PHOSPHORYLATION AND PROTEOLYSIS REGULATE THE NAD-DEPENDENT GLUTAMATE DEHYDROGENASE FROM *SACCHAROMYCES CEREVISIAE*

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1. Introduction

Protein phosphorylation and degradation are recognized as two of the most important mechanisms for controlling the activity of key regulatory enzymes in eukaryotic cells [1,2]. Both of these control mechanisms are involved in the regulation of enzyme activity in the yeast [3,8].

It is firmly established that the NAD-dependent glutamate dehydrogenase (NAD-GDH) from *Candida utilis* is regulated by phosphorylation [6] but there is no evidence suggesting a role for proteolysis in this system. In fact, unpublished studies (B.A.H.) have shown that the phosphorylated enzyme from *C. utilis* is stable in vivo. In contrast to the regulation of NAD-GDH from *C. utilis* many questions remain unanswered regarding the regulation of the NAD-GDH from *Saccharomyces cerevisiae* (review in [9]). On the basis of activity levels found in *S. cerevisiae* grown on different growth media, it is concluded that the NAD-GDH functions primarily in a catabolic capacity; the specific activity of the NAD-GDH is highest during growth on glutamate– or aspartate–glucose medium and lowest during growth on ammonium salts. However, these experiments do not distinguish between changes in the rate of enzyme synthesis, degradation and activity modulation.

Preliminary results indicated that the NAD-GDH is rapidly inactivated in *S. cerevisiae* during glutamate starvation [10]. Also, it is noted that transfer of yeast from pre-sporulation medium to sporulation medium resulted in a rapid and extensive loss of NAD-GDH activity [11]. In view of the fact that the NAD-GDH from *C. utilis* is regulated by phosphorylation–dephosphorylation mechanism, I re-examined the enzyme inactivation observed with *S. cerevisiae*. From these experiments, it is concluded that the inactivation of NAD-GDH activity in *S. cerevisiae* occurs initially from phosphorylation and subsequently by proteolytic degradation.

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2. Materials and methods

2.1. *Organism*

Saccharomyces cerevisiae 288c was used in all experiments (obtained from Dr E. Cabib, National Institutes of Health, Bethesda, MD). For the purification of the NAD-GDH the protease deficient strain, *pep 4-3* (kindly provided by Dr Elizabeth W. Jones, Carnegie-Mellon University, Pittsburgh, PA) was used.

2.2. *Growth conditions*

Cells were grown at 30°C on 2% (w/v) potassium acetate, 0.5% (w/v) yeast-extract, and 0.5% (w/v) bactopeptone. For labelling experiments, 2–4 μCi/mol L-[4,5-^{3}H]leucine was added to this growth medium. To label the enzyme with [^{32}P]phosphate, the cells were grown on the above medium supplemented with 28.5 μCi [^{32}P]orthophosphate/ml.

To initiate enzyme inactivation, cells were harvested by centrifugation, washed with ice cold distilled water and resuspended in 2% (w/v) potassium acetate. When the cells were prelabeled with [^{3}H]leucine, 1 mM cold L-leucine was added to the potassium acetate.

For the purification of the NAD-GDH, *S. cerevisiae pep 4-3* was precultured on the following medium: 1% (w/v) glucose, 1% (w/v) yeast extract and 2% (w/v) bactopeptone, in a 10 liter fermentor. The entire cul-

ture was then transferred to a 350 liter fermentor containing the following: 1% (w/v) potassium acetate, 1% (w/v) yeast extract, 2% (w/v) bactopeptone, 1% (w/v) sodium glutamate and 0.01% (w/v) trytophan. The cell yield was ~4 g/l.

2.3. *Preparation of extracts and determination of NAD-GDH activity*

Frozen cell pellets were resuspended into 3 ml 0.1 M sodium phosphate (pH 7.5) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl-fluoride (PMSF), and passed 3 times through a French pressure cell. Unbroken cells and debris were removed by centrifugation at 40 000 × *g* at 4°C for 20 min. The resulting supernatant was used for enzyme assay and immunoprecipitation.

