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Evidence for the Degradation of Nicotinamide Adenine Dinucleotide Phosphate-Dependent Glutamate Dehydrogenase of *Candida utilis* During Rapid Enzyme Inactivation

BRIAN A. HEMMINGS†

Biochemisches Institut der Universität und Institut für Biochemie der Gesellschaft für Strahlen- und Umweltforschung, 7800 Freiburg im Breisgau, West Germany

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The nicotinamide adenine dinucleotide phosphate-dependent glutamate dehydrogenase (NADP-GDH) from the food yeast *Candida utilis* was found to be rapidly inactivated when cultures were starved of a carbon source. The addition of glutamate or alanine to the starvation medium stimulated the rate of inactivation. Loss of enzyme activity was irreversible since the reappearance of enzyme activity, following the addition of glucose to carbon-starved cultures, was blocked by cycloheximide. A specific rabbit antibody was prepared against the NADP-GDH from *C. utilis* and used to quantitate the enzyme during inactivation promoted by carbon starvation. The amount of precipitable antigenic material paralleled the rapid decrease of enzyme activity observed after transition of cells from NH_4^+-glucose to glutamate medium. No additional small-molecular-weight protein was precipitated by the antibody as a result of the inactivation, suggesting that the enzyme is considerably altered during the primary steps of the inactivation process. Analysis by immunoprecipitation of the reappearance of enzyme activity after enzyme inactivation showed that increase of NADP-GDH activity was almost totally due to de novo synthesis, ruling out the possibility that enzyme activity modulation is achieved by reversible covalent modification. Enzyme degradation was also measured during steady-state growth and other changes in nitrogen and carbon status of the culture media. In all instances so far estimated, the enzyme was found to be very stable and not normally subject to high rates of degradation. Therefore, the possibility that inactivation was caused by a change in the ratio of synthesis to degradation can be excluded.

Many examples of selective inactivation of enzymes in vivo, from procaryotes and simple eucaryotes, have been reported in the literature (for a review, see reference 32). The mechanisms whereby a rapid decrease in the catalytic activity of certain enzymes is achieved fall into two broad categories, reversible and irreversible. In the reversible-type mechanisms, there are such examples as the adenylylation-deadenylylation system of glutamine synthetase from *Escherichia coli* (30) and the acetylation-deacetylation of citrate lyase from *Rhodopseudomonas gelatinosa* (9, 10). However, with the many examples (32) where the loss of enzyme activity is irreversible, the underlying biochemical mechanisms are poorly understood. It has been suggested (16) that selective proteolysis may be primarily, or at least secondarily, responsible for the loss of enzyme activity.

† Present address: Laboratory of Biochemistry, National Heart, Lung and Blood Institute, Bethesda, MD 20014.

The nicotinamide adenine dinucleotide phosphate-dependent glutamate dehydrogenase (NADP-GDH) from *Candida utilis* has a molecular weight of 276,000 and is composed of six apparently identical subunits (25). The enzyme undergoes reversible conformational transition, which affects the overall catalytic activity (25) and is regulated by feedback inhibition (8). Recently an additional control mechanism has been described for this enzyme from *Aspergillus nidulans*; during carbon starvation or transfer to amino acids as sole nitrogen, carbon, and energy source, the enzyme is rapidly inactivated (17, 18). A similar rapid inactivation of the NADP-GDH from *C. utilis* was noted when cells were starved for carbon or transferred to amino acids as sole nitrogen, carbon, and energy source. In this paper, experiments are described in which a specific antibody against NADP-GDH was used to quantitate enzyme amounts during inactivation and steady-state growth. The rapid

loss of enzyme activity, promoted after the removal of carbon or resuspension into glutamate medium, was paralleled by a decrease of antigenic cross-reacting material. Analysis of the degradation rate during steady-state growth revealed that the enzyme normally had a low turnover rate. Therefore, it is proposed that enzyme inactivation is achieved by the selective proteolytic degradation of the NADP-GDH after the onset of carbon starvation.

MATERIALS AND METHODS

Chemicals. L-[4,5-^{3}H]leucine (46 Ci/mmol), L-[*U*-^{14}C]leucine (348 Ci/mol), and tritiated amino acid mixture (7 mCi/ml, TRK 440) were obtained from Amersham-Buchler (Braunschweig, Germany). NCS tissue solubilizer was from Amersham/Searle Corp. (Arlington Heights, Ill.). Bovine serum albumin was obtained from Behringwerke AG (Marburg, a.d. Lahn, Germany). α-Ketoglutarate (disodium salt), reduced NADP, and reduced NAD were obtained from Boehringer Mannheim GmbH (Mannheim, Germany); *N,N'*bisacrylamide and *N,N,N',N'*-tetramethylethylenediamine were from Fluka AG (Bucks, Switzerland); carrier ampholyte, pH 3 to 10, was from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade and obtained from Serva (Heidelberg, Germany), E. Merck AG (Darmstadt, Germany), and Carl Roth OHG (Karlsruhe, Germany).

Organism and growth conditions. *C. utilis* NCYC 737 was obtained from A. P. Sims, Norwich, England.

The cells were cultured at 30°C on the following medium, containing (per liter): KH_2PO_4, 8.0 g; $K_2HPO_4 \cdot 3H_2O$, 1.0 g; Na_2SO_4, 1.5 g; $MgCl_2 \cdot 6H_2O$, 240 mg; $CaCl_2 \cdot 6H_2O$, 80 mg; $FeCl_3 \cdot 6H_2O$, 2 mg; $ZnSO_4$, 2 mg; $MnCl_2 \cdot 4H_2O$, 2 mg; H_3BO_3, 2 mg; $CuSO_4 \cdot 5H_2O$, 0.2 mg; KI, 0.2 mg; $NaMoO_3$, 0.2 mg; glucose, 10 g; and a nitrogen source (as indicated in legends) at a final concentration of 12 mM. Glutamate and alanine, when employed as sole nitrogen, carbon, and energy source, were used at a final concentration of 20 mM. Cultures were grown at 29°C on a Braun LSR rotary shaker (Braun Co., Melsungen, Germany) at 200 cycles/min. Any changes in the nitrogen or carbon status of the culture medium are explicitly mentioned in the legends.

