A Study of Derepression of NAD-specific Glutamate Dehydrogenase of *Neurospora crassa*

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Transfer of *Neurospora crassa* mycelium from a 1% (w/v) sucrose medium to carbon-free or 1% (w/v) glutamate medium results in the onset of derepression of the catabolic NAD-specific glutamate dehydrogenase (NAD-GDH), within 30 min of the shift. Immunoprecipitation of *in vivo* pulse-labelled NAD-GDH demonstrated that this enzyme was synthesized *de novo*, correlating with increasing enzyme activity in shifted cells. Derepression was shown to be under transcriptional control by using the RNA synthesis inhibitor, picolinic acid, and by immunoprecipitation of the *in vitro* translation products of poly(A)-containing mRNA from repressed and derepressed cells. A brief (5 min) shift to derepression medium followed by a return to 1% (w/v) sucrose medium was sufficient to trigger synthesis of abundant NAD-GDH transcripts and low levels of the active enzyme. A secondary level of translational control is proposed to account for the discrepancy between the detectable levels of NAD-GDH transcripts and protein, following transient derepression.

INTRODUCTION

Glutamate dehydrogenase (GDH) is a key regulatory enzyme linking nitrogen and carbon metabolism, catalysing the formation of glutamate by reductive amination of 2-oxoglutarate or that of 2-oxoglutarate and ammonia from glutamate by oxidative deamination. While GDH in the *Enterobacteriaceae* is specific for the coenzyme NADP (Coulton & Kapoor, 1973; Sakamoto *et al.*, 1975), in *Neurospora crassa*, *Saccharomyces cerevisiae* and several other fungi, two distinct species of GDH are produced – one specific for NAD and another for NADP (Sanwal & Lata, 1961; Holzer & Schneider, 1957). NAD-GDH (EC 1.4.1.2) is believed to serve a catabolic function and NADP-GDH (EC 1.4.1.4) a biosynthetic one.

The NAD-GDH of *N. crassa* belongs to a class of enzymes subject to carbon catabolite repression. It is derepressed by carbon limitation to an extent of approximately 10-fold (Sanwal & Lata, 1961; Kapoor & Grover, 1970), the level of activity being further enhanced if L-glutamate is provided as the sole carbon and nitrogen source. If, however, urea is utilized as a nitrogen source instead of ammonia, this enzyme is partially derepressed even in the presence of a rich carbon source (Strickland, 1969). It has been demonstrated that urea-induced NAD-GDH derepression does not function in the *nit-2* mutant (defective in nitrogen catabolite repression) although the latter responds normally toward carbon derepression (Dantzig *et al.*, 1979). In contrast, NADP-GDH activity appears to be increased in the presence of a rich carbon source and repressed in media containing glutamate or ammonia. This apparently inverse regulation of the two enzymes by carbon and nitrogen metabolites does not prevail under all growth conditions (Kapoor & Grover, 1970).

The NADP-GDH of *N. crassa* has been extensively investigated biochemically and its structural gene cloned and sequenced (Kinnaird *et al.*, 1982; Kinnaird & Fincham, 1983). Although much of the amino acid sequence of the NAD-GDH has been published (Austen *et al.*, 1980), virtually no genetic information is available. The molecular mechanism of regulation of these two enzymes is also not understood.

In this communication, we report the results of an investigation of protein synthesis in *N. crassa* cultures raised under carbon catabolite repression as well as derepression conditions. Monitoring the levels of NAD-GDH by enzyme activity measurements, immunoprecipitation of pulse-labelled cells and *in vitro* translations of mRNA isolated from repressed and derepressed cultures confirm carbon catabolite repression to be the primary control governing the synthesis of this enzyme.

METHODS

Growth and labelling of cultures. *Neurospora crassa* (wild-type, FGSC no. 262) cultures were grown in Vogel's minimal medium (Vm; Vogel, 1956), supplemented with 0.5–1% (w/v) sucrose, for 20–24 h in Fernbach flasks at 28 °C with rotary shaking at 180 r.p.m. The mycelium was harvested by vacuum filtration on four layers of cheesecloth, rinsed with Vm medium and cut into thin pads (0.25 g wet weight). These pads were quickly transferred to 50 ml of the appropriate derepression medium in 250 ml Erlenmeyer flasks. The onset and progress of derepression of NAD-GDH was monitored by enzyme activity assays and visualization of the immunoprecipitated enzyme on SDS-PAGE gels as described below.