NAD-GDH activity was assayed at pH 8.0 and pH 6.9, as in [10], and is expressed as specific activities (μmol . min^{-1} . mg protein^{-1}). Protein was determined by the Bradford method [12] with bovine serum albumin as a standard.

2.4. *Purification of NAD-GDH from* S. cerevisiae (pep 4-3)

NAD-GDH was purified from *pep 4-3* by the procedure used for the enzyme from *C. utilis* in [6]. With the following modifications pure enzyme was obtained employing two additional steps after the final Ultrogel AcA22 chromatography.

The pooled fractions from the Ultrogel AcA22 filtration step were dialyzed into 50 mM sodium phosphate (pH 7.5), 5 mM EDTA and 0.2 mM NAD, then applied to a column of DEAE-BioGel A (1.6 × 8.0 cm) equilibrated with the same buffer. The enzyme was eluted from the column by applying a gradient of KCl in equilibration buffer from 0–0.4 M. The fractions of highest specific activity were pooled and concentrated by precipitation with solid ammonium sulfate to 60% saturation and dialyzed against 0.1 M sodium phosphate (pH 7.5), 0.1 M KCl, 5 mM EDTA, 0.2 mM NAD and 1 mM PMSF. The protein was then passed through a column of BioGel A0.5m (2.5 × 40 cm) equilibrated with the same buffer. The NAD-GDH eluted as an apparently homogeneous protein, fractions with the highest specific activity were pooled and stored at 4°C in 60% ammonium sulfate.

2.5. *Preparation of antibody against NAD-GDH*

Sheep antibody against NAD-GDH was prepared as follows: 0.8 mg protein was mixed with an equal volume of Freund's complete adjuvant and was injected subcutaneously. This was repeated 2 weeks later. One week after the second injection, 500 ml blood were taken and serum was prepared. The centrifuged serum was stored at −20°C until used. Control serum was taken before injection of antigen.

2.6. *Immunoprecipitation and electrophoresis*

To 1 ml radioactively-labeled crude extract (5–7 mg protein) Triton X-100, KCl and Tris–HCl (pH 7.5) were added to give final concentrations of 1.2% (v/v), 1.2 M and 0.1 M, respectively, followed by the addition of 100 μl antisera (2–3-fold excess). The mixture was incubated for 60 min at room temperature followed by a further 90 min at 4°C. Precipitation was >97% complete at this stage, as judged by determination of enzyme activity.

Immunoprecipitates were washed and solubilized as in [3]. The dissociated immunoprecipitates were separated by electrophoresis on 10% SDS–polyacrylamide gels (0.5 × 10 cm). After electrophoresis, the gels were cut into 1.5 mm slices and radioactivity in the protein band corresponding to NAD-GDH was quantitated as in [5].

2.7. *Phosphate analysis*

The alkali-labile phosphate analysis of the purified NAD-GDH was performed as in [13] using the Ames method [14]. This method was also used to determine P_i in the acetate/yeast extract/bactopeptone medium and found to be 1.33 mM.

2.8. *Sedimentation equilibrium studies on NAD-GDH from* S. cerevisiae pep 4–3

To determine the M_r of the NAD-GDH by sedimentation equilibrium centrifugation protein (2.1. mg . ml^{-1}) was dialyzed against 0.1 M sodium phosphate (pH 7.5), 1 mM EDTA for 24 h prior to centrifugation. The density of the buffer was 1.0112 g/ml. For the calculation of the M_r the $\bar{\nu}$ was assumed to be 0.736 (calculated from the amino acid composition of *C. utilis* NAD-GDH).

2.9. *Materials*

All radioactive isotopes were obtained from New England Nuclear or the Radiochemical Center, Amersham. DEAE-cellulose (DE-52) was from Whatman Ltd. and Ultrogel AcA22 was from LKB Instr. Affigel Blue, DEAE-BioGel A, and BioGel A0.5m were purchased from BioRad Labs. NAD, NADH, and

the disodium salt of α-ketoglutaric acid were obtained from Boehringer. Phenylmethylsulfonylfluoride (PMSF) and NAD were from Sigma. All electrophoresis chemicals were from BioRad Labs. Yeast nitrogen base, bactopeptone and Freund's complete adjuvant were from Difco.