Labeling conditions. Typically, 250 ml of ammonium chloride-glucose medium was supplemented with L-[4,5-^{3}H]leucine (usually 1 μCi/ml); carrier leucine was not added since the cells were found to efficiently utilize the radioactive label.

Cultures were grown in the above medium from a small inoculum to mid or late log phase with a doubling time of about 95 min. By this stage around 90% of the label is utilized by the organism. The entire culture was harvested in a Sorvall refrigerated centrifuge at 6,000 × *g* for 1 min. The cell pellet was washed with fresh medium containing 1 mM leucine and recentrifuged. The resulting pellet was then resuspended into an equal volume of fresh medium containing 1 mM leucine and 20 mM glutamate (or as indicated in the legends).

Samples of 40 to 50 ml were removed from the main culture and centrifuged as described above. The resulting pellet was washed once with ice-cold distilled water, centrifuged, and rewashed with 100 mM sodium phosphate (pH 7.4)–0.5 mM ethylenediaminetetraacetic acid (EDTA) buffer. The cells were then resuspended in 3.0 ml of the same buffer and stored at −20°C for up to 24 h before cell disruption. Storage of cells at this temperature did not result in any significant change of enzyme activity. The same activity levels were found if the cells were disrupted immediately or after 2 to 3 days of storage at −20°C.

Preparation of purified NADP-GDH. Partially purified NADP-GDH from *C. utilis* was purchased from the Oriental Yeast Co. (Tokyo, Japan). The enzyme was purified to homogeneity by preparative polyacrylamide gel electrophoresis using the method described by Betz et al. (1). Typically 1.5 mg of the commercial lyophilized enzyme (containing ca. 450 μg of protein) in 10% sucrose was layered on a 5% polyacrylamide gel (1.3 by 11.0 cm) at 4°C, and electrophoresis was performed at a constant current of 10 mA per gel until the tracking dye was 0.5 cm from the bottom of the gel. Protein was located by staining with Coomassie brilliant blue by the technique of Chrambach et al. (3). The region of gel corresponding to NADP-GDH was cut out from unstained gels. Protein was extracted by sonic treatment of the gel slices in 20 ml of 0.1 M sodium phosphate, pH 7.3, containing 0.5 mM EDTA buffer. Pieces of acrylamide were removed by centrifugation, and the resulting supernatant was concentrated by lyophilization.

Immunization of rabbits. Immunization was carried out basically as described by Mason et al. (21). Each rabbit received three injections, at weekly intervals, of the sonically treated gel (containing 0.7 to 0.8 mg of NADP-GDH protein) from the preparative gel electrophoresis; the gel had been emulsified with an equal volume (1.0 ml) of Freund complete adjuvant. The emulsified antigen was injected, in 0.2-ml aliquots, subcutaneously in the back. For the third injection (on the 21st day), purified protein without polyacrylamide present was mixed with Freund incomplete adjuvant and injected subcutaneously in the back. On the 28th day a booster injection (1.0 mg of NADP-GDH in 1.0 ml of 0.9% saline) was administered intravenously. On the 6th, 8th, and 10th days after the booster injection, blood was collected from the ear vein and allowed to clot. The serum was centrifuged and stored in 1.5-ml aliquots with 0.1% $NaNO_3$ at −20°C. Control serum was taken from the rabbits before injection of antigen.

Immunodiffusion analysis. Double diffusion according to Ouchterlony was performed on microscope slides. Antiserum (5 to 10 μl) was added to center wells, and antigen was added to peripheral wells. Maximal precipitates developed in 1 to 2 days at 4°C.

Immunoprecipitation of radioactively labeled NADP-GDH. Radioactively labeled yeast, harvested as described above, was resuspended in 3.0 ml of ice-cold 0.1 M sodium phosphate (pH 7.2)–0.5 mM EDTA buffer and passed four times through a French press at 20,000 lb/in^2. Cell debris was removed by centrifugation at 4°C for 90 min at 40,000 × *g*. The supernatant, which contained 80% of radioactive label, was then used for immunoprecipitation.

Usually duplicates of 1.3-ml (5 to 9 mg of protein) portions from each sample from the crude protein extracts were incubated with an excess of antiserum (normally 80 μl/1.3 ml) for 30 min at 25°C, at which time precipitation was maximal (>95%). The antigen-antibody complex was pelleted by centrifugation at 8,000 × *g* for 2 min, washed with 1.0 ml of 0.1 M sodium phosphate (pH 7.2) buffer containing 1.2 M KCl, 1% Triton X-100, and 0.5 mM EDTA, and centrifuged as above, followed by 2 × 1.0-ml water washes. The washed immunoprecipitate was dissociated with sodium dodecyl sulfate (SDS) as described below. For the experiment described in Table 1 the anti-NADP-GDH serum was partially purified by the method of Steinbuch and Audran (31), omitting the diethylaminoethyl-Sephadex step.

Electrophoresis and counting procedures. Polyacrylamide gel electrophoresis in the presence of SDS was performed at 20°C in cylindrical gels (10 by 0.5 or 6 by 0.6 cm) containing 10% acrylamide, 0.27% bisacrylamide, and the buffer system according to Laemmli (19). The washed immunoprecipitates were dissociated by heating for 30 min at 95°C with 1% SDS and 5% mercaptoethanol in 62.5 mM tris-(hydroxymethyl)aminomethane-hydrochloride (pH 6.8) buffer. Dissociated immunoprecipitates were loaded onto the upper gel, and electrophoresis was carried out at 1 mA/gel until the bromophenol blue had migrated through the stacking gel; the current was then increased to 3 mA/gel.