In *in vivo* labelling experiments, [^{35}S]methionine (Amersham; sp. act. > 1000 Ci mmol $^{-1}$) was added to 30 ml culture medium (final concn 5 $\mu\text{Ci ml}^{-1}$, 185 kBq ml $^{-1}$) and left for 30 min. Mycelial pads were harvested by vacuum filtration, rinsed thoroughly with ice-cold sterile H $_2$ O, pressed dry, frozen rapidly on dry ice in 1.5 ml microfuge tubes and freeze-dried.

Protein extraction and enzyme assays. Freeze-dried mycelium to be assayed for enzyme activity was pulverized with a Teflon microhomogenizer in 400 μl of the GDH extraction buffer (100 mM-(NH $_4$) $_2$ HPO $_4$, pH 8.0, 1 mM-EDTA, 2.5 mM-glutamate, 1 mM-2-mercaptoethanol) employed by Veronese *et al.* (1974). *In vivo* labelled cultures were similarly homogenized in 200 μl 10 mM-(NH $_4$) $_2$ HPO $_4$, pH 8.0, 1 mM-2-mercaptoethanol, 0.2 mM-PMSF (phenylmethylsulphonyl fluoride). Extracts were clarified by two consecutive 10 min centrifugations in a Fisher model 235A microcentrifuge at 4 °C.

Glutamate dehydrogenase activity was assayed by reductive amination of 2-oxoglutarate as described previously (Kapoor & Grover, 1970). Specific activity is expressed in katal [1 kat corresponds to the conversion of 1 mol substrate s $^{-1}$ (mg protein) $^{-1}$]. Protein concentration was estimated spectrophotometrically by the procedure of Kalb & Bernlohr (1977).

Purification of NAD-GDH. Typically, 40 g freeze-dried mycelium was pulverized briefly in a Waring blender and resuspended in 700 ml ice-cold extraction buffer with vigorous stirring for 45 min. The resulting suspension was homogenized with three strokes of a Teflon pestle powered by a motorized homogenizer (A. H. Thomas Co.). The resulting extract was centrifuged at 1400 *g* for 10 min, and the supernatant was filtered through two layers of cheesecloth and centrifuged again at 23000 *g* for 30 min. NAD-GDH was purified to apparent homogeneity by a modification of the method of Veronese *et al.* (1974). Briefly, this procedure employed sequentially: ammonium sulphate fractionation and DEAE-cellulose chromatography, as described by Veronese *et al.* (1974), an additional step involving affinity chromatography through a Blue Matrex column (Amicon) and finally gel filtration through a Sephacryl S-300 column (Pharmacia).

Precipitation of antisera and purification of IgG. Two young female New Zealand rabbits were injected with 250 μg purified NAD-GDH in Freund's adjuvant and boosted twice with 500 μg and 1000 μg each at 3 week intervals. Blood was withdrawn weekly by cardiac puncture and the titre of the antiserum monitored by Ouchterlony double diffusion. The IgG fraction was enriched by ammonium sulphate fractionation and ion exchange chromatography according to the method of Palmiter *et al.* (1971). Anti-GDH IgG was further purified by passage through an immunoaffinity column, prepared by coupling 2 mg purified NAD-GDH to Affi-Gel-10 (Bio-Rad) according to the manufacturer's instructions. The identity of this antibody was confirmed by enzyme inactivation.

Immunoprecipitation and gel electrophoresis. Typically, 2.5 $\times 10^6$ c.p.m. (20–40 μl) of translation products or *in vivo*-labelled crude extracts [4–20 $\times 10^3$ c.p.m. ($\mu\text{g protein}$) $^{-1}$] were diluted to 400 μl with RIPA buffer (10 mM-Tris/HCl, pH 7.2, 150 mM-NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM-methionine and 0.02% NaN $_3$) according to Gilead *et al.* (1976). This mixture was pre-adsorbed with 60 μl of a 10% suspension of *Staphylococcus aureus* cells (Pansorbin, Calbiochem) previously washed with RIPA buffer. The *S. aureus* cells were removed by centrifugation at 10000 *g* for 15 min at 4 °C and the supernatants incubated with 8 μg of immunopurified anti-GDH IgG for 8–16 h at 4 °C, followed by addition of 60 μl of *S. aureus* cells for 20 min. The immunoprecipitates were collected by centrifugation through a 1 M-sucrose cushion and washed as described by Siebert *et al.* (1982). ^{35}S -labelled antigens were released by boiling for 5 min in 80 μl SDS electrophoresis buffer (Laemmli, 1970) and centrifugation at 10000 *g* for 10 min at room temperature.