3. Results

3.1. *Properties of NAD-GDH from* S. cerevisiae

The enzyme was purified from *S. cerevisiae pep 4-3*, a mutant yeast with very low levels of proteinases A and B, and carboxypeptidase Y [15]. Using the modified procedure (section 2) 46 mg pure NAD-GDH was obtained from 1 kg yeast, with a 4% recovery. The enzyme eluted from the final gel filtration step as an apparently homogeneous enzyme with a specific activity of ~190 units/mg. The NAD-GDH subunit was 117 000 M_r by SDS–polyacrylamide gel electrophoresis. From sedimentation equilibrium studies the enzyme was calculated to be 460 000 ± 4000 M_r. These results indicate that the NAD-GDH from *S. cerevisiae* is a tetramer composed of 4 apparently identical subunits.

The NAD-GDH from *C. utilis* has been shown to contain alkali-labile phosphate [6]. Similar analysis of the *S. cerevisiae* enzyme showed that the enzyme contained 0.6 mol phosphate/mol subunit.

3.2. *Inactivation of NAD-GDH in vivo*

Because growth of *S. cerevisiae* on acetate/yeast extract/bactopeptone (acetate-YEP) results in the derepression of NAD-GDH activity, acetate-adapted cells were used to investigate the mechanism of enzyme inactivation. Transfer of acetate-YEP cells to growth conditions lacking any nitrogen source promote a rapid loss of NAD-GDH activity (fig.1). The inactivation of NAD-GDH was associated with a change in the properties of the enzyme; the pH optimum of the enzyme stifted from pH 8.0–6.9 during the course of inactivation.

The reactivation of the NAD-GDH after addition of yeast extract and bactopeptone to the acetate medium was incomplete. After 60 min in the starvation medium, the NAD-GDH activity is 62% of the control value; addition of yeast extract and bactopeptone promoted a partial reactivation of enzyme to 88% of the control activity. After 180 min in the starvation medium, the activity of NAD-GDH was 18% of

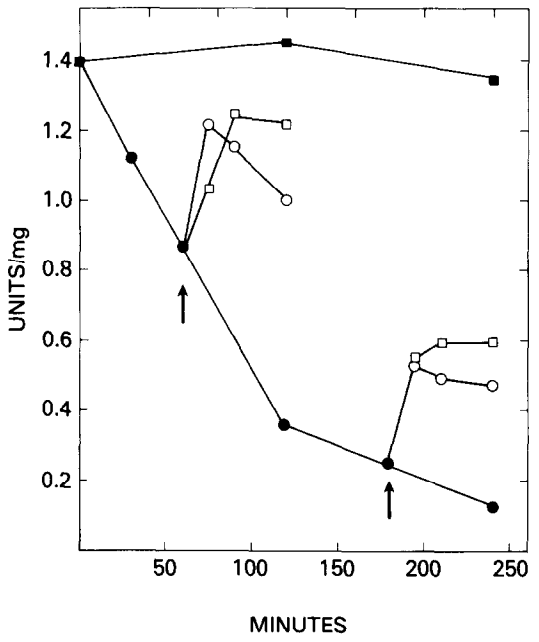

Fig.1. Loss of NAD-GDH activity during nitrogen starvation. Yeast was cultured (1 liter) on potassium acetate, yeast extract and bactopeptone (■) to a cell density of ~100 Klett units. The cells were then harvested and resuspended into potassium acetate (●) to promote enzyme inactivation, at 60 and 120 min yeast extract and bactopeptone was added to an aliquot of the culture medium with (○) and without (□) 25 μg cyclohexide/ml. Samples of 80 ml were harvested at the times indicated and stored at −70°C until used for estimation of enzyme activity. NAD-GDH activity was assayed as in section 2.

the control value; addition of yeast extract and bactopeptone resulted in an increase of enzyme activity to 42% of the control (fig.1). In both cases, the addition of 25 μg cycloheximide/ml did not block reactivation; suggesting the increase of enzyme activity was due to enzyme inter-conversion not synthesis; at this concentration of inhibitor, synthesis of NAD-GDH is completely blocked (unpublished).