Protein was stained as described by Laemmli (19) and quantitated by scanning the gels at 575 nm with a Gilford spectrophotometer.

Gels containing radioactivity were sliced into 1- or 2-mm segments and incubated at 50°C overnight with 0.7 ml of NCS tissue solubilizer containing 10% (vol/vol) water. Radioactivity in the polyacrylamide gel slices was counted in 10 ml of a toluene-based scintillator [4 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis-(5-phenyloxazolyl)benzene (POPOP) per liter of toluene], using a Packard scintillation counter with an efficiency of 43%. For the determination of NADP-GDH degradation rate, the radioactivity corresponding to the position of the enzyme on the gel was totaled.

Isoelectric focusing. Isoelectric focusing was performed at 4°C for 5 h in cylindrical polyacrylamide gels (0.6 by 9 cm) containing 5% acrylamide, 6 M urea, and 0.2% Ampholine (pH 3 to 10). Immunoprecipitates were solubilized by incubation with 6 M urea and 25 mM dithiothreitol for 30 min at 95°C, after which the samples were made 2.5% (vol/vol) with ampholyte and 10% (wt/vol) with sucrose. Gels were overlayered with 100 μl of 5% (wt/vol) sucrose and 2.5% (vol/vol) ampholyte solution and prerun for 20 min at 0.5 mA/gel before the solubilized immunoprecipitates were loaded.

After electrofocusing, the gels were cut into 2-mm slices and 0.5 ml of water was added for pH determination. Protein in the gel slices was hydrolyzed by heating the slices in 0.5 ml of water with 0.5 ml of 0.1 M NaOH for 1 h. Radioactivity was counted by transferring the hydrolysates to a Triton X-100–toluene (1:2)-based scintillator as described earlier.

Enzyme and protein assays. NADP-GDH and NAD-GDH activity was estimated according to the method of Ferguson and Sims (4). Protein was estimated by the method of Lowry et al. (20), using bovine serum albumin as standard. Nitrate reductase was assayed by the method of Nason and Evans (24).

All enzyme activities are expressed as specific activities (micromoles per minute per milligram of protein).

RESULTS

Effect of glucose starvation on NADP-GDH activity. A rapid loss of NADP-GDH activity was observed following transfer of NH_4^+-glucose-grown *C. utilis* to a medium lacking carbon or carbon and nitrogen (Fig. 1). The rate of inactivation was enhanced by the addition of L-glutamate or L-alanine to starvation medium (minus N and C; see Fig. 1). The difference in rates of inactivation observed with the two treatments (i.e., minus N and C versus glutamate or alanine) cannot be attributed to dilution of protein by growth: during the initial 80-min period after transfer, little if any growth occurred. Addition of glucose to the starvation medium (minus N and C) completely blocked the inactivation of NADP-GDH. Ammonia afforded no protection against inactivation (Fig. 1). Resuspension of cells into the original or fresh NH_4^+-glucose medium did not result in any loss of activity (Fig. 1). A similar glucose starvation-promoted inactivation has been reported for the malate dehydrogenase from glucose-repressed *Schizosaccharomyces pombe* (6, 7) and glutamine synthetase from *C. utilis* (5).

Essentially the same rapid rate of loss of NADP-GDH activity was observed when cells were initially grown on nitrate or glutamate as nitrogen source, with glucose or sucrose as car-

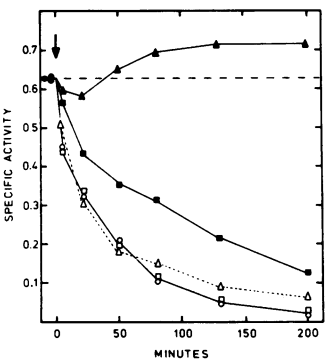

FIG. 1. *Effects of carbon starvation on NADP-GDH activity from C. utilis previously grown on ammonia-glucose medium. Cells were grown on ammonia-glucose* (●) *medium to mid-log phase, harvested, and resuspended into minimal medium (minus carbon and nitrogen) with the following addition:* (■) *none;* (▲) *glucose (55 mM);* (△) *NH_4Cl (12 mM);* (○) *glutamate (20 mM);* (□) *alanine (20 mM).*

bon source, and transferred to glutamate medium. Also in these cases, addition of glucose to starvation medium was found to block inactivation. In the experiment where nitrate was employed as nitrogen source, the rate of loss of nitrate reductase activity following the transfer to glutamate medium (nitrogen, carbon, and energy source) was measured. Decrease of nitrate reductase activity was considerably slower (20% decrease in 90 min) when compared with the loss of NADP-GDH activity (90% in 90 min). In the same experiment, malate dehydrogenase and glucose-6-phosphate dehydrogenase were assayed and found to be stable and not inactivated, suggesting that the observed loss of NADP-GDH is probably specific.

Reversibility of NADP-GDH inactivation. Two different growth conditions were employed to ascertain whether the loss of enzyme activity was reversible. Yeast was grown initially on NH_4^+-glucose and glutamate-glucose and then transferred to medium containing L-glutamate. In both cases, this resulted in a rapid loss of enzyme activity (Fig. 2). After 120 min in the inactivating medium, ammonia and glucose were added back to the culture previously grown on ammonia-glucose and glucose to the culture previously grown on glucose-glutamate; NADP-GDH activity returned to both cultures (Fig. 2). The addition of cycloheximide blocked the reappearance of NADP-GDH activity in both experiments. Cycloheximide has no effect on the stability of the enzyme in vivo; this result therefore suggests that the antibiotic exerts its effect by inhibiting protein synthesis. There was a small increase in activity in the presence of cycloheximide after the readdition of glucose (Fig. 2B). This may indicate either that the loss of activity is a two-step process, the first being reversible and the other irreversible, or that protein synthesis is not totally inhibited by the drug under these conditions. (Under these conditions protein synthesis is inhibited by 92%; the remaining incorporation could be due to protein synthesis occurring in the mitochondria).

