Purification and Partial Kinetic and Physical Characterization of Two Chloroplast-Localized NADP-Specific Glutamate Dehydrogenase Isoenzymes and Their Preferential Accumulation in *Chlorella sorokiniana* Cells Cultured at Low or High Ammonium Levels

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**ABSTRACT**

Two ammonium-inducible, chloroplast-localized NADP-specific glutamate dehydrogenase isoenzymes were purified to homogeneity from *Chlorella sorokiniana*. These isoenzymes were homopolymers of either \( \alpha \)- or \( \beta \)-subunits with molecular weights of 55,500 or 53,000, respectively. The \( \alpha \)-isoenzyme was preferentially induced at low ammonium concentrations (2 millimolar or lower), whereas only the \( \beta \)-isoenzyme accumulated after cells were fully induced (120 minutes) at high ammonium concentrations (29 millimolar). Purification of isoenzymes was achieved by (NH₄)₂SO₄ fractionation, gel-filtration, anion-exchange fast protein liquid chromatography, and affinity chromatography. The \( \alpha \)- and \( \beta \)-isoenzymes were separated by their differential binding to Type 4 nicotinamide adenine dinucleotide phosphate-Sepharose. Both isoenzymes bound to an antibody affinity column to which purified antibody (prepared against \( \beta \)-isoenzyme) was covalently attached. Peptide mapping of the subunits showed them to have a high degree of sequence homology. Both subunits were synthesized in *vitro* from precursor protein(s) with a molecular weight of 58,500. Although the subunits have similar chemical, physical, and antigenic properties, their holoenzymes have strikingly different ammonium \( K_a \) values. The ammonium \( K_a \) of the \( \beta \)-isoenzyme remained constant at approximately 75 millimolar, whereas this \( K_a \) of the \( \alpha \)-isoenzyme ranged from 0.02 to 3.5 millimolar, depending upon nicotinamide adenine dinucleotide phosphate concentration.

Earlier research from this laboratory revealed that *Chlorella sorokiniana* cells, cultured for 6 to 12 h in ammonium medium (29 mM), contain a chloroplast-localized ammonium inducible NADP-GDH1 composed of six identical subunits that have a mol wt of 53,000 (4, 8, 10, 11, 17–19, 22). Prunkard *et al.* (17) and Bascomb *et al.* (3) obtained evidence that this subunit is synthesized on cytosolic polysomes as a precursor protein (\( M_r = 58,500 \)) that is presumably processed to the mature subunit upon entry into the chloroplast. This NADP-GDH was shown to have different physical, chemical, and antigenic properties than the mitochondrion-localized NADP-GDH (8, 13, 22) that is composed of four identical subunits (\( M_r = 45,000 \)).

In a recent study, Prunkard *et al.* (18) compared the kinetics of induction of NADP-GDH catalytic activity with the accumulation of NADP-GDH antigen induced in cells under different cultural conditions. The apparent mol wt of the catalytically active NADP-GDH holoenzyme forms were estimated by use of an enzyme activity stain after native polyacrylamide slab-gel electrophoresis. The NADP-GDH antigens were examined after native and SDS slab-gel electrophoresis by use of a Western blot/immunodetection procedure. Native gel electrophoresis of extracts, from cells harvested during a 3 h induction time-course in continuous light in 29 mM ammonium medium, revealed that the number of electrophoretic forms of NADP-GDH holoenzyme(s) that were catalytically-active (and antigenic) increased from several forms, at the earliest induction times, to seven forms (\( M_r = 290,000-360,000 \)) by the end of the 3 h induction period. When these same cell extracts were examined by SDS gel electrophoresis, two NADP-GDH antigens were detected with mol wt of 55,500 and 53,000. The 53,000 D NADP-GDH antigen increased continuously throughout the 3 h induction period. The 55,500 D antigen increased in concentration only during the first 1.5 h of the induction period and decreased in concentration thereafter. In fact, this larger antigenic species was barely detectable by the end of the 3 h induction period and essentially was absent from cells induced for 6 h. The 55,500 D antigen was shown to be associated only with NADP-GDH holoenzyme-size proteins. Therefore, it was assumed to be a subunit of NADP-GDH holoenzyme(s). The 55,500 D and 53,000 D antigens were designated as being \( \alpha \)- and \( \beta \)-subunits of NADP-GDH, respectively. Depending upon the cultural conditions (*i.e.* autotrophic *versus* heterotrophic; light *versus* dark) and the length of the induction periods, a wide variation was observed in the \( \alpha: \beta \) subunit ratio and in the number and sizes of the NADP-GDH holoenzymes.

Tischner (23) cultured *C. sorokiniana* cells at low and high concentrations of ammonium. By use of an enzyme kinetic approach, he concluded that cells, cultured in medium with an
initial concentration of 5 mM ammonium, contained forms of NADP-GDH with high and low affinities for ammonium, whereas cells cultured at a high ammonium concentration contained a single form of NADP-GDH, with a low affinity for ammonium. Although the different forms of NADP-GDH were not purified nor even separated from each other before the kinetic studies were performed, the biphasic nature of the Hanes plot, obtained with ammonium as the variable substrate, provided reasonably convincing evidence that C. sorokiniana cells can contain more than one form of NADP-GDH under certain cultural conditions. However, since these forms of NADP-GDH were not purified and characterized, it was unclear whether they were different isoforms, resulting from covalent modification of an enzyme composed of a single type of subunit, or whether a new type of subunit was induced at low ammonium concentrations resulting in formation of new isoenzyme(s) of NADP-GDH.

The aforementioned observations made by Tischner (23) prompted us to consider the possibility that the accumulation of the α- and/or β-subunits of NADP-GDH, observed under different cultural conditions and induction times by Prunkard et al. (18), might be related to the accumulation of NADP-GDH isoenzymes with different affinities for ammonium. Therefore, the objectives of the present study were (a) to attempt to establish cultural conditions for the preferential accumulation of NADP-GDH isoenzymes containing either α- or β-subunits, (b) to purify and compare the kinetic and physical properties of the α- and β-isoenzymes, (c) to determine if the α-isoenzyme is chloroplast-localized as was shown earlier (17) for the β-isoenzyme, and (d) to gain further insight into the possible roles that these isoenzymes might play in nitrogen assimilation in C. sorokiniana.