One-dimensional SDS-PAGE was done using 7–15% (w/v) linear polyacrylamide gradients essentially according to the method of Laemmli (1970). High *M_r* protein markers (BRL) were: myosin H chain, 200000;

phosphorylase b, 97400; bovine serum albumin, 68000; ovalbumin, 43000; α -chymotrypsin, 25700; β -lactoglobulin, 18400; and lysozyme, 14300. Gels were stained, dried and subjected to autoradiography using Kodak X Omat film.

RNA extraction, preparation of poly(A)-containing RNA and *in vitro* translation. Total RNA was extracted by grinding fresh or frozen mycelium with acid-washed sand in 1 vol. 100 mM-Tris, pH 9.0, 1% SDS and 2 vols phenol/chloroform/isoamyl alcohol (P/C/I; 24:24:1 by vol.) (Brawerman, 1973) followed by two or three further extractions with equal volumes of P/C/I and finally 1 vol. C/I (24:1, v/v). RNA was precipitated with an equal volume of 4 M-LiCl and re-precipitated twice more with 2.5 vols ethanol in the presence of 0.3 M-sodium acetate, pH 5.2.

Poly(A)-containing RNA was prepared from total RNA by fractionation on an oligo(dT)-cellulose (BRL) column essentially as described by Aviv & Leder (1972). Poly(A)-containing RNA (4 μ g) was translated in 60 μ l reaction volumes of a micrococcal nuclease-treated rabbit reticulocyte lysate (BRL) with [35 S]methionine, according to the manufacturer's recommendations. Incorporation of labelled TCA-precipitable radioactivity was determined as described previously (Devchand & Kapoor, 1984).

RESULTS

Derepression of NAD-GDH

The specific activity curve of derepression of NAD-GDH during growth under carbon limitation conditions is presented in Fig. 1. On transfer of mycelium to derepressing medium containing 1% (w/v) glutamate, a rapid increase in specific activity occurred following a lag of approximately 30 min. Derepression proceeded at a rapid rate for 2 h and then levelled off substantially. As permeability problems were encountered with actinomycin D, the chelating agent picolinic acid was used to assess whether derepression of NAD-GDH was regulated at the transcriptional level. At a concentration of 20 mM, this compound has been shown to preferentially block transcription, following rapid uptake by *N. crassa* cells (Martegani, 1981). The cells were initially grown for 21 h in Vm supplemented with 1% (w/v) sucrose, and were resuspended in Vm supplemented with 1% (w/v) glutamate as the sole carbon source. Picolinic acid (1 M), pH 5.5, was added to certain culture flasks (final concn 20 mM) either immediately after the nutritional shift, or 60 min post-transfer. Derepression of NAD-GDH was demonstrated by determining the specific activity at 30 min intervals after transfer.

When picolinic acid was added at the time of the shift to Vm supplemented with 1% (w/v) glutamate, derepression was completely blocked. Furthermore, when this inhibitor was added 60 min after transfer, derepression of NAD-GDH activity began to plateau within 20 min, rather than continuing to increase for an additional 60 min as seen in the control cultures. These results suggested that derepression of NAD-GDH is transcriptionally controlled and that RNA synthesis must be maintained for continued increases in specific activity.