Immunochemical investigation of NADP-GDH inactivation. From the preceding data it was concluded that the NADP-GDH is irreversibly denatured, possibly as a result of proteolysis, during inactivation. In an attempt to elucidate the biological mechanism of inactivation, antibodies were raised against the purified enzyme so that the fate of the NADP-GDH could be unambiguously monitored and quantitated.

Specificity of antiserum raised against NADP-GDH. The specificity of the antiserum raised against *C. utilis* NADP-GDH was tested by the Ouchterlony double-diffusion method.

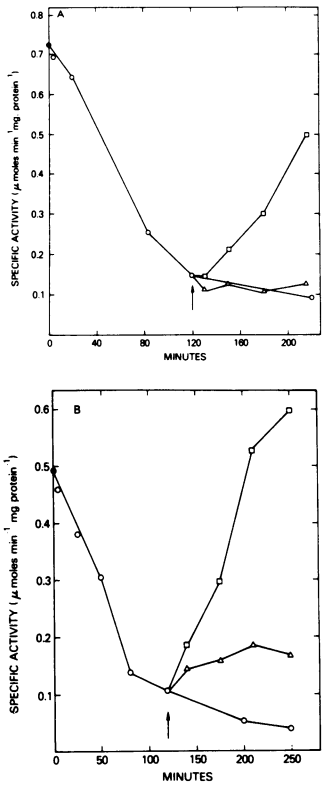

FIG. 2. *Effect of cycloheximide on the reappearance of NADP-GDH activity in C. utilis. (A) Cells were previously grown on ammonia-glucose* (●) *medium, harvested, and resuspended into minimal medium containing 20 mM glutamate* (○). *After 120 min, the culture was divided and the following additions were made:* (□) *ammonium chloride (12 mM) and glucose (55 mM);* (△) *ammonium chloride, glucose, and cycloheximide (25 μg/ml). (B) Cells grown on glucose-glutamate* (●) *medium were harvested at zero time and resuspended into minimal medium containing 20 mM glutamate* (○). *After 120 min, the culture was divided and the following additions were made:* (□) *glucose (55 mM);* (△) *glucose (55 mM); and cycloheximide (25 μg/ml).*

With crude, partially purified and with pure NADP-GDH, a single precipitin band was produced (Fig. 3). Control serum failed to produce any reaction. The antibody was also tested against the NAD-GDH (250-fold purified) from *C. utilis* and failed to cross-react.

Specificity was also verified by analysis of immunoprecipitates by SDS-polyacrylamide gel electrophoresis (Fig. 4). The antiserum was found to precipitate a single major radioactive protein from extracts containing high levels of NADP-GDH activity and with extracts from cells 105 min after the onset of inactivation; the mobility of the peaks of radioactivity corre-

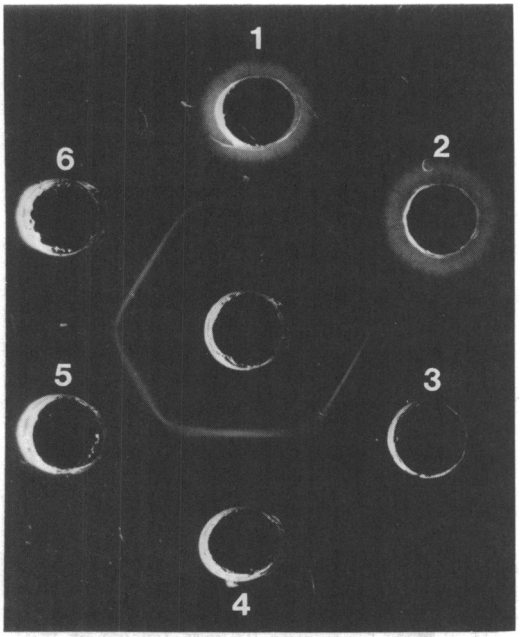

FIG. 3. *Ouchterlony double immunodiffusion of NADP-GDH against anti-NADP-GDH serum. Center well contained 10 μl of antiserum. The peripheral wells contained 10 μl of each of the following (clockwise from the top): 1 and 2, C. utilis extracts from NH_4^+-glucose grown cells (500 μg); 3 and 4, partially purified NADP-GDH (10 μg); 5 and 6, pure NADP-GDH (10 μg).*

sponds to that of purified NADP-GDH. A variable amount of small-molecular-weight material was also precipitated but judged to be due to nonspecific precipitation.

Analysis of mixed immunoprecipitates, from ^{14}C-labeled active cells and ^{3}H-labeled cells 70 and 140 min after the start of inactivation by SDS-polyacrylamide gel electrophoresis, showed that the ^{3}H- and ^{14}C-labeled protein precipitated by the antiserum migrated at exactly the same rate, indicating there is no significant change in the molecular weight of the GDH subunits pre- and post-enzyme inactivation. The ratio of ^{3}H to ^{14}C was constant through the peak.

Titration of NADP-GDH with anti-NADP-GDH serum. The data in Table 1 show the amount of antiserum necessary to cause 50% inhibition of NADP-GDH taken from cells harvested after different periods of time in the inactivating medium. Assuming that in all the extracts an equivalent amount of antibody is needed to inhibit the same amount of enzyme activity, the activity of NADP-GDH decreases at a rate parallel to that of loss of antigen. These results suggest that there is little, if any, accumulation of cross-reacting inactive enzyme during inactivation. These results were confirmed by labeling cells with radioactive leucine and analyzing the decrease of isotope in immunoprecipitates.