The results described in this paper indicate that cells cultured in medium in which the ammonium concentration was maintained at approximately 1 mM, accumulated a chloroplastic NADP-GDH isoenzyme that is a homopolymer of α-subunits. This isoenzyme is an allosteric enzyme with a high affinity for ammonium. In contrast, cells cultured for extended periods in 29 mM ammonium accumulated a chloroplastic NADP-GDH isoenzyme, composed of only β-subunits, that has a low affinity for ammonium. These findings are consistent with the α- and β-holoenzymes functioning in nitrogen assimilation at low and high ammonium concentrations, respectively. The purified isoenzymes were shown to have similar peptide maps and precursors-proteins of identical mol wt, suggesting a possible common origin for the α- and β-subunits.

**MATERIALS AND METHODS**

**Materials.** NADP, NADPH, Sephacryl S-300, CNBr-activated Sepharose 4B, Sepharose 4B CL, NADP-Sepharose (AGNADP type 3 and type 4) were obtained from Pharmacia; electrophoresis reagents, Bio-Rad; [35S]methionine, [125I]protein A, Amersham; nitrocellulose and Nytran membranes, Schleicher and Schuell; rabbit reticulocyte lysate, Green Hectares; oligo(dT)12-18-cellulose, Collaborative Research; SDS, highest purity, Gallard-Schlessinger Chemical Corp.; phenyl isothiocyanate (sequenation grade), adipic acid dihydrazide, Eastman-Kodak; acetonitrile (HPLC grade), pyridine (certified grade), Fisher Scientific; cyanogen bromide, Sigma Chemical Co.; periodate, Pierce. All other chemicals and materials were of the highest grade available from Calbiochem or Sigma Chemical Co. or as described earlier (25, 28).

**Organism and Growth Conditions.** Chlorella sorokiniana cells were cultured as previously described (4, 10, 26) except that a filter-sterilized modified culture medium (18) was used in the studies described herein. Media flasks, culture tubes, and other glassware were autoclaved with steam from an electric steam-generator (model LB-20, American Sterilizer Co.). The cells were synchronized by three light-dark cycles (9 h:7 h). The light intensity was adjusted to support a growth rate at which each cell would divide into four progeny at the end of each cell cycle. Culture turbidity was measured at a wavelength of 640 nm in a Beckman DB spectrophotometer.

**Enzyme and Ammonium Assay Procedures.** The NADP-GDH catalytic activity was measured in the deaminating or aminating direction by a spectrophotometric procedure as previously described (26). In this paper all enzyme assays were performed in the deaminating direction, except where noted. One unit of enzyme activity was defined as the amount of enzyme required to reduce or oxidize 1 μmol of coenzyme/min at 38.5°C.

The level of ammonium in the culture medium was measured by the procedure of Harwood and Kuhn (9).

**Polyacrylamide Gel Electrophoresis and Western Blotting Procedures.** Western blots were prepared from polyacrylamide gels, after electrophoresis of NADP-GDH antigen(s) under denaturing (12) or non-denaturing (7) conditions, as described by Prunkard et al. (18). In the Western blot procedure, the NADP-GDH antigen(s) were detected on the nitrocellulose membranes by use of purified anti-NADP-GDH IgG (28) and 125I-labeled Protein A. The dried nitrocellulose membranes were exposed to Kodak X-Omat AR film for 36 h at −70°C to reveal the position of the NADP-GDH antigen(s).

**Synthesis of Type 4 NADP-Sepharose and Anti-NADP-GDH IgG-Sepharose.** During development of the purification procedure from the α- and β-isoenzymes, the substrate affinity resin, NADP-Sepharose (type 4), was purchased from Pharmacia. For later routine purification work, the affinity resin was synthesized to link NADP to Sepharose through the nicotineamide-ribose moiety and a 6 carbon linker (equivalent to Pharmacia, type 4). The resin was prepared according to the method of Wilchek and Lamed (27).

For preparation of the anti-NADP-GDH IgG-Sepharose, the anti-NADP-GDH IgG (i.e. antibody prepared against β-isoenzyme) was purified by antigen affinity chromatography, and then coupled to CNBr-activated Sepharose as described by Yeung et al. (28).

**Purification of NADP-GDH Isoenzymes Composed of α- or β-Subunits.** For purification of the α- or β-isoenzymes, previously uninduced cells were cultured in either 1 or 29 mM ammonium medium for 3 or 6 h, respectively. Cells cultured in 1 mM ammonium medium accumulated the NADP-GDH isoenzyme composed only of α-subunits, whereas cells cultured in 29 mM ammonium medium for 6 h accumulated predominantly the β-isoenzyme (i.e. approximately 1–2% of the α-isoenzyme was also present).

Because of the sensitivity of the NADP-GDH isoenzymes to irreversible inactivation by oxidation during their purification, all buffers and resins were degassed and bubbled with argon before use, and all buffers contained 2 mM DTT. Approximately 5 g fresh weight of cells were harvested from the 1 and 29 mM cultures and processed separately. The cells were washed twice with 10 mM Tris (pH 8.25), resuspended in 5 ml of cell breakage buffer (1 mM Tris, pH 8.25), and frozen at −20°C. After thawing, the cells were broken in a French pressure cell at 20,000 p.s.i., and the homogenates were frozen overnight at −20°C. The thawed homogenates were centrifuged at 27,000g for 30 min, and the supernatants were subjected to 35 to 70% (NH4)2SO4 fractionation as described earlier (8, 28). The precipitates from the (NH4)2SO4 fractionation step were dissolved in 25 mM Tris (pH 7.4), and dialyzed against the same buffer for 12 h. After dialysis and concentration by ultrafiltration (Diaflow YM-10 membrane), the preparations were subjected to gel-filtration chromatography in a column (2.5 × 65 cm) of Sephacryl S-300 through which buffer (25 mM Tris, pH 7.4, 150 mM NaCl) was pumped at 0.75 ml/min. Fractions from the column, containing NADP-GDH activity, were pooled, and applied to a Mono Q
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column (anion-exchange) in a Pharmacia FPLC. Proteins were eluted from the column with a gradient of 0.15 to 0.35 M NaCl in 25 mM Tris (pH 7.4). Fractions from the Mono Q column that contained more than 5% of the total NADP-GDH activity were combined and applied to a 25 ml NADP-Sepharose (type 3) substrate-affinity column that had been equilibrated with 10 mM sodium phosphate (pH 6.5) at 4°C. After binding of the enzyme to the affinity column, the column was washed with 50 ml of phosphate buffer, 50 ml of 2 mM NAD in the same buffer, and finally with 50 ml of the phosphate buffer without NAD. The NADP-GDH isoenzymes were eluted from their individual columns with 2 mM NADP in the phosphate buffer. Although both isoenzymes bound to the type 3 NADP-Sepharose, only the α-isozyme binds to the type 4 NADP-Sepharose. Therefore, after elution of the α-isozyme (contaminated with the β-isozyme) from the type 3 column, the NADP was removed by dialysis and the enzyme preparation was passed through the type 4 column. The flow-through from this column contained only the β-isozyme. After the substrate affinity column step(s), the α- and β-isozyme preparations were dialyzed into the anion-exchange buffer and recycled through the Mono Q anion-exchange chromatography step. After this purification step, the α- and β-isozyme preparations were judged to be homogenous by silver staining after SDS-denaturing and native polyacrylamide slab-gel electrophoresis. These purified α- and β-isozyme preparations were used for enzyme kinetic studies. However, to ensure their maximal purity before use in peptide mapping studies, the isoenzymes were bound to anti-NADP-GDH IgG-Sepharose columns to remove traces of possible contaminating proteins. After binding of the isoenzymes to separate columns, the columns were washed extensively with 10 mM phosphate (pH 7.2), and the isoenzymes (inactivated) were eluted with 100 mM sodium acetate (pH 2.5).