These results suggested that the two mechanisms of enzyme regulation were involved in the loss of NAD-GDH activity: (i) enzyme interconversion; (ii) proteolysis.

3.3. *Immunochemical investigation of inactivation*

For these experiments the NAD-GDH was purified from *pep 4-3*, a yeast possessing very low levels of carboxypeptidase and proteases A and B [15], to reduce the problems of proteolysis during isolation.

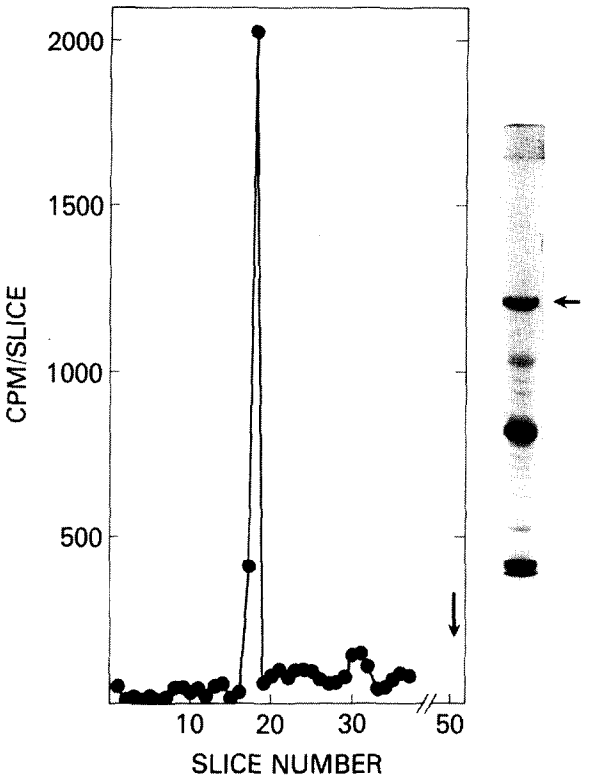

Fig.2. SDS–polyacrylamide gel profile of an anti-NAD-GDH immunoprecipitates from [^{3}H]leucine-labeled *S. cerevisiae*. For the experiment illustrated, yeast was cultured on glutamate (0.4% w/v), glucose (2% w/v) and yeast nitrogen base (0.145% w/v) medium containing 1.2 μCi [^{3}H]leucine/ml. Similar immunoprecipitate profiles were obtained when the enzyme was labeled during growth on potassium acetate, yeast extract and bactopeptone. Immunoprecipitation, SDS–polyacrylamide gel electrophoresis, and preparation of extracts were as in section 2. The insert shows a photograph of the SDS–polyacrylamide gel stained with Coomassie blue. The arrow indicates the position of NAD-GDH (M_r 117 000).

The purified protein was used to elicit antibody production.

The antisera precipitated a single protein from [^{3}H]leucine-labeled yeast (fig.2). The mobility of the precipitated protein corresponded to that of purified NAD-GDH and had 117 000 M_r in SDS–polyacrylamide gels.

The NAD-GDH was shown to be regulated by phosphorylation–dephosphorylation by labeling cells with [^{32}P]orthophosphate prior to the initiation of enzyme inactivation. Enzyme was isolated by immunoprecipitation from cells taken at 0, 60 and 120 min after transfer of cells to the starvation medium. The incorporation of phosphate into protein was determined after SDS–polyacrylamide gel electrophoresis (table 1). These data demonstrate that enzyme inactivation is paralleled by an increase in the amount of ^{32}P associated with the GDH protein.