Protein synthesis during derepression

Changes in protein synthesis patterns during derepression were assessed by a comparison of the SDS-PAGE profiles of polypeptides synthesized *in vivo* (Fig. 2) in cells under carbon catabolite repression (lane 1) and derepression conditions (lanes 2, 3 and 4). Transferring *N. crassa* mycelium from a carbon rich medium [Vm + 1% (w/v) sucrose] to conditions of carbon starvation with urea (lane 2) or ammonia (lane 3) as the sole nitrogen source, or to a medium with a poor carbon source, such as 1% (w/v) glutamate (lane 4), resulted in the appearance of several labelled polypeptides. In addition to NAD-GDH, a number of other proteins were observed to be derepressed concurrently by these alterations in the growth regimen. The four prominent polypeptides (indicated by arrows in lane 2) synthesized under carbon starvation were also synthesized when glutamate was provided as the sole carbon source. No pronounced differences were apparent in the polypeptide composition of cells cultured in carbon-deficient media in the presence of urea or ammonia as the sole nitrogen source. The identity of the other catabolite-repressed proteins is not known at present although it is possible that they represent some of the other enzymes implicated in carbon assimilation, such as catabolic enzymes or permeases.

Fig. 1

Fig. 1. Time course of NAD-GDH derepression in the presence and absence of the transcriptional inhibitor picolinic acid. Cells were grown for 21 h in Vm + 1% (w/v) sucrose and were transferred to Vm + 1% (w/v) glutamate. ●, No picolinic acid; ▽, □, picolinic acid (final concn 20 mM) added to the treated cultures either at the time of transfer to Vm (▽), or 60 min after transfer to Vm + 1% (w/v) glutamate (□).

Fig. 2

Fig. 2. Autoradiographs of proteins pulse-labelled with [^{35}S]methionine and resolved by SDS-PAGE. Cultures were grown for 21 h in Vm + 1% (w/v) sucrose and transferred to the following media and pulse-labelled 60–90 min thereafter: lane 1, Vm + 1% (w/v) sucrose; lane 2, 150 mM-urea in carbon- and ammonium-free Vm; lane 3, carbon-free Vm; lane 4, Vm + 1% (w/v) glutamate.

Demonstration of de novo synthesis of NAD-GDH during derepression

The dramatic increase in the specific activity of NAD-GDH during derepression could theoretically result either from the activation of a constitutive, inactive form of the enzyme or as a consequence of *de novo* synthesis. To distinguish between these two possibilities *N. crassa* was cultivated for 21 h in Vm + 1% (w/v) sucrose and transferred to three different derepressing media: nitrogen-free Vm + 150 mM-urea, carbon-free Vm and Vm + 1% (w/v) glutamate. [^{35}S]Methionine was added 60 min after transfer and the cells were harvested 30 min later. Concurrently, the control cells, maintained in 1% (w/v) sucrose, under catabolite repression, were labelled without transfer for 30 min, 22 h after inoculation. Immunoprecipitation with anti-GDH IgG was used to quantify the amount of NAD-GDH produced under derepressing conditions. The crude extract of repressed cells (Fig. 3, lane 1) did not yield any immunoprecipitable material (lane 2) while crude extracts of derepressed cells (lanes 3, 5 and 7) showed one prominent, labelled band corresponding to newly synthesized NAD-GDH. As the 60–90 min period following transfer corresponds to the period during which enzyme activity increases most rapidly, derepression is directly dependent upon *de novo* synthesis of the NAD-GDH subunit polypeptide, rather than the activation of a pre-existing NAD-GDH zymogen.

In order to examine the course of NAD-GDH subunit synthesis during derepression, 21 h cultures grown under carbon catabolite repression [Vm + 1% (w/v) sucrose] were resuspended in Vm + 1% (w/v) glutamate and pulse-labelled with [^{35}S]methionine for various times during derepression (Fig. 4a, lanes 1, 3, 5, 7). The greatest incorporation of label into GDH occurred 30–60 min after transfer (Fig. 4b, lane 6). By 150 min post-transfer, incorporation of label into GDH had declined appreciably (Fig. 4b, lane 8). This phase corresponds to the plateau in enzyme activity evident in the data of Fig. 1.

Fig. 3. Autoradiographs of crude extracts and immunoprecipitates labelled *in vivo* with anti-GDH IgG and resolved by SDS-PAGE. Crude extracts (odd-numbered lanes) were obtained from cells pulse-labelled for 30 min as described in Fig. 2; immunoprecipitates from these extracts are shown in the adjacent, even-numbered lanes. Lanes 1 and 2, Vm + 1% (w/v) sucrose; lanes 3 and 4, 150 mM-urea in carbon- and ammonium-free Vm; lanes 5 and 6, carbon-free Vm; lanes 7 and 8, Vm + 1% (w/v) glutamate.

