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Regulation of *Saccharomyces cerevisiae* Nicotinamide Adenine Dinucleotide Phosphate-Dependent Glutamate Dehydrogenase by Proteolysis During Carbon Starvation

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Inactivation of the nicotinamide adenine dinucleotide phosphate-dependent glutamate dehydrogenase from *Saccharomyces cerevisiae* during carbon starvation occurs with a simultaneous loss of enzyme protein and enzyme activity.

Microorganisms respond to changes in environmental conditions by rapid inactivation of certain enzymes (12). Yeast cultures transferred from glucose-deficient medium to medium containing glucose show a rapid decline in the activities of malate dehydrogenase, fructose-1,6-bisphosphatase, phosphoenol pyruvate carboxykinase, and α-isopropylmalate synthase (5, 12). This rapid loss of enzyme activities, which appears to be due to proteolysis (9), has been termed "catabolite inactivation" by Holzer (5).

The NADP-dependent glutamate dehydrogenase (NADP-GDH) from *Saccharomyces cerevisiae* and glutamine synthetase from *Candida utilis* are inactivated during carbon starvation (2, 7). Both enzymes are thought to be inactivated by a proteolytic mechanism. Support for this idea comes from observations made with the NADP-GDH from *C. utilis*, where it was demonstrated that specific degradation of NADP-GDH occurred during transfer from NH_4-glucose to glutamate media (i.e., transfer from carbon-sufficient to carbon-limiting conditions).

Demonstration that proteolysis is an important regulatory mechanism during enzyme inactivation requires that the enzyme protein be quantitated during the loss of activity. We have employed immunological techniques to demonstrate that the loss of NADP-GDH activity in *S. cerevisiae* during carbon starvation occurs concomitantly with a decline of the enzyme protein.

S. cerevisiae X2180 was used for these studies (R. Mortimer, Donner Laboratory, Berkeley, Calif.) and cultured as described earlier (7). Extracts were prepared using a French pressure cell (4). Assay for NADP-GDH activity has been described elsewhere (7). For purification of NADP-GDH, *S. cerevisiae* X2180 was cultured on 0.7% (wt/vol) yeast nitrogen base and 2% (wt/vol) glucose in a 12-liter fermentor at 30°C. These conditions lead to maximal derepression of enzyme activity. The enzyme was purified by a modified procedure of Doherty (1). Purification involved ammonium sulfate precipitation and chromatography on DEAE-cellulose and Ultrogel AcA34 (LKB Labs). The final purification step was chromatography on DEAE-BioGel, and elution was achieved using a shallow NH_4Cl gradient. The specific activity of the purified enzyme preparation was 136.4 units per mg (174-fold purification), approximately the same as that reported by Grisolia et al. (3) and Doherty (1), and was apparently homogeneous as judged by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Goat antibody against NADP-GDH was prepared as follows: 0.55 mg of pure enzyme was mixed with an equal volume of Freund complete adjuvant (Difco) and injected subcutaneously. Two weeks later this was repeated. One week after the second injection, 600 ml of serum was taken, and the γ-globulin fraction was purified by ammonium sulfate fractionation (10). The goat was given a further booster injection (0.55 mg of protein) 1 month later, and serum was taken 1 week after the booster injection. Other immunochemical techniques have been given elsewhere (4).

Specificity of the goat anti-NADP-GDH was verified by the method of Ouchterlony, immunoprecipitation of [^{3}H]leucine-labeled NADP-GDH, and immunotitration experiments. The double-diffusion method of Ouchterlony gave a single precipitin band (data not shown). Analysis of immunoprecipitates by SDS-polyacrylamide gel electrophoresis revealed that the antiserum precipitated a single major radioactive protein from cell-free extracts (prepared from cells

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grown on [^{3}H]leucine medium) containing high levels of NADP-GDH activity (Fig. 1). The position of the radioactive peak corresponds to the subunit molecular weight of the purified enzyme with a value of about 54,000. From these experiments it was calculated that the NADP-GDH constituted about 0.5% of the soluble protein in the cell-free extracts used for immunoprecipitation.

