased in a step pattern between 6 and 8 h during the Chlorella cell cycle in culture medium with nitrate as the sole source of nitrogen. To show whether the increase in activity of the NAD-GDH might also be light-dependent, synchronized cells growing in nitrate medium

Light requirement for induction of Chlorella NADP-GDH by ammonia (3 mM) in uninduced synchronous daughter cells. NADP-GDH antigen also was measured, as described in Figure 1, and observed to increase coincident with catalytic activity at the onset of the light period at the 3rd h (data not plotted). Zero time was explained in Figure 1. Catalytic activity (O-O); total cellular protein (●-●); ammonium uptake (▲-▲).

Light requirement for the continuous accumulation of Chlorella NADP-GDH in induced synchronous cells growing in ammonium medium (2 mM). The cells were placed into the dark at the third hour of the cell cycle. Zero time was explained in Figure 1. Catalytic activity (O-O); total cellular protein (●-●); ammonium uptake (▲-▲).

Absence of light requirement for the normal step increase in activity of Chlorella NADP-GDH during the cell cycle of synchronous cells growing in nitrate medium (30 mM). One of the two synchronous cultures which were growing in parallel was placed into the dark at the 8th h of the cell cycle. The initial levels of NAD-GDH and cell number per ml culture were 120 units and 190 × 10^8 cells, respectively. Culture in continuous light: NADP-GDH (O-O) and cell number (▲-▲); culture subjected to light to dark to light transitions: NADP-GDH (●-●) and cell number (▲-▲).
FIG. 5. Light requirement for induced accumulation of NADP-GDH but not for constitutive accumulation of the NAD-GDH in synchronous Chlorella cells growing in ammonium medium (30 mm). The cells were placed in the dark at the 6th h of the cell cycle. NADP-GDH (O-○); NAD-GDH (■—■); total cellular protein (●—●).

DISCUSSION

The induction of activity of the NADP-GDH was paralleled by a coincident increase in enzyme antigen (Fig. 1). The induction and continuous accumulation of this GDH isozyme was clearly dependent upon both ammonia (10) and light (Figs. 2, 3, and 5). The reason for the lack of induction of NADP-GDH activity in the dark is unclear. The same amounts of ammonia absorbed by cells in light and dark resulted in induction of NADP-GDH activity only in lighted cells (Fig. 2). The failure to observe (Figs. 2, 3, and 5) a net increase in total cellular protein in the dark does not indicate that enzyme synthesis cannot occur under these conditions. It was previously shown (1) that synthesis of isocitrate lyase occurs within 15 to 20 min after transfer of cells of this species of Chlorella to the dark in the absence of exogenous organic substrates at any time during the cell cycle. The constitutive NAD-GDH isozyme was apparently synthesized during the dark in either nitrate or ammonium medium (Figs. 4 and 5).

In higher plants, there is quite an extensive literature (5, 24) dealing with the light induction of a number of enzymes and other proteins of which some are localized within and others outside of the chloroplast. Some of the induced activities are related to activation by light-dependent redox systems (5) whereas others are undoubtedly related to de novo enzyme synthesis (24) as appears to be the case for Chlorella NADP-GDH antigen (Fig. 1). The present studies with the Chlorella NADP-GDH have not ruled out the possibility that an inactive nonantigenic precursor of the enzyme is converted to an active antigenic form by a light-dependent redox reaction (5) or by some photosynthetic-linked reaction (24). Alternatively, the synthesis of the NADP-GDH subunits (7) or their assembly into holoenzyme might be closely coupled to photophosphorylation (20). Moreover, unless cellular compartmentalization is altering the accessibility or rate of flow of ATP within the cells in the dark, the ATP level per se is unlikely to be rate-limiting for the synthesis of NADP-GDH. The total cellular levels of ATP have been shown to decrease only momentarily and then to increase to equal or higher levels in Chlorella cells transferred from light to darkness (4).

Although the constitutive NAD-GDH has been shown (15) to be situated in the mitochondrion of Chlorella, the intracellular location of the NADP-GDH is currently unknown. In certain higher plants (13) and in another unicellular green alga (6) which contain more than one GDH isozyme, the NADP-GDH isozyme has been shown to be localized in the chloroplast. Because the activity of the mitochondrial NAD-GDH increases in the dark (Figs. 4 and 5) whereas the accumulation of the NADP-GDH is light-dependent (Figs. 2, 3, and 5) in Chlorella, an important step in the elucidation of the mechanism of the light-dependent induction of the Chlorella NADP-GDH will be to determine whether it is a chloroplast enzyme.

In this laboratory, Bascomb (2) has recently shown that subunits of NADP-GDH are continuously synthesized and then undergo rapid covalent modification and degradation in uninduced Chlorella cells. Research is currently in progress with specific immunological procedures to determine the rates of synthesis and degradation of the NADP-GDH subunits in cells in ammonium medium in the dark and during light to dark transitions.

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