Cyanogen Bromide Peptide-Mapping of α- and β-Subunits of NADP-GDH Isoenzymes. The purified α- and β-subunits were cleaved by cyanogen bromide, and the resulting peptides were derivatized with PITC as described in the peptide mapping procedure of Chang et al. (6). The derivatized peptides were separated on a C18 reverse phase column (Pharmacia PepRPC) with 0.035 M sodium acetate (pH 5.5) and acetonitrile. The derivatized peptides were detected by their absorbance at 260 nm. For each separation, 20 pmol of derivatized peptide were used.

Isolation and in Vitro Translation of Poly(A)*RNA. To increase the yield and purity of the poly(A)*RNA from C. sorokiniana, several modifications were made from the procedure used previously in this laboratory (18, 25). Cells (1 g fresh weight) were harvested by centrifugation at 8,000 rpm for 5 min, resuspended in 10 ml of 100 mM Tris (pH 8.5), 400 mM LiCl, 10 mM EGTA, 5 mM EDTA, 100 units/ml heparin, and broken by passage through a French pressure cell at 20,000 p.s.i. The homogenate was mixed with 0.05 volume of 0.2 M EDTA (pH 8.0) and 0.1 volume of 20% SDS for 10 min at room temperature. The homogenate was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and twice with chloroform:isoamyl alcohol (24:1, v/v). The RNA was precipitated by addition of an equal volume of 2X LiCl-urea buffer (4 M LiCl, 4 M urea, 2 mM EDTA) at 0°C overnight. The RNA was pelleted by centrifugation and washed with 1X LiCl-urea buffer, resuspended in 10 mM Tris (pH 8.0) and 1 mM EDTA, and precipitated with 2.5 volumes of ethanol and 0.3 M sodium acetate. The RNA was pelleted and washed with 70% ethanol and dissolved in water to give 10 to 15 μg units/ml. The RNA in 10 ml was adjusted to 0.1% SDS and heated at 65°C for 5 min. After quick-chilling in an ice-ethanol bath, the solution was adjusted to 0.5 M NaCl, heated at 65°C for 30 s to redissolve the SDS, and the poly(A)*RNA was isolated by oligo(dT)25-34-cellulose chromato-
tography as described earlier (25). By this modified procedure, routinely 30 μg of poly(A)*RNA was obtained from 1.0 g fresh weight of cells.

The poly(A)*RNA was translated in vitro in a mRNA-dependent rabbit reticulocyte system (15) modified as described earlier (25). The in vitro translation mixture was further modified to contain 2 mm of each amino acid except methionine which was the radioactive amino acid used.

In Vitro Processing of NADP-GDH Precursor Protein(s). After in vitro translation for 60 min, the total translation products (0.05 ml) were added to 0.05 ml of freshly prepared cell-free extract from uninfected C. sorokiniana cells. The cell-free extracts were prepared from 1.5 g fresh weight of Chlorella cells harvested from cultures induced for either 80 min or 180 min in 29 mM ammonium medium. The cells were broken in 3 ml of 250 mM Tris (pH 7.5) containing 25 mM KCl, 25 mM MgCl₂, 2 mM DTT, and the resulting homogenate was centrifuged for 3 h at 100,000g. After addition of the Chlorella cell-free extract (i.e. 100,000g supernatant) to the total translation products to initiate the in vitro processing reaction, samples were taken at zero time, 10 min, and 30 min, and the reaction was stopped by the addition of an equal volume (0.05 ml) of immunoprecipitation buffer (10 mM Tris, 150 mM NaCl, 2% Triton X-100, 5 mg/ml BSA, pH 7.2). The samples were centrifuged at 15,000g for 15 min and the supernatants were saved for immunoprecipitation of NADP-GDH antigen(s).

The NADP-GDH antigen(s) were recovered by an indirect immunoprecipitation procedure described earlier (4, 17, 28). The immunoprecipitates were dissolved in 20 μl of Laemmli incubation buffer (12) and subjected to SDS denaturing electrophoresis in 10% polyacrylamide gels. After electrophoresis, the proteins were electrophotoretically transferred to Nytran membranes. The membranes were dried and placed directly against Kodak X-Omat AR film for autoradiography.

Intracellular Localization of the α-Subunit NADP-GDH Isoenzyme. C. sorokiniana cells were cultured in 2 mM ammonium medium for 80 min. Under these conditions, only the α-subunit NADP-GDH isozyme accumulated. The membranes of these cells were then selectively permeabilized to small molecules by a freeze-thaw procedure, and the intracellular location of the catalytically active NADP-GDH was revealed by use of an activity staining procedure which has been described in detail by Prunkard et al. (17). The same control experiments described by Prunkard et al. (17), such as, substituting NAD for NADP and the use of uninduced cells in the staining reaction, were also performed in the present study.