Table 1
Correlation of enzyme activity with phosphate incorporation during nitrogen starvation[a]

Time (min)	Enzyme act.[b] (units/mg)	pH act.[c] ratio	mol PO_4/mol subunit incorp.[d]
0	2.19	0.46	0.17
60	0.58	1.2	0.65
120	0.36	1.4	0.56

[a] Yeast was grown on acetate/yeast extract/bactopeptone medium containing 28.5 μCi [^{32}P]orthophosphate/ml. At t = 0 cells were harvested by centrifugation and resuspended into potassium acetate

[b] Enzyme activity was measured at pH 8.0

[c] pH activity ratio is the enzyme activity measured at pH 6.9 divided by the activity measured at pH 8.0

[d] The stoichiometry of phosphorylation was calculated by assuming that the specific activity of the dephosphorylated enzyme is 440 units/mg by analogy with the *C. utilis* enzyme [6]. Corrections were made for enzyme degradation using the data from fig.3

At zero time GDH contained ~0.17 mol phosphate/mol subunit; after 60 min in the starvation medium this value increased to ~0.65 mol phosphate/mol subunit. The 4-fold increase of phosphate content of GDH is paralleled by a 75% reduction of enzyme activity. The phosphate content of GDH after 120 min in the starvation medium was ~0.56 mol/mol subunit.

The introduction of the phosphate group is also associated with a change in the catalytic properties of the NAD-GDH as illustrated by the shift in the ratio of enzyme activity measured at pH 6.9 and 8.0 (table 2). After 60 min in the inactivation medium the ratio changes from 0.46–1.2.

The rate of degradation of NAD-GDH was measured immunochemically during enzyme inactivation promoted by nitrogen starvation (fig.3). The results of this experiment showed that there is a differential loss of enzyme activity and enzyme protein (crossreacting material) during inactivation. The $t_{1/2}$ for loss in enzyme activity was 56 min whereas the enzyme protein level remained constant during the first 30 min enzyme inactivation before declining with $t_{1/2}$ 100 min. This experiment demonstrates that the introduction of the phosphate group results in a loss of enzyme

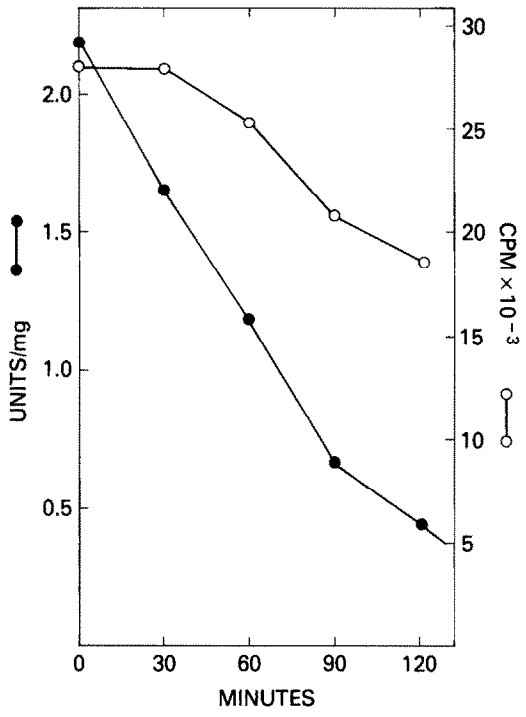

Fig.3. Loss of NAD-GDH crossreacting material (○) and enzyme activity (●) during nitrogen starvation. Yeast was cultured on potassium acetate (2% w/v), yeast extract (0.5% w/v) and 0.5% bactopeptone containing 3.64 μCi [^{3}H]leucine/ml to a cell density of 115 Klett units. Cells were then harvested by centrifugation at 4°C and resuspended into an equivalent volume of potassium acetate (2% w/v) and 1 mM leucine. Samples (100 ml) were harvested at the times indicated and stored at −70°C until used for preparation of extracts. Immunoprecipitation, SDS–polyacrylamide gel electrophoresis, and preparation of extracts were as in section 2. Each point represents the total cpm incorporated into NAD-GDH from 50 ml culture medium.

activity. Whether the phosphorylation of the NAD-GDH is a necessary pre-requisite for proteolysis remains to be established.