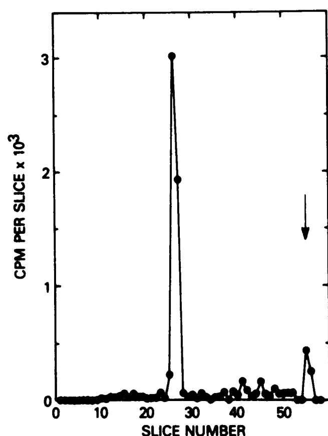

FIG. 1. *SDS-polyacrylamide gel profile of immunoprecipitates from [^{3}H]leucine-labeled S. cerevisiae. Yeast was cultured on yeast nitrogen base (0.7%, wt/vol) and glucose (2%, wt/vol) medium containing 1 µCi of [^{3}H]leucine per ml. A sample of 230 ml was harvested at a cell density of 180 Klett units and frozen. The frozen cell pellet was suspended in 10 ml of 0.1 M sodium phosphate buffer (pH 7.5), containing 2 mM phenylmethyl sulfonyl fluoride, and passed three times through a French pressure cell at 20,000 lb/in^2. The cell-free extracts were centrifuged at 4°C for 60 min at 40,000 × g. Duplicate immunoprecipitations were carried out by incubating 1.0 ml (6 to 7 mg of total protein containing about 30 µg of NADP-GDH) of cell-free extract with 200 µl of anti-NADP-GDH (7.48 mg) at 30°C for 60 min. Precipitates were collected by centrifugation for 4 min at 7,700 × g. The immunoprecipitates were washed with 0.5 ml of 50 mM Tris-chloride buffer (pH 7.5), containing 1.2 M KCl, 1.2% Triton X-100, and 0.5 mM EDTA, and again centrifuged as above. Immunoprecipitates were solubilized by suspending in 100 µl of 8 M urea and 100 µl of 0.125 M Tris-chloride buffer (pH 6.8) containing 4% SDS, 10% mercaptoethanol, and 20% glycerol, followed by heating at 96°C for 3 min. Electrophoresis was then carried out on 10% polyacrylamide gels (0.5 by 10 cm). After electrophoresis, gels were either stained or sliced into 1.5-mm disks and solubilized with 1.0 ml of NCS tissue solubilizer containing 10% water. Solubilized gels were then counted in 10 ml of a toluene scintillator (4 g of 2,5-diphenyloxazole per liter of toluene). Radioactivity loaded onto the gel represents the amount of [^{3}H]-leucine incorporated into 8.5 µg of NADP-GDH. Migration was from left to right; the arrow indicates the position of the tracking dye.*

Quantitation of NADP-GDH levels during enzyme inactivation was carried out using immunoprecipitation techniques. For these experiments cells were precultured on yeast nitrogen base and glucose medium containing 1 µCi of [^{3}H]leucine per ml and then transferred to medium without glucose supplemented with 0.5 mM leucine. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, and the radioactivity in the NADP-GDH band was quantitated. These experiments showed that the NADP-GDH activity and cross-reactive material declined at a parallel rate during carbon starvation (Fig. 2). This suggests that the NADP-GDH is either destroyed or rendered totally unrecognizable to the antibody during enzyme inactivation. The data presented in Fig. 2 show that there is a slight lag in the loss of cross-reacting material compared to the loss of enzyme activity, indicating that a small amount of inactive NADP-GDH is produced during inactivation and is precipitated by the antibody. A similar parallel loss of cross-reactive material and enzyme activity was observed when cells were starved for both carbon and nitrogen. However, in this instance, a delay of 60 min was observed before the onset of enzyme inactivation and loss of cross-reactive material. These data were corroborated by immunotitration of NADP-GDH activity with varying amounts of antibody. Using cell-free extracts taken at different times during enzyme inactivation, it was found that the neutralization ratio (units of NADP-GDH activity inhibited per microliter of anti-NADP-GDH) remained constant. Consequently, it is assumed that during inactivation the NADP-GDH is not converted to an inactive enzyme species and no substantial amount of immunoreactive fragments of GDH is produced.