RESULTS

Effect of Different Concentrations of Ammonium on Induction of α- and β-Subunit NADP-GDH Isoenzymes. To examine the effect of different concentrations of ammonium on the induction of NADP-GDH holoenzymes composed of α- and/or β-subunits, synchronized daughter cells were transferred from nitrate medium in the dark into ammonium medium (i.e. 1, 2, or 3.4 mM ammonium) in continuous light. Synchronized daughter cells were used so that the kinetics of enzyme induction could be measured in the absence of gene replication. After addition of ammonium to the uninduced cells, samples were taken for measurements of ammonium uptake from the culture medium and accumulation of total NADP-GDH activity in the cells. The NADP-GDH holoenzymes in crude cell-homogenates were dissociated and subjected to SDS-slab-gel electrophoresis and the mol wt of the NADP-GDH subunits were determined by use of a Western blot/immunodetection procedure.

In the culture in which the initial ammonium concentration was 3.4 mM, the ammonium concentration decreased to 1.4 mM during the course of the 120 min induction period (Fig. 1A).
After a 50-min lag period, enzyme activity accumulated in a linear manner until approximately 90 min into the induction period; thereafter, an acceleration in the rate of accumulation of the enzyme was observed (Fig. 1A). During the period of linear increase in enzyme activity, the α-subunit was the only NADP-GDH antigen that accumulated in the holoenzyme (Fig. 1D). However, coincident with the acceleration in rate of accumulation of enzyme activity at 90 min, the β-subunit began to accumulate in NADP-GDH holoenzyme(s).

In the cultures initiated with 2 mM ammonium, the ammonium concentration decreased to 0.15 mM in 90 min, and ammonium was exhausted from the culture medium by 120 min (Fig. 1B). In this culture, NADP-GDH activity also began to accumulate after a 50-min lag period; however, enzyme activity accumulated at a lower rate than in the culture with 3.4 mM ammonium medium (Fig. 1B). When the ammonium concentration decreased below 0.15 mM, a loss in enzyme activity was observed. Only the α-subunit was observed in the NADP-GDH holoenzyme in cells cultured in 2 mM ammonium medium (Fig. 1E).

The appearance of the α-subunit coincided with the beginning of accumulation of NADP-GDH activity, and the loss of this subunit was accompanied by a loss in the enzyme activity.

In the 1 mM ammonium culture, the cells absorbed most of the ammonium from the medium by 50 min (i.e. time corresponding to end of induction lag period); thus, only a small increase in enzyme activity was observed before it began to decay (Fig. 1C). The α-subunit was the only type of NADP-GDH antigen detected in the holoenzyme (Fig. 1F).

Since the cells accumulated only the α-subunit NADP-GDH in the 1 and 2 mM ammonium cultures, before ammonium was depleted from the medium, it seemed possible that the α-holoenzyme might be synthesized continuously (in the absence of β-subunits) if the ammonium concentration in the medium could be maintained between 1 and 2 mM. Therefore, uninduced synchronized daughter cells were added to 1 mM ammonium medium. An attempt was made to maintain this ammonium concentration by the frequent addition of ammonium to the culture medium to match its rate of uptake (i.e. 18 μmol/min-L) that was determined in the previous experiment (Fig. 1, B and C). Although ammonium was added at the predetermined rate, the concentration of ammonium in the medium actually decreased from 1 to 0.65 mM during the first 3 h of the induction period (Fig. 2A, inset). In this culture, NADP-GDH activity accumulated continuously in a nonlinear manner following a 50-min induction lag period (Fig. 2A). The α-holoenzyme was the only form of the NADP-GDH that accumulated during the initial 3 h induction period (Fig. 2B).

To determine if an increase in the ammonium concentration would result in induction of the β-holoenzyme, the ammonium concentration was increased from 0.65 to 20 mM at 3 h into the induction period (Fig. 2A). This abrupt increase in ammonium concentration caused a 50-min cessation in accumulation of NADP-GDH activity (Fig. 2A); thereafter, enzyme activity began to accumulate at a rate comparable to the rate reported earlier (4, 25) for cells induced initially in 29 mM ammonium medium. With the resumption of accumulation of enzyme activity, the β-subunit began to accumulate in NADP-GDH holoenzyme(s) (Fig. 2B).

Because it was unclear as to whether the decelerating rate of accumulation of NADP-GDH activity (i.e. α-holoenzyme) during the first 3 h of the induction period was caused by changing external factors (i.e. decreasing ammonium concentration in the culture medium and/or decreasing effective light intensity per cell as culture turbidity increased) or by changing intracellular factors (e.g. nitrogen catabolite repression), the induction experiment was repeated and the ammonium concentration was held nearly constant (i.e. approximately 0.85 mM) for the last 3 h of the 5 h induction period (Fig. 3, upper inset). During the first 2 h of the induction period, the ammonium concentration ranged between 1.1 and 0.82 mM. After the induction lag period, NADP-GDH activity increased in a linear manner for the remaining 4 h of the 5 h induction period (Fig. 3). Only the α-isoenzyme was present for the duration of the 5 h induction experiment (Fig. 3, lower inset).

Prunkard et al. (18) and Bascomb et al. (2) showed that cells induced in 29 mM ammonium medium, accumulated both α- and β-holoenzymes during the first 2 h of the induction period; however, after 2 h only the β-holoenzyme accumulated. In fact, after 6 h into the induction period, the α-subunit was barely detectable, and the β-subunit NADP-GDH still continued to accumulate in a linear manner. Thus, from the earlier and present induction studies, cultural conditions have been established in which C. sorokiniana cells will accumulate either the α- or β-isoenzymes.
Fig. 2. Induction of the \(\alpha\)-subunit isoenzyme of NADP-GDH in synchronized daughter cells of *C. sorokiniana* cultured for 3 h in continuous light in medium in which the ammonium concentration was maintained between 1 and 0.65 mm followed by induction of the \(\beta\)-subunit isoenzyme after the ammonium concentration in the medium was increased to 20 mm. After the culture was adjusted to 20 mm ammonium at the 3rd h, the induction was continued for an additional 2 h without further additions of ammonium. The initial cell number and culture turbidity were 140 × 10^6 cells/ml and 4.0 \((A_{600})\), respectively. The ammonium concentration was maintained by the addition of 0.2 ml of a solution of 0.54 m ammonium chloride every 2 min to a 3 L culture \((i.e. 18 \mu\text{mol/min.L})\). Panel A, NADP-GDH activity \(\bigcirc\) and ammonium concentration in the medium \(\bigcirc\). The inset is an expanded scale which shows the change in ammonium concentration in the culture medium during the 1st 3 h of induction. Panel B, \(\alpha\)- and \(\beta\)-subunit antigens were visualized by a Western blot/immunodetection procedure after SDS polyacrylamide slab-gel electrophoresis. Lane 1, 60 min; 2, 70 min; 3, 80 min; 4, 90 min; 5, 100 min; 6, 120 min; 7, 140 min; 8, 160 min; 9, 180 min; 10, 190 min; 11, 200 min; 12, 210 min; 13, 220 min; 14, 230 min; 15, 240 min; 16, 250 min; 17, 260 min; 18, 280 min. The autoradiogram for lanes 1 through 9 was exposed for 60 h, and the autoradiogram for lanes 10 through 18 was exposed for 18 h to obtain proper intensity of all radioactive bands.