Derepression of NAD-GDH can be initiated by a brief carbon limitation

The lag period preceding an appreciable increase in NAD-GDH activity, upon transfer to a derepression medium, was found to be approximately 30 min in duration (Fig. 1). A brief immersion in carbon-free Vm and subsequent return to repression conditions should demonstrate whether derepression occurs only after depletion of some intracellular carbon catabolite or represents an immediate response to extracellular deprivation. This experiment was done by transferring repressed cells from Vm + 1% (w/v) sucrose, immersion in carbon-free Vm for 5 min and transfer back to Vm + 1% (w/v) sucrose. The cultures were pulse-labelled with [³⁵S]methionine for 30 min periods following the return of cells to a carbon-rich medium, and the resulting polypeptide profiles and anti-NAD-GDH immunoprecipitates were analysed (Fig. 4*a* and *b*, lanes 2, 4, 6 and 8).

Some incorporation of the label into the NAD-GDH subunit was evident in these autoradiographs. However, most of it occurred within the first 30 min of incubation in the repression medium and declined to basal levels thereafter. It is noteworthy that a brief exposure to carbon-free medium, preceding the transfer to a carbon-rich medium, appeared to result in a transient stimulation of GDH subunit synthesis.

In a parallel experiment, NAD-GDH activity in cells subjected to a 5 min immersion in carbon-free Vm as above was also monitored. A transient, two- to threefold increase in specific activity was detected during the first 60–90 min (Fig. 5). Treatment of cells undergoing such a transfer with 20 mM-picolinic acid or with 0.2 µg cycloheximide ml⁻¹ indicated that both RNA and protein synthesis were required for the occurrence of transient derepression of NAD-GDH. The requirement for protein synthesis is consistent with the results of pulse-labelling and immunoprecipitation experiments discussed earlier.

In vitro translation of poly(A)-containing RNA

To confirm that transcription of the NAD-GDH structural gene is essential for derepression, total poly(A)-containing RNA was extracted from representative cultures and translated *in vitro*.

Fig. 4. Autoradiographs of (a) crude extracts and (b) immunoprecipitates labelled *in vivo* and resolved by SDS-PAGE. Cells were grown for 21 h and were then either transferred immediately to Vm + 1% (w/v) glutamate (lanes 1, 3, 5, 7) or were harvested, immersed in carbon-free Vm for 5 min and transferred to Vm + 1% (w/v) sucrose (lanes 2, 4, 6, 8). Cells were then pulse-labelled with [35 S]methionine at the following times after transfer: lanes 1 and 2, 0–30 min; lanes 3 and 4, 30–60 min; lanes 5 and 6, 90–120 min; lanes 7 and 8, 150–210 min.

The SDS-PAGE profiles of translation products of mRNA from derepressed and repressed cultures (Fig. 6, lanes 1 and 3) revealed the presence of a wide range of labelled polypeptides. It can be inferred from the size distribution that the majority of transcripts comprising the mRNA fraction were less than 3.5 kb in length. Immunoprecipitation with anti-GDH IgG specifically selected a polypeptide corresponding to NAD-GDH from the translation products of poly(A)-containing RNA from derepressed cultures (lane 2). In contrast, only a faint band of immunoprecipitable material from the translation products of mRNA from carbon-rich, repressed cultures was observed (lanes 3 and 4). The latter was detected in amounts consonant with a basal level of gene expression.

Fig. 5. Increase in the level of NAD-GDH activity in a carbon-rich medium following transient derepression. Cells grown in Vm + 1% (w/v) sucrose for 21 h were harvested, immersed for 5 min in carbon-free Vm and transferred to fresh Vm + 1% (w/v) sucrose medium (●). Some of the cultures contained 20 mM-picolinic acid (□) or 0.2 µg cycloheximide ml⁻¹ (■). Enzyme activity was determined as described in Methods.