It was shown earlier (7) that protein synthesis is necessary for restoration of NADP-GDH activity in cells after inactivation in vivo. This communication has shown that material cross-reactive with NADP-GDH declines during inactivation of the enzyme in vivo. Taken together, these data suggest that the NADP-GDH from *S. cerevisiae* is selectively proteolyzed during starvation for glucose. Thus, inactivation of the NADP-GDH from *S. cerevisiae* and *C. utilis* is apparently regulated in a similar manner during carbon starvation (4, 7). Transfer of cultures of yeast to a medium deficient in a readily utilizable carbon source results in the activation of a specific NADP-GDH-degrading system. However, in both cases the detailed mechanism for the loss of cross-reactive material is unclear. As yet no substantial amounts of GDH fragments (presumably from endoproteolytic cleavage) have been detected.

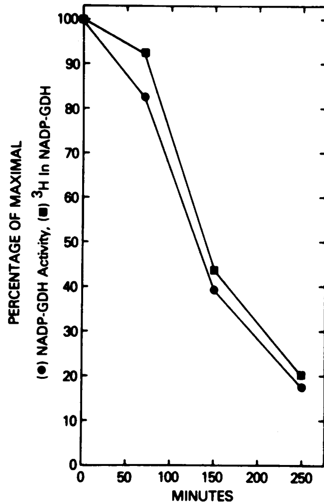

FIG. 2. *Loss of NADP-GDH cross-reacting material and enzyme activity during carbon starvation. Yeast was cultured (1 liter) on yeast nitrogen base (0.7%, wt/vol) and glucose (2%, wt/vol) medium containing 1 μCi of [^{3}H]leucine per ml to a cell density of about 180 Klett units. Cells were then harvested by centrifugation at 4°C and suspended in 1 liter of carbon starvation (minus glucose) medium containing 0.7% (wt/vol) yeast nitrogen base and 0.5 mM leucine. Samples of 230 ml were harvested at the times indicated and stored at −70°C until used for preparation of extracts. (During starvation of the yeast cultures no appreciable increase of cell mass was observed.) Immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and preparation of extracts were carried out as described in Fig. 1. The total radioactivity incorporated into the NADP-GDH band at t_0 time point was 6,000 cpm, and the enzyme specific activity of NADP-GDH was 0.75 μmol/min per mg.*

The role of the known yeast proteases in the regulation of GDH could be examined by the use of yeast mutants deficient in specific protease activities. Recently it was reported that *S. cerevisiae* strains lacking protease B activity still inactivated the cytoplasmic malate dehydrogenase after addition of glucose to carbon-deficient cultures (14).

Similar results were obtained by Zubenko and Jones (Abstr. 9th Int. Congr. on Yeast Genetics, Abstr. 314, p. 98, 1978), who demonstrated that protease B-deficient *S. cerevisiae* strains inactivated fructose-1,6-bisphosphatase, malate dehydrogenase, and phosphoenol pyruvate carboxykinase at a rate comparable to the wild type. It has been suggested that protease B is responsible for the in vivo inactivation of these enzymes based on their in vitro susceptibility to yeast proteases (5). The results obtained using protease B-deficient mutants indicate that unrecognized protease(s) are involved in these regulatory systems.

There are several examples of the in vivo inactivation of microbial enzymes, in which it has been demonstrated that cross-reactive protein disappears from cells at the same rate as enzymatic activity: glycogen phosphorylase from *Dictyostelium discoideum* (13), phosphoribosylanthranilate isomerase-indoleglycerol phosphate from *Escherichia coli* (8), aspartate transcarbamylase from *Bacillus subtilis* (6), malate dehydrogenase from *S. cerevisiae* (9), and NADP-GDH from *C. utilis* (4). In all these systems no evidence for the production of fragments derived from the native enzyme was found. This suggests that proteolysis proceeds extremely quickly once initiated. Also, it is not known how these proteins become "tagged" for rapid proteolysis during the particular nutritional or morphological transitions.

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