**Purification of \(\alpha\)- and \(\beta\)-Subunit NADP-GDH Isoenzymes.** The \(\alpha\)- and \(\beta\)-holoenzymes were purified from cells that were accumulating only one or the other of these isoenzymes. The purification procedure was similar for both isoenzymes and involved \((\text{NH}_4)_2\text{SO}_4\) fractionation, gel filtration chromatography, FPLC anion-exchange chromatography, and substrate affinity chromatography. An important finding was that, after FPLC anion-exchange chromatography, both isoenzymes would bind to a substrate affinity resin to which NADP was covalently linked to Sepharose through carbon 8 of its adenine ring \((i.e. \text{Sepharose} 4B-\text{NH}-(\text{CH}_2)_3-\text{NH}-\text{C}^8\text{-NADP}; \text{Pharmacia AGNADP, type} 3)\), whereas only the \(\alpha\)-isoenzyme would bind to a resin to which NADP was linked to Sepharose through its nicotinamide-ribose moiety \((i.e. \text{Sepharose} 4B\text{-NHNH-}[\text{CH}_2]_3\text{-CO-NHNH-}[\text{nicotinamide ribose}]\text{NADP}; \text{Pharmacia AGNADP, type} 4)\). To remove the \(\alpha\)-holoenzyme from preparations of the \(\beta\)-holoenzyme, advantage was taken of this differential binding of the two isoenzymes to the type 4 NADP-Sepharose resin. For example, the \(\beta\)-holoenzyme was purified from cells cultured in 29 mm ammonium medium for 6 h, and these cells still contained a small amount of the \(\alpha\)-holoenzyme \((i.e. \alpha/\beta \text{ subunit ratio of approximately 1.99})\) that was removed by the type 4 resin.

After the aforementioned purification steps, the \(\alpha\)- and \(\beta\)-holoenzyme preparations were shown by silver staining of gels (after native and SDS slab-gel electrophoresis) to be free of detectable contaminating proteins. These isoenzyme preparations were used in enzyme kinetic studies. For peptide mapping experiments, these apparently homogeneous isoenzyme preparations were subjected to another purification step by binding the isoenzyme to individual antibody affinity columns composed of purified anti-NADP-GDH IgG covalently linked to Sepharose. During elution of the isoenzymes from this type of affinity column, they were inactivated by the acetate buffer at pH 2.5, and therefore could not be used in enzyme kinetic experiments.

**Kinetic Characterization of \(\alpha\)- and \(\beta\)-Subunit NADP-GDH Isoenzymes.** As shown in Figure 4, the \(K_m\) for ammonium for the \(\beta\)-holoenzyme was 77 mm. This \(K_m\) value is similar to the \(K_m\) for ammonium determined for the NADP-GDH which was

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**Fig. 3.** Induction of the \(\alpha\)-subunit isoenzyme of NADP-GDH in synchronized daughter cells cultured for 5 h in continuous light in medium in which the ammonium concentration was maintained between 0.82 and 1.1 mm by 2 min additions of ammonium chloride. The initial cell number and culture turbidity were 156 × 10^6 cells/ml and 4.34 \((A_{600})\), respectively. For the first 60 min, ammonium was added at the rate of 18 \(\mu\text{mol/min.L}\), thereafter it was added at the rate of 18.2 \(\mu\text{mol/min.L}\). The ammonium concentration in the culture medium is shown in the upper inset. The \(\alpha\)-subunit antigen was visualized by a Western blot/immunodetection system after SDS slab-gel electrophoresis as shown in the lower inset. Lane 1, 1 h; 2, 2 h; 3, 3 h; 4, 4 h.
purified and characterized previously from cells cultured in 29 mM ammonium medium for 12 h (8, 28). The \( K_m \) values for glutamate and NADP, for the \( \alpha \)-holoenzyme, were determined to be 32.3 and 0.031 mM, respectively (Lineweaver-Burk plots not shown). The \( K_m \) for NADPH for the \( \beta \)-holoenzyme was 0.14 mM. As also shown in Figure 4, the ammonium \( K_m \) of the \( \alpha \)-holoenzyme was approximately 3.3 mM. The \( K_m \) values of glutamate and NADPH were 38.2 and 0.040 mM, respectively (Lineweaver-Burk plots not shown). The \( K_m \) for NADPH for the \( \alpha \)-holoenzyme was 0.10 mM.

When NADP-GDH activity was induced in cells in 29 mM ammonium medium, the \( \alpha \) and \( \beta \)-isoenzymes were observed to accumulate during the first 120 min of the induction period (2, 18). As shown by a Western blot/immunodetection procedure, the cells contained a 1:1 ratio of \( \alpha \beta \) subunits at approximately 80 to 90 min into the induction period. When the \( K_m \) for ammonium was determined for a crude enzyme preparation of cells, having equal amounts of \( \alpha \)- and \( \beta \)-subunits, the resulting double reciprocal plot was nonlinear (Fig. 5A). This nonlinear double reciprocal plot could have been due to the presence of two NADP-GDH holoenzymes which were homopolymers of either \( \alpha \)- or \( \beta \)-subunits with each homopolymer having different \( K_m \) values for ammonium. Alternatively, the crude enzyme mixture could have contained a mixture of homopolymers and heteropolymers of \( \alpha \)- and \( \beta \)-subunits, with each type of subunit maintaining its unique kinetic properties whether situated in a homopolymer or heteropolymer. To determine if a mixture of homopolymers of \( \alpha \)- and \( \beta \)-isoenzymes would have the same kinetic properties as a mixture of isoenzymes in a crude enzyme preparation, solutions of \( \alpha \)- or \( \beta \)-holoenzymes (homopolymers) were mixed in a 1:1 ratio and the ammonium \( K_m \) of this mixture was measured. The double-reciprocal plot obtained with this in vitro 1:1 mixture of homopolymers was almost identical to the one observed for in vivo 1:1 mixture of \( \alpha \)- and \( \beta \)-subunits in NADP-GDH isoenzymes of unknown subunit composition (Fig. 5B).