Fig. 6. Autoradiographs of translation products labelled *in vitro* with [³⁵S]methionine and resolved by SDS-PAGE. Lanes 1, 3 and 6, translation products of total poly(A)-containing RNA from derepressed, repressed and transiently derepressed cells, respectively. Lanes 2, 4 and 5, anti-NAD-GDH precipitable translation products from derepressed, repressed and transiently derepressed cultures, respectively. For transient derepression, cells were grown in Vm + 1% (w/v) sucrose for 21 h, transferred to carbon-free Vm for 5 min and then shifted to a fresh Vm + 1% (w/v) sucrose medium. *In vitro* translations were done in rabbit reticulocyte lysate as described in Methods. Approximately 3×10^5 c.p.m. of total translation products were loaded per lane and 2.5×10^6 c.p.m. was used in the immunoprecipitation reaction.

To ascertain whether RNA specific for NAD-GDH was detectable in transiently derepressed cells, total poly(A)-containing RNA was isolated from mycelium that had been briefly immersed in a carbon-free medium but otherwise maintained in a medium with a rich carbon source (Fig. 6, lane 6). Immunoprecipitation of the translation products (lane 5) indicated that even at 60 min after transfer to a carbon-rich medium, transcripts coding for NAD-GDH were present at levels comparable to the fully derepressed state.

DISCUSSION

The results of experiments documented in this report confirm that the synthesis of NAD-GDH of *N. crassa* is controlled primarily by carbon catabolite repression. By immunoprecipitation of *in vivo*-labelled cell extracts, we have demonstrated that the NAD-GDH subunit is synthesized *de novo* during derepression. The detectable levels of this polypeptide, at various times during derepression, correlate well with changes in specific activity. Furthermore, experiments utilizing picolinic acid and translation of total poly(A)-containing RNA, described here, suggest that NAD-GDH derepression is regulated at the transcriptional level. This observation is consistent with most other carbon catabolite-repressed fungal systems examined so far (Hoosein & Lewin, 1984). The rapidity of the derepression response upon brief immersion of mycelium in a carbon-deficient medium was a surprising observation, as filamentous fungi are known to have a considerable storage capacity for carbohydrates (Blumenthal, 1975). However, our results demonstrate that *N. crassa* cells register a dramatic response toward changes in the composition of their extracellular environment, before depletion of the internal reserves can be expected to have occurred. In view of these observations, it is possible that carbon catabolite repression is mediated, at least in part, by a membrane-bound signalling apparatus, perhaps analogous to the adenylate cyclase mediation of catabolite repression encountered in *Escherichia coli* and some other prokaryotes (Botsford, 1981).

An adenylate cyclase activity resembling that in mammalian systems has been reported recently in *N. crassa* (Rosenberg & Pall, 1983). In addition, Pall (1977) demonstrated that simply washing *N. crassa* cells with water or the growth medium for 2 min, followed by filtration, resulted in as much as a 10-fold increase in cyclic AMP levels. Therefore, although a pleiotropic role for cAMP in the fungi has been largely rejected (Pall, 1981), the results of our work and studies with enzymes such as δ -aminolaevulinic dehydratase of *S. cerevisiae* (Mahler & Lin, 1978) suggest its involvement in these catabolite-repressed systems.

Our experiments showed that immersion in a carbon-free medium was sufficient to stimulate the production of NAD-GDH transcripts. The latter appeared to persist for up to 60 min, following a return to a carbon-rich medium, as evidenced by *in vitro* translation. However, it has also been shown that after 60 min, enzyme activity in such transiently derepressed cells had already reached a plateau. Likewise, the amount of immunoprecipitable, *in vivo*-labelled GDH had also declined considerably by this time.

It is interesting that the rapid transcriptional response elicited by transient derepression leads to the formation of normal, stable, NAD-GDH transcripts that are translated *in vitro*, but evidently remain untranslated *in vivo*. The existence of a translational control mechanism responsible for the prevention of the synthesis of additional GDH subunits in the presence of a preferred carbon source is suggested by these results. The relaxation of this translational regulation *in vitro* is attributable to the functioning of a regulatory factor(s) indigenous to *N. crassa* cells, but probably not available in the heterologous rabbit reticulocyte *in vitro* translation system. An analysis of translational control of repression/derepression systems should be feasible using the homologous *in vitro* translation system, based on a *N. crassa* lysate, recently developed in our laboratory (Devchand & Kapoor, 1984).

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