Quantitation of NADP-GDH antigen by antibody precipitation during enzyme inactivation. For these experiments, the cells were routinely grown on ^{3}H-labeled leucine NH_4^+-glucose medium and transferred to gluta-

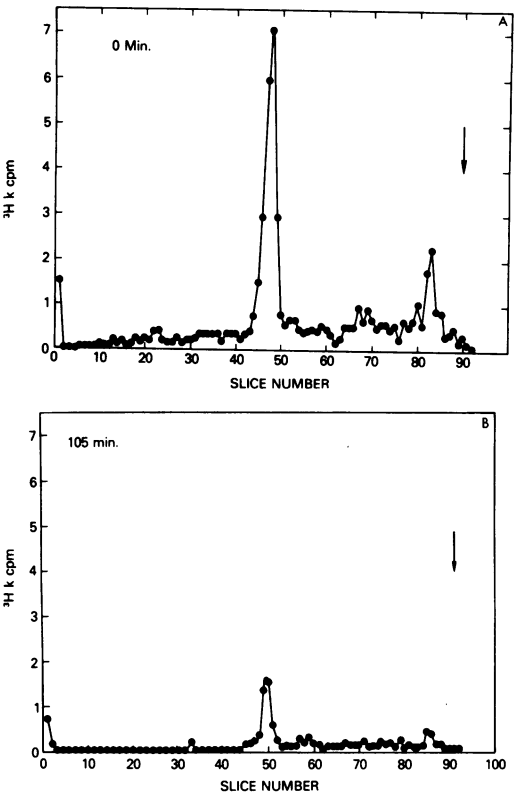

FIG. 4. *Immunoprecipitation of radioactive NADP-GDH from crude cell-free extracts before and after enzyme inactivation. Cells were cultured as described in the text in the standard NH_4^+-glucose medium in the presence of 1.3 μCi of L-[3,5-^{3}H]leucine per ml. At zero time, a control sample of 50 ml of culture was harvested, and the remaining 50 ml of culture was transferred to a minimal salt medium containing 24 mM L-glutamate and 0.5 mM L-leucine for 105 min before harvesting. Cell-free extracts were prepared, and immunoprecipitation and SDS-polyacrylamide gel electrophoresis were carried out as detailed in the text. The radioactive profile from the gels represents the amount of material precipitated from 7.0 ml of culture. (A) Active enzyme. Specific activity, 0.47 μmol/min per mg. Total radioactivity loaded, 18.0 × 10^3 cpm. (B) Inactive enzyme. Specific activity, 0.11 μmol/min per mg. Total radioactivity loaded, 3.0 × 10^3 cpm. Migration was from left to right; the arrow indicates the position of the tracking dye.*

TABLE 1. *Quantitation of NADP-GDH during enzyme inactivation by immunotitration*[a]

Time (min)	NADP-GDH sp act (U/mg of protein)	μl of antiserum[b] needed to inhibit 50% enzyme activity
0	0.99 (100)[c]	73 (100)
30	0.79 (81)	61 (84)
60	0.52 (51)	46 (63)
110	0.24 (24)	19 (26)
160	0.10 (10)	13 (18)

[a] Cells were cultured as described in Fig. 1.

[b] Immunotitration was performed as follows: 0.25 ml of crude extract was incubated with varying amounts of antiserum in a final volume of 0.35 ml. The extracts were incubated at 30°C for 1 h and then allowed to stand at 4°C for a further 2 h before enzyme activity was measured. The initial concentration of partially purified anti-NADP-GDH used for immunotitration was adjusted to 0.4 mg/ml.

[c] Numbers in parentheses are percentages.

mate as sole nitrogen, carbon, and energy source to promote enzyme inactivation. This was found to produce the most rapid and reproducible inactivation time course. The cells were ^3H labeled and manipulated as described above. A parallel loss of antigenically precipitated ^3H-labeled NADP-GDH and enzyme activity was observed (Fig. 5), similar to the results described above. A similar rapid loss of ^3H-labeled antigen and enzyme activity was observed when cells were transferred to total starvation conditions (minus N and C). These results suggest that the NADP-GDH is considerably altered, rendering the antigen unrecognizable to the antibody.

Quantitation of antigen levels after immunoprecipitation by staining of protein by Coomassie blue after SDS-polyacrylamide gel electrophoresis showed that total antigen levels decreased at the same rate as loss of enzyme activity and radioactivity (data not shown). This demonstrates that synthesis of NADP-GDH is very low or stopped completely during enzyme inactivation or that any enzyme that is synthesized after the initiation of inactivation is rapidly degraded.

Analysis of immunoprecipitates by isoelectric focusing in 6 M urea (Fig. 6) also showed that the NADP-GDH rapidly decreased, the antigen precipitated was homogeneous with a pI of around 4.8 to 4.9, and no additional significant protein peaks were detected. Detailed examination of the complete SDS-polyacrylamide gel profiles (data not shown) of the immunoprecipitates (washed with water only) taken at different times after the onset of inactivation showed that no new low-molecular-weight protein species were present. Additionally, no increase in the width of the peak of radioactivity corresponding to NADP-GDH was observed. If proteolysis is involved in the mechanism of inactivation, these results suggest the initial steps involve an endoproteolytic and not an exoproteolytic cleavage.