To determine whether the \( K_m \) values for the NADP-GDH holoenzymes, composed of either \( \alpha \)- or \( \beta \)-subunits, are maintained in a population of holoenzymes composed of both \( \alpha \)- and \( \beta \)-subunits, five different ratios of \( \alpha \)- and \( \beta \)-holoenzymes (homopolymers) were prepared. The results are shown in an Eadie-Hofstee plot (Fig. 6). To determine if these kinetic data obtained from mixtures of \( \alpha \)- and \( \beta \)-subunit holoenzymes could be simulated by computer modeling, the measured values for \( K_m \) and \( V_{max} \) for the \( \alpha \)- and \( \beta \)-subunit holoenzymes (homopolymers) were used in an equation (20) which mathematically describes the total velocity of an enzyme preparation composed of two isoenzymes with different \( K_m \) and \( V_{max} \) values for the same substrate. For these calculations, ammonium \( K_m \) values (average of several experiments) of 3.5 and 70 mM were used for the \( \alpha \)- and \( \beta \)-holoenzymes, respectively. The \( V_{max} \) values of the \( \alpha \)- and \( \beta \)-holoenzymes were 160 and 480 \( \mu \)mol/min, respectively. As can be seen in Figure 6 (inset), a very close correlation was obtained between the Eadie-Hofstee plots determined experimentally and by computer simulation. These kinetic results obtained with mixtures of homopolymers, along with the ability of the type 4 NADP-Sepharose resin to separate homopolymers of \( \alpha \)- and \( \beta \)-subunits from each other, support the inference that homopolymers rather than heteropolymers of \( \alpha \)- and \( \beta \)-subunits exist for the NADP-GDH isoenzymes in C. sorokiniana.

Fig. 4. Lineweaver-Burk plot of initial velocities (\( \mu \)mol/min) measured at varied concentrations of ammonium for NADP-GDH isoenzymes (i.e., homopolymers) composed of \( \alpha \)- or \( \beta \)-subunits. Each point represents the average of two spectrophotometric assays. The concentrations of 2-oxoglutarate and NADPH were 26 and 0.37 mM, respectively. The slope and intercept values were determined by linear regression analysis.
PURIFICATION OF NADP-GLUTAMATE DEHYDROGENASE ISOENZYMES

Figure 6. Eadie-Hofstee plot of initial velocities (μmol/min) measured at varied concentrations of ammonium using different ratios of α-subunit and β-subunit NADP-GDH isoenzymes. The lines were generated from the total NADP-GDH activities of mixtures of isoenzymes resulting from the combination of preparations containing only the α-isoenzyme or β-isoenzyme. Ratios used: 100 α-subunit:0 β-subunit (A); 20 α-subunit:80 β-subunit (Δ); 50 α-subunit:50 β-subunit (Δ); 20 α-subunit:80 β-subunit (O); 100 β-subunit:0 α-subunit (O). The inset shows the theoretical initial velocities that were generated by an equation in which a mixture of two isoenzymes with different Km and Vmax values were assumed to contribute to the total initial velocity. The ammonium Km and Vmax values used in the calculations for the α-isoenzyme were 3.5 mM and 160 μmol/min, respectively. The ammonium Km and Vmax values used for the β-isoenzyme were 70 mM and 480 μmol/min, respectively. Line 1, 100 α-subunit:0 β-subunit; 2, 80 α-subunit:20 β-subunit; 3, 50 α-subunit:50 β-subunit; 4, 20 α-subunit:80 β-subunit; 5, 100 β-subunit:0 α-subunit.

Table 1. Km Values for Ammonium for Chlorella sorokiniana α- and β-Subunit NADP-GDH Isoenzymes (Homopolymers) at Different NADPH Concentrations

<table>
<thead>
<tr>
<th>Isoenzyme Type</th>
<th>Relative Specific Activity*</th>
<th>Concentration of NADPH (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Km for ammonium</td>
</tr>
<tr>
<td>α-Subunit</td>
<td>1.0</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.37</td>
</tr>
<tr>
<td>β-Subunit</td>
<td>3.0</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77</td>
</tr>
</tbody>
</table>

* The relative specific activities (units activity/mg enzyme) were determined at 0.37 mM NADPH.

The Km value for ammonium for the α-holoenzyme was observed to be sensitive to changes in NADPH concentration whereas the ammonium Km of the β-holoenzyme was unaffected by changes in concentration of this reduced pyridine nucleotide (Table 1). When the ammonium Km values were measured over an NADPH concentration range of 0.03 to 3.5 mM, the Km of the α-holoenzyme ranged from 0.02 to 3.5 mM, whereas the Km of the β-holoenzyme remained essentially constant at an average value of 75 mM.

Comparison of Peptide Maps of the α- and β-Subunit NADP-

GDH Isoenzymes. To compare the peptide maps of the α- and β-subunits, the purified NADP-GDH homopolymers of these subunits were treated with cyanogen bromide to cleave the protein chains at methionine residues. The resulting peptides were then derivatized with PITC, and the peptide derivatives were separated by FPLC reverse phase chromatography. Although all of the peptides produced by cyanogen bromide cleavage, of a given type of subunit, appeared to be derivatized by PITC, the relative amounts of the different peptide-derivatives varied between replicate PITC derivatizations of even the same peptide preparation. Therefore, it was important to compare the α- and β-subunit maps on the basis of the presence or absence of peptide derivatives rather than on the relative amounts (i.e., peak heights) of the different peptides present. As shown in Figure 7, the peptide maps of the α- and β-subunits were very similar, with 36 peptide derivatives in common and four peptides that were different.