4. Discussion

The rapid inactivation of the NAD-GDH observed during nitrogen starvation is probably mediated primarily by phosphorylation secondarily by proteolysis. This is corroborated by:

(i) The loss of enzyme is partially reversible;

(ii) The rate of loss of enzyme activity is twice as fast as the rate of degradation of GDH.

The GDH inactivation system described here would appear to be one of the first examples in which an enzyme is covalently modified prior to degradation. However, further experiments are necessary to delineate whether there is a sequential connection between the two processes.

The use of growth conditions where protein phosphorylation occurs independent of protein degradation will enable one to establish the exact correlation between the extent of phosphorylation and loss of enzyme protein. Early experiments [10], using glutamate–glucose-adapted cultures, showed that the loss of enzyme activity observed during glutamate starvation was completely reversible (even in the presence of cyclohexide). The levels of enzyme produced under these conditions was ~20% of those observed in this study and thus, could make quantitation difficult. Further analysis of the rate of degradation of NAD-GDH under different growth conditions could establish a link between enzyme phosphorylation and degradation. It would be of interest to know the rates of degradation under conditions where the enzyme is maximally de-repressed (dephosphorylated) and where the enzyme is fully de-repressed (phosphorylated?).

The fact that the NAD-GDH is phosphorylated prior to degradation could be helpful in elucidating the mechanism of proteolysis of eukaryotes. An additional marker, such as a phosphate group on a protein, could probably be employed to identify fragments which are generated by protease action (provided the phosphorylated fragments are not dephosphorylated). Here, two additional proteins containing phosphate were identified when immunoprecipitates were electrophoresed on SDS–polyacrylamide gels; their mobilities corresponded to 34 000 and 10 000 M_r. The amount of radioactivity in the two fragments increased progressively during the inactivation process. Whether these proteins are proteolytic fragments generated from the NAD-GDH during the inactivation process remains to be established.

Several enzymes from *S. cerevisiae* are rapidly inactivated in vivo [16,17] and, in [7] a proteolytic mechanism has been proposed based on immunochemical data. A parallel loss of enzyme activity and enzyme protein has been demonstrated for the NADP-GDH and the cytoplasmic malate dehydrogenase [7,8]. This relationship has not been established for the other inactivation systems. With all of these enzyme inactivation systems there is, as yet, no data available to ascertain whether covalent modification occurs or

not. The extremely rapid inactivation of fructose 1,6-bisphosphatase, observed after the addition of glucose to acetate grown cells, would appear to be a good candidate for a closer re-examination for covalent modification.

These results clearly demonstrate that two different forms of post-translational modification operate to regulate the level of NAD-GDH activity. Therefore, caution should be used in the interpretation of data on the levels of enzyme observed during growth on different carbon and nitrogen sources. The different activity levels observed could be due to the NAD-GDH existing in either fully active or less active forms, and not due to different rates of enzyme synthesis.

It is also possible that mutants which have been isolated defective in NAD-GDH synthesis, may not result from mutations in the structural gene for NAD-GDH but could possibly be due to mutations in the genes which code for the proteins responsible for phosporylation and dephosphorylation. However, much of the above discussion will remain speculative until the proteins responsible for phosphorylation and dephosphorylation have been identified and characterized.

The subunit M_r of 117 000 of the *S. cerevisiae* NAD-GDH is the same as that found for the enzyme isolated from *Neurospora crassa* [18] and *C. utilis* [6]. It would be of interest to know whether the NAD-specific GDHs found in other organisms, especially prokaryotes, are composed of similarly large subunits. Generally the GDHs isolated from other sources which are NADP-specific, exist as hexamers composed of subunits with M_r ~50 000 [19]. The possible role of phosphorylation in the regulation of other NAD-GDHs would be of considerable interest.

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