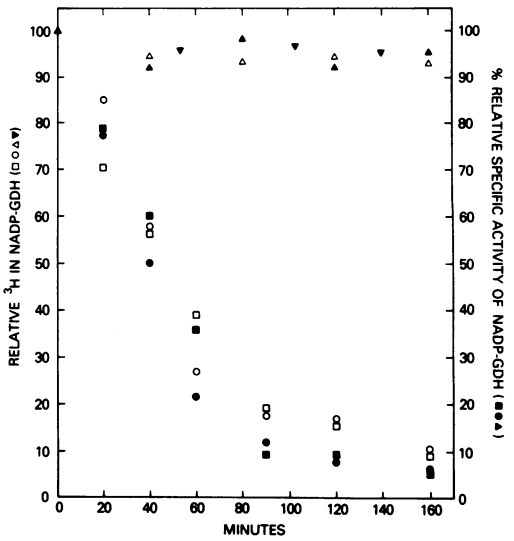

FIG. 5. *Decrease of NADP-GDH cross-reacting material and enzyme activity following transfer of NH_4^+-glucose medium containing 1.0 μCi of [^3H]leucine per ml and then transferred to glutamate (24 mM) medium containing 0.5 mM leucine or fresh NH_4^+-glucose medium containing 0.5 mM leucine. Cell-free extracts, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis were carried out as described in the text. Each point represents the total number of counts per minute from 50 ml of culture in the three to four slices (2 mm) of an SDS-polyacrylamide gel of antibody-purified material corrected for the local background in the gel. Symbols: (●, ■) percent relative NADP-GDH activity after transfer to glutamate medium; (▲) percent relative NADP-GDH activity after transfer to NH_4^+-glucose medium; (○, □) percent relative [^3H]leucine in NADP-GDH after transfer to glutamate medium; (△, ▼) percent relative [^3H]leucine in NADP-GDH after transfer to NH_4^+-glucose medium. The data in the figure are from two separate experiments (●, ○, ▲, △, experiment I; ■, □, ▼, experiment II).*

Experiments with denatured purified enzyme, prepared by incubation with guanidine hydrochloride, SDS, urea, strong acid and alkali, and heat treatment (Table 2) showed that the antiserum was able to precipitate the enzyme quantitatively after all these treatments, apart from the alkali-denatured enzyme. This result suggests that antibody can recognize subunits, but harsh treatment with alkali reduces the antigenic response. Consequently, it is concluded that antiserum can probably precipitate NADP-GDH subunits from crude extracts. A second antibody from goat directed against rabbit im-

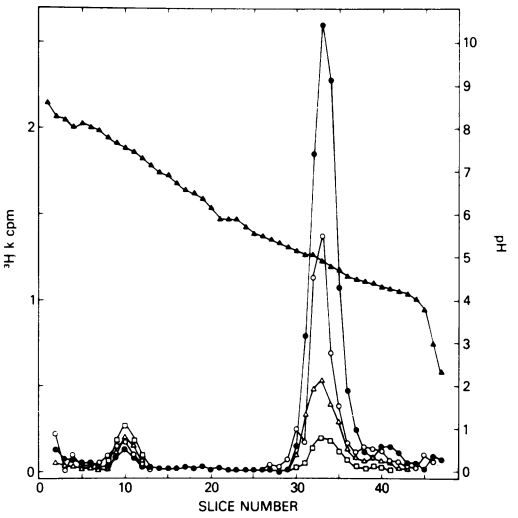

FIG. 6. *Isoelectric focusing of immunoprecipitates in 6 M urea. Cells were grown on NH_4^+-glucose medium plus 0.75 μCi of L-[3,4-^{3}H]leucine; at zero time the culture was harvested and transferred to glutamate medium. Samples were harvested at the times indicated; cell-free extracts, immunoprecipitation, and isoelectrofocusing were carried as described in the text. Symbols: (●) 0 min; (○) 40 min; (■) 80 min; (△) 120 min after transfer to glutamate medium.*

munoglobulin G was used to precipitate any soluble antibody-NADP-GDH complexes. No evidence was found to suggest that a modified nonprecipitable GDH was produced during inactivation.

Ouchterlony double-diffusion analysis of concentrated media, taken at different times during inactivation, failed to produce any precipitin bands. Therefore, it appears that the enzyme is not secreted into the medium during inactivation.

The results presented in this section are consistent with the hypothesis that the enzyme is rapidly and probably completely destroyed during inactivation because no heterogeneity of the antigen was detected by SDS-polyacrylamide gel electrophoresis and isoelectric focusing. No evidence suggests that an inactive species of intact enzyme is produced by the inactivation process. The above results, however, do not exclude the possibility that the enzyme has a high turnover rate in normal growing cells, with the loss of enzyme activity resulting from a cessation of enzyme synthesis at the onset of carbon starvation. Therefore, experiments were carried out to determine the degradation rate of NADP-GDH in NH_4^+-glucose-grown cells. For these experiments, the same experimental approach was used as in the study of enzyme inactivation. Cells were cultured on NH_4^+-glucose containing ^{3}H-labeled leucine and transferred to fresh NH_4^+-glucose containing unlabeled leucine as chase. The enzyme was found to be very stable during normal growth, with about a 5% loss of the radioactivity in 60 min (Fig. 5). From these experiments it was calculated that NADP-GDH constituted about 0.3 to 0.5% of the total labeled material of the cell.

Immunological investigation of the reversibility of inactivation. The data presented on the inactivation and subsequent reappearance of enzyme activity suggest that inactivation is an almost totally irreversible process; cycloheximide completely blocked the reappearance of enzyme activity. With the availability of the specific antiserum, the reappearance of enzyme activity was reexamined. By prelabeling the NADP-GDH prior to inactivation, one can determine whether any steps in the inactivation process are reversible.

When the cells were initially grown on NH_4^+-glucose and inactivated by transfer to glutamate, the amount of radioactivity in the immunoprecipitates did not increase when the cells were given glucose, but continued to decline slowly (Fig. 7). However, if the cells were initially grown on glucose-glutamate medium, some increase in

TABLE 2. *Effect of NADP-GDH denaturation on precipitation by anti-NADP-GDH serum*

Treatment[a]	% Protein precipitated[b]
Control	100
SDS (0.1%)	85.2
Urea (6 M)	100
Guanidine hydrochloride (2 M)	84.0
Acid (6 M HCl)	71.0
Alkali (6 M NaOH)	35.0
Heat (30 min at 95°C)	85.0

[a] All enzyme solutions were incubated at 25°C for 1 h with the indicated compound and then dialyzed at 4°C for 12 h against 1 liter of 0.1 M sodium phosphate buffer (pH 7.2)–0.5 mM EDTA. The heat-denatured enzyme was solubilized by the addition of 0.1% SDS and then incubated and dialyzed as above. With the alkali and acid treatments, the protein solutions were neutralized prior to dialysis. After dialysis there was no significant reactivation of enzyme activity, although this does not exclude the possibility of subunit reassociation.