Intracellular Localization of the α-Subunit NADP-GDH Isoenzyme. Prunkard et al. (17) showed, by use of an enzyme activity staining procedure, that the active β-holoenzyme was localized in the chloroplast of cells cultured for at least 6 h in 29 mM ammonium. In addition to confirming the localization of the antigen of active β-holoenzyme in the chloroplast, an immunoelectron microscopy procedure also was used to show that inactive antigen of this isoenzyme was in the cytosol, presumably as nascent chains of its precursor-protein being synthesized on cytosolic 80S ribosomes. In the present study, because the α-holoenzyme was shown to have similar physical, chemical, and antigenic properties to the β-holoenzyme, it seemed possible that the α-isoenzyme might also be localized in the chloroplast.

C. sorokiniana cells were cultured under conditions in which only the α-holoenzyme accumulated (i.e., 2 mM ammonium medium for 80 min). The membranes of cells were then selectively permeabilized to small molecules by a freeze-thaw procedure. After this procedure, small molecules, such as substrates, products, soluble dyes, etc., can diffuse across the plasma, chloroplastic, and mitochondrial membranes; however, large molecules, such as enzymes and other proteins, remain in their respective cellular compartments. After permeabilization, the cells were incubated in a buffered reaction mixture containing NADP, glutamate, phenazine methosulfate, and nitroblue tetrazolium. When the active α-holoenzyme catalyzed the oxidation of the glutamate in a NADP-dependent reaction in the permeobilized cells, the soluble and colorless nitroblue tetrazolium was reduced and converted to an insoluble blue formazan dye that precipitated only in the chloroplast of the cells. The intense blue staining of the chloroplast was identical to that observed earlier for cells containing only the β-holoenzyme (color photograph not shown; Ref. 17). Thus, the active holoenzymes of both the α- and β-subunit NADP-GDH isoenzymes are localized in the chloroplast of the C. sorokiniana cell.

Molecular Weight of Precursor Protein(s) of α- and β-Subunits of NADP-GDH Isoenzymes and in Vitro Processing of Precursor Proteins. Prunkard et al. (17) showed that the β-subunit of the NADP-GDH was synthesized as a precursor-protein with a mol wt of 58,500. Because the α-holoenzyme also was shown to be localized in the chloroplast, it seemed likely that the α-subunit also might be synthesized as a precursor-protein with a transit-peptide sequence to direct its entry into the chloroplast.

To compare the mol wt of the precursor-proteins of the α- and β-subunits, poly(A)°RNA was isolated from cells that were accumulating both types of subunits (i.e., 29 mM ammonium medium and 80 min induction period) or from cells which were accumulating only the β-subunit (i.e., 29 mM ammonium medium and 3 h induction period). After the poly(A)°RNAs from the two different induction periods were translated in vitro in a mRNA-dependent rabbit reticulocyte lysate system, the NADP-
GDH antigen(s) were immunoprecipitated and their mol wt estimated after SDS polyacrylamide slab-gel electrophoresis. Only a single band of NADP-GDH antigen was produced in each translation mixture, and surprisingly the antigen(s) in each band had essentially the same (if not identical) mol wt of 58,500 (Fig. 8A).

Prankard et al. (17) used a cell-free extract from fully-induced Chlorella cells (i.e. induction in 29 mm ammonium medium for 6 h) to process in vitro the NADP-GDH precursor-protein, produced by in vitro translation of poly(A)+RNA from fully induced cells, to the β-subunit. Therefore, in the present study, fresh cell-free extracts were prepared from cells, induced for 80 min and 3 h in 29 mm ammonium medium, and were added to the precursor-proteins synthesized from poly(A)+RNA isolated from these same induction times. When the 58,500 D precursor protein(s), produced from 80 min poly(A)+RNA, was processed with cell extract prepared from 80 min cells, the primary product was an antigen the size of the α-subunit (Fig. 8B). However, a small amount of antigen the size of the β-subunit was also formed. When the 58,500 D precursor protein(s) from the 3 h poly(A)+RNA was processed by cell extract from 3 h induced cells, a large amount of antigen the size of the β-subunit and a small amount of α-subunit was formed (Fig. 8C).

**DISCUSSION**

Although the purification of the α- and β-isoenzymes involved the use of (NH₄)₂SO₄ fractionation, gel-filtration, and anion-exchange and affinity chromatography, it was the high resolving power of the anion-exchange column (Pharmacia Mono Q column) used with a Pharmacia FPLC that greatly facilitated the rapid purification of these isoenzymes from other contaminating cellular proteins. By taking advantage of their differential binding to a type 4 NADP-Sepharose affinity column, the separation of the two isoenzymes from each other was accomplished.

Since it was shown earlier (8) that the NADP-GDH purified from fully induced cells contained 15 methionine residues, it was anticipated that 16 possible peptides would result from cyanogen bromide cleavage of the β-subunit. Although PITC reacts primarily to form single derivatives with many of the amino acids at the N-termini of peptides, it also forms secondary reaction products with a number of amino acids (e.g. serine, threonine, lysine, etc.). Therefore, it was predicted that more than 16 PITC-derivatives would be formed from the peptides produced by cyanogen bromide cleavage. Of the 40 PITC peptide-derivatives obtained, from the peptides produced by cleavage of each subunit, there were 36 peptide derivatives in common and 4 peptide derivatives that were different (Fig. 7). Thus, a high degree of sequence homology exists between the α- and β-subunits.

When the ammonium $K_m$ and $V_{max}$ values for the purified α- and β-holoenzymes were used in an equation that describes the velocity for two independent enzymes catalyzing the same reaction, the theoretical kinetic data generated by computer matched the experimental kinetic data for different ratios of the α- and β-isoenzymes in a mixture (Fig. 6). Moreover, a Lineweaver-Burk plot for reaction velocity versus ammonium concentration for an in vivo mixture of NADP-GDH isoenzymes, having a 1:1 ratio of α- and β-subunits, was identical to the plot obtained for a mixture in which a 1:1 ratio of α- and β-holoenzymes (heteropolymers) prepared in vitro (Fig. 5). These kinetic data strongly suggest that C. sorokiniana cells contain homopolymers rather than heteropolymers of α- and β-subunits. However, if heteropolymers do exist, the two types of subunits appear to retain their individual kinetic properties within the heteropolymers.

Unequivocal evidence that heteropolymers do not exist in extracts of this organism would be the complete separation, without significant loss of total NADP-GDH activity, of an in vivo mixture of NADP-GDH isoenzymes (e.g. 1:1 ratio of α- and β-subunits in holoenzymes) into only two holoenzymes fractions containing either α- or β-subunits. Although the type 4 NADP-Sepharose affinity column has been used to resolve completely...
the α-isoenzyme from the β-isoenzyme in partially purified enzyme preparations (e.g. after purification through the FPLC anion-exchange chromatography step), the α-isoenzyme in crude cell extracts binds very poorly to the affinity resin. It is likely that the many other dehydrogenases in the crude cell extract outcompete the α-isoenzyme for NADP binding-sites on the affinity resin. The same phenomenon has been observed with the type 3 NADP-Sepharose which has the ability to bind both isoenzymes; neither the α- nor β-isoenzyme in crude cell extracts will bind efficiently to this affinity resin. Because partial purification of the α- and β-isoenzymes through the third step of the purification procedure (i.e. FPLC chromatography) usually results in a 20 to 30% loss in total NADP-GDH activity, it is possible that heteropolymers might have been denatured and lost in the purification steps preceding the type 4 NADP-Sepharose affinity chromatography step. Therefore, although the results from the kinetic experiments and the isoenzyme fractionations with the type 4 affinity resin are consistent with this species of Chlorella containing only NADP-GDH homopolymers of α- and β-subunits, the possible existence of heteropolymers cannot be excluded at this time.

From the ammonium nutrition studies (Figs. 1-3), it can be concluded that a threshold ammonium concentration (i.e. between 2 and 3.4 mM) exists below which the β-isoenzyme does not accumulate. In contrast, the accumulation of the α-isoenzyme seems to be inhibited at high ammonium concentrations in a time-dependent process. For example, when uninduced cells are placed in medium at a high concentration of ammonium, the cessation of accumulation of the α-isoenzyme does not occur until 120 min into the induction period (2, 18). However, when cells are already accumulating the α-isoenzyme, as in a culture maintained at 1 mM ammonium, the addition of a high concentration of ammonium (20 mM) results in immediate cessation of accumulation of the α-isoenzyme (Fig. 2). This type of time-dependent negative response suggests that ammonium or one of its metabolites increases to an intracellular level which inhibits or represses (i.e. nitrogen catabolite repression) the accumulation of the α-isoenzyme.

When ammonium was added to nitrate-cultured cells of C. sorokiniana, Tischner and Lorenzen (24) observed rapid and major (i.e. greater than 50%) losses in total glutamine synthetase and glutamate synthase activities. The loss in activities of these two enzymes was accompanied by the induction of high levels of NADP-GDH activity. Ahmad and Hellebust (1) observed, in another species of Chlorella (i.e. C. autotrophica), that ammonium induction of NADP-GDH activity was also accompanied by an almost total loss of glutamine synthetase activity in these cells. Since C. sorokiniana (5) and other species of Chlorella (21) appear to have two glutamine synthetase isoenzymes that probably play roles in nitrogen assimilation and in recycling of ammonium produced in photosynthesis (5) in nitrate-cultured cells, as observed in higher plants (14), the major losses in glutamine synthetase and glutamate synthase activities and induction of NADP-GDH activity suggests that the major route of entry of nitrogen into organic metabolism shifts from the glutamate synthase/glutamate synthase pathway to the NADP-GDH in Chlorella cells shifted from nitrate to ammonium medium.

From the results obtained in the present study, it appears that when C. sorokiniana cells are placed into an environment, containing a low ammonium concentration (i.e. 1-2 mM), the assimilation of ammonium is catalyzed by an ammonium-inducible NADP-GDH isoenzyme (α-holoenzyme) that has a high affinity for ammonium (i.e. Km ranges from 0.02 to 3.5 mM, depending upon NADP concentration). When the ammonium concentration is increased to 3.4 mM and above, another NADP-GDH isoenzyme (β-holoenzyme) is induced that has a low affinity for ammonium (i.e. Km of 75 mM) and accumulation of the high affinity isoenzyme is inhibited or repressed. The reason that the responsibility for ammonium assimilation should shift from a NADP-GDH isoenzyme with a high affinity to one with a low affinity for ammonium is unclear. However, it seems possible that the high affinity NADP-GDH isoenzyme could drastically lower NADPH levels in the chloroplast, if this were the form of isoenzyme that persisted in the chloroplast of cells transferred into medium containing high levels of ammonium.

As observed previously for the β-isoenzyme, the catalytically active α-holoenzyme also was shown to be localized in the chloroplast, and its subunit to be synthesized as a precursor-protein (Fig. 8, A and B). A surprising observation was that, although the α- and β-subunits have different mol wt (i.e. 55,500
versus 53,000, respectively), their putative precursor proteins have the same apparent mol wt (i.e. 58,500). Because the native α- and β-subunits have similar physical, chemical, and antigenic properties, and have putative precursor-proteins of the same size, an attractive hypothesis is that these subunits are derived by differential processing of the same precursor-protein. However, the results of the in vitro processing experiments, performed with the precursor-protein(s) synthesized from poly(A)^+ RNA isolated from cells accumulating both α- and β-isoenzymes or only the β-isoenzyme, are actually consistent with two hypotheses, i.e. only one precursor-protein exists and the specificity of the processing enzyme (s) changes during induction, or two different precursor-proteins of the same size exist that are processed specifically. To distinguish between these hypotheses, it will be necessary to perform additional in vitro processing experiments in which each of the cell extracts (i.e. extracts from cells accumulating α- or β-isoenzymes) are mixed with the different precursor-protein preparations.

In another eukaryotic microorganism, it has been recently observed (16) that, in response to nutritional changes, a single gene can give rise to different mRNAs coding for intracellular and extracellular invertases. In view of this observation, it is possible that changes in the nitrogen nutritional status of Chlorella cells could alter the expression of possibly one or two structural genes coding for the α- and β-isoenzymes of NADP-GDH. Therefore, although current research in this laboratory involves a continuation of the in vitro processing studies with the precursor-protein(s), it also includes the use of a radioactive NADP-GDH DNA hybridization probe in experiments (involving Northern and Southern blotting analyses) aimed at determining how many genes and mRNAs code for the two NADP-GDH isoenzymes in Chlorella.

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