[b] For immunoprecipitation, the treated enzyme solutions were divided into two equal portions; a fivefold excess of antiserum (as judged by activity measurement of the control) was added to one and an equal volume of control serum was added to the other. The protein precipitated by the control serum was subtracted from that precipitated by the anti-NADP-GDH serum. Immunoprecipitation was carried out as described in the text. Protein was determined by the method of Lowry et al. (20) after solubilization with 0.1 M NaOH for 1 h at 56°C.

the radioactivity was observed after the readdition of glucose. This, again, may indicate that the initial step of inactivation is reversible or, alternatively, the increase of radioactivity could represent the assembly of undegraded labeled subunits into active enzyme with newly synthesized subunits.

Degradation of NADP-GDH during steady-state growth and following changes in culture conditions. The in vivo stability of the enzyme was also investigated during the transition of NH_4^+-glucose-adapted yeast to other carbon and nitrogen sources (Table 3). Changing the carbon source to 10% glycerol did not affect the stability of the enzyme, although there was a 40% increase, over 2.0 h, of the specific activity. Moreover, transfer of cells to a complex medium containing a mixture of several amino acids, i.e., glutamate, glutamine, arginine, and alanine, did not increase the steady-state degradation rate of the enzyme. Under such growth conditions the enzyme would be redundant, because the amino acid requirements of the cell could be furnished directly or by transamination of glutamate. Similar results were obtained when cells were transferred to a medium containing Casamino Acids as nitrogen source. During the adaption of yeast to nitrate as sole nitrogen source, degradation of the NADP-GDH was again barely detectable; there was, however, a 80% derepression of enzyme activity during this period. These results are consistent with the work of Halvorson (14) for the turnover rate of total cellular protein, in that under normal steady-state growth the degradation of NADP-GDH is very low, similar to that observed for total protein.

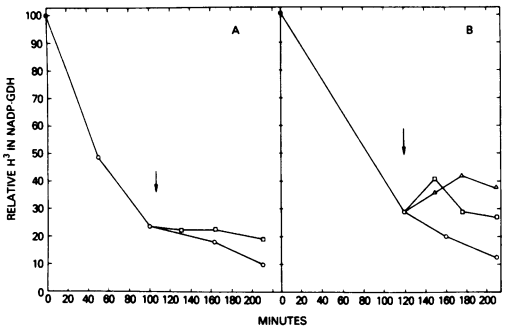

FIG. 7. *Immunological quantitation of NADP-GDH during reactivation of enzyme activity. (A) NH_4^+-glucose (●)-grown cells (0.86 μCi of L-[3,4-^{3}H]-leucine per ml) were transferred to glutamate medium containing 1 mM L-leucine (○). At 105 min the culture was divided in two parts; one part was centrifuged and resuspended into NH_4^+-glucose plus 1 mM cold L-leucine medium (□), and the other part served as the control (○). At the time indicated, samples were taken and analyzed for enzyme activity and antigen levels. For typical enzyme activity data, see Fig. 2. Preparation of cell-free extracts, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis were carried out as described in the text. (B) Glutamate-glucose (●) medium containing 0.72 μCi of L-[3,4-^{3}H]leucine per ml. At zero time the cells were harvested, transferred to cold glutamate medium (plus 1 mM cold leucine) lacking glucose for 2 h (○), and then divided into three portions: (○) control, no additions; (△) plus glucose (10%); and (□) plus glucose (10%) and cycloheximide (25 μg/ml).*

However, if yeast was transferred from NO_3^-- to NH_4^+-containing medium, a slight increase of the degradation rate (7% per h) was observed concomitant with a 40% decrease in the enzyme activity as the cell adjusted to the increased NH_4^+ supply (26). This difference in the rate of change of activity and ^{3}H in the immunoprecipitates, following transfer to NH_4^+, probably reflects a decrease in the rate of synthesis of NADP-GDH and a slight increase in the rate of degradation relative to the other cellular protein; hence the specific activity fails. It has been shown that the steady-state levels in the NADP-GDH on NH_4^+ and NO_3^- are different (5). The degradation rate is still very low compared with that during inactivation. Interestingly, under these conditions the loss of nitrate reductase activity was quite extensive (80% in 160 min), somewhat analogous to the results of Sorger and

TABLE 3. *Degradation of NADP-GDH during exponential growth*[a]

Expt no.	Initial conditions	New growth conditions	% change of NADP-GDH activity[b]	% loss of radioactivity in the immunoprecipitate[b]
1	NH_4^+-glucose	NH_4^+-glucose	−5	<5
2	NH_4^+-glucose	NH_4^+-glycerol, 10%	+23	<3
3	NO_3^--glucose	NH_4^+-glucose	−40	14
4	NH_4^+-glucose	Alanine, arginine, glutamate, glutamate-glucose[c]	−13	<3
5	NH_4^+-glucose	NO_3^--glucose	+77	<3

[a] Experimental conditions as in Fig. 5 except that the yeast was cultured on the medium described above. All other procedures are detailed in the text.

[b] Loss of activity or radioactivity after transfer to fresh growth medium for 120 min.

[c] Amino acids used at 5 mM final concentration.

co-workers for the inactivation of nitrate reductase from *Neurospora crassa* (29), and could also represent another example of proteolytically mediated enzyme inactivation.

Effect of a number of inhibitors on NADP-GDH inactivation. A general feature of intracellular protein degradation and of many enzyme inactivation systems (12) is the requirement for metabolic energy. Therefore, the sensitivity of the NADP-GDH inactivation to such compounds was examined.

Both azide, an inhibitor of the terminal oxidase, and 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, were found to inhibit loss of enzyme activity and GDH protein (Table 3). Cyanide, however, was only partially inhibitory, and arsenate was completely without effect (Table 4). It is interesting to note that in the case of cyanide, a concomitant decrease of cross-reacting material and loss of enzyme activity was observed, suggesting that this inhibitor causes a reduction of rate.

With the inactivation of NADP-GDH observed in *A. nidulans* (17), cycloheximide was found to completely block inactivation; this result could not be confirmed with *C. utilis* (Table 4). The fact that phenylmethylsulfonyl fluoride had only a slight effect on inactivation suggests that a serine proteinase is not involved in the inactivation mechanism.

DISCUSSION

The results presented are consistent with the rapid loss of NADP-GDH activity being mediated by selective proteolysis during carbon starvation. However, definitive proof of degradation is incomplete because no partially degraded forms of the enzyme have been isolated. Although the specific mechanism is unclear, there are some points which are worth noting. First, no smaller-molecular-weight protein(s) is precipitated by the antiserum, indicating that there is no accumulation of any large precipitable products resulting from proteolytic cleavage. Second, no increase in the band width of the peak of radioactivity, after SDS-polyacrylamide gel electrophoresis, was observed. This result argues that the NADP-GDH is subject to endoproteolytic attack.

The degradation rate of NADP-GDH is specifically increased during carbon limitation. In view of the observations here, the selective inactivation of NADP-GDH may occur as follows: following the decrease in the availability of carbon, the enzyme undergoes a specific, effector-promoted conformational change, is covalently modified, or is proteolytically nicked, resulting in conformational change in the enzyme which renders it susceptible to further proteolytic attack. Such a degradation would need to proceed very quickly, because as yet no small-molecular-weight proteins have been detected by immunoprecipitation. The relationship between the irreversible inactivation of NADP-GDH and the requirement for metabolic energy is not clear. However, Goldberg and St. John have suggested

TABLE 4. *Effect of metabolic inhibitors on the inactivation of NADP-GDH from C. utilis*

Pregrowth conditions	Inactivation conditions	% loss of NADP-GDH activity	% loss of CRM[a]
Part 1[b]			
NH_4^+-glucose	Glutamate	76	74
NH_4^+-glucose	Glutamate + potassium cyanide (1.5 mM)	46	45
NH_4^+-glucose	Glutamate + sodium azide (0.5 mM)	2	2
NH_4^+-glucose	Glutamate + sodium arsenate (1.0 mM)	75	73
NH_4^+-glucose	Glutamate + 2,4-dinitrophenol (1.0 mM)	8	13
Part 2[c]			
NH_4^+-glucose	Glutamate	90	ND[d]
NH_4^+-glucose	-N,-C	44	ND
NH_4^+-glucose	Glutamate + PMSF[e] (1.5 mM)	80	ND
NO_3^--glucose	Glutamate	82	ND
NO_3^--glucose	Glutamate + cycloheximide (25 μg/ml)	43	ND
NO_3^--glucose	Glutamate + azide (0.5 mM)	13	ND

[a] CRM, Cross-reacting material precipitated by anti-NADP-GDH serum.

[b] Part 1: Cultures of *C. utilis* were grown on NH_4^+-glucose containing 0.5 μCi of L-[3,4-^{3}H]leucine medium per ml, harvested, and transferred to glutamate medium with 0.5 mM leucine chase plus the different inhibitors. A control sample was removed at zero time; all other samples were harvested after 95 min. Cell-free extracts, immunoprecipitation, and SDS-gel eletrophoresis were carried out as described in the text. Changes in CRM and NADP-GDH are calculated with respect to the values of the control at zero time.

[c] Part 2: Nonradioactive cultures were grown on the nitrogen source indicated, harvested, and transferred to the indicated medium. Results are documented as in part 1.

[d] ND, Not determined.

[e] PMSF, Phenylmethylsulfonyl fluoride.

(12) that uptake of proteins into vacuoles may require an energy source, and the yeast proteinases have been shown to be almost exclusively localized in the vacuole system (15).

The rapid degradation of the β-galactosidase X90 protein and the deletion-mutated protein of the *lac* repressor from *E. coli* (11, 26) are very similar to the system described in this paper. Both proteins are rapidly degraded upon the removal of their respective inducers from the culture medium; in both cases the native protein is completely stable in the absence of its inducer. The X90 fragment was found to have a half-life of a few minutes, and the altered *lac* repressor had a half-life of 20 min. A similar rapid degradation of the bacteriophage lambda repressor has also been reported (27) during prophage induction. Recently (11) a protein fragment, with a molecular weight 30,000 less than that of the X90, has been isolated by using the auto-X complementation assay of Bukhari and Zipser (2); this protein appears to be an intermediate in the process and results from an endoproteolytic cleavage.

Similar parallel losses of enzyme activity and antigen levels have been reported for the inactivation of glutamine synthetase from cultured Chinese hamster cells (22), aspartate transcarbamylase from *Bacillus subtilis* (35), malate dehydrogenase from *Saccharomyces cerevisiae* (J. Neff and D. Mecke, Abstr. Int. Congr. Biochem., Abstr. 07-8-11, p. 419, 1976), and glycogen phosphorylase from *Dictyostelium discoideum* (33). In all these cases, no evidence was found for a degradation product resulting from the cleavage of the native protein or for an inactive form of the enzyme accumulating during inactivation. This suggests that a similar control mechanism of selective proteolysis is invoked during the appropriate physiological transition. Attempts to construct an in vitro inactivating system have so far been unsuccessful.

The physiological function of the inactivation of NADP-GDH would appear to be twofold. (i) During transfer to growth on glutamate (as sole nitrogen, carbon, and energy source) the NADP-GDH is redundant and antagonistic to the efficient catabolism of the amino acid by the catabolic GDH (15a). (ii) The NADP-GDH also represents a valuable source of amino acids and carbon which could be utilized for synthesis of new proteins or to provide energy.

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