Carbon Source Regulation of Nicotinamide Adenine Dinucleotide (Phosphate) Glycohydrolase in *Neurospora crassa*: Induction and Repression of Enzyme Synthesis

By JOÃO A. JORGE¹* AND HÉCTOR F. TERENCE²

*Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto¹ and
Departamentos de Fisiologia e Bioquímica, Faculdade de Medicina de Ribeirão Preto²,
Universidade de São Paulo, 14100 – Ribeirão Preto, Estado de São Paulo, Brasil*

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Synthesis and release of NAD(P)ase by *Neurospora crassa* wild type was studied in experiments in which mycelia grown in Vogel minimal medium were transferred to media containing protein as the only carbon source. Several results are presented suggesting that the NAD(P)ase may be induced by the presence of protein in the culture medium. Low concentrations of sucrose or glucose (0.1%), Casamino acids or some amino acids such as methionine, cysteine, phenylalanine and tryptophan strongly repressed the enzyme synthesis. Under induction conditions NAD(P)ase and alkaline protease appeared together in the culture medium. It would appear that NAD(P)ase and alkaline protease are coordinately regulated by a common control mechanism related to carbon catabolism.

INTRODUCTION

Nicotinamide adenine dinucleotide (phosphate) glycohydrolase (NAD(P)ase; EC 3.2.2.6) was first described, in *Neurospora crassa*, as an enzyme that has its level increased in mycelia grown in a zinc-deficient medium (Nason *et al.*, 1951). Following this discovery Zalokar & Cochrane (1956) found that NAD(P)ase activity increased sharply during conidiation. Stine (1968) showed that NAD(P)ase activity appeared together with the formation of aerial hyphae, accumulating in conidia and decreasing very fast as the mature conidia germinated. Combépine & Turian (1970) concluded that NAD(P)ase was important to conidiogenesis. A mutant that was temperature sensitive for conidiation was also temperature sensitive for NAD(P)ase formation (Hochberg & Sargent, 1974). Urey (1971) was also able to separate the conidiophores from the mycelial pad and to demonstrate that newly formed NAD(P)ase activity was confined to developing aerial tissue. We showed (Jorge & Terenzi, 1980) that the cyclic AMP-deficient, hyperconidiating mutant, strain *cr-1* (*crisp-1*) continuously produces and secretes NAD(P)ase, and that the enzyme level decreases when the mutant is cultivated in the presence of cyclic AMP, which also corrects the morphology defect. However, conidiation does not seem to be a prerequisite for the production of NAD(P)ase, since the aconidial strain *fluffy* makes large amounts of the enzyme (Stine, 1968). On the other hand, the absence of NAD(P)ase did not affect the growth of aerial hyphae, conidiation or conidial germination in five defective mutant strains (*nada*) studied (Nelson *et al.*, 1975).

Nelson *et al.* (1976) concluded that NAD(P)ase is developmentally regulated, but is not required for cell differentiation. Thus, NAD(P)ase is in some way correlated with aerial hyphae formation and conidiogenesis, but its physiological role is still obscure.

The objective of this study was to investigate the nutritional conditions which influence NAD(P)ase level. We found that NAD(P)ase production was increased when the mycelium was

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Abbreviation: PMSF, phenylmethylsulphonyl fluoride.

incubated in liquid minimal medium with protein as the sole carbon source. Under these conditions, NAD(P)ase production occurred even in agitated cultures. We also provide evidence suggesting that NAD(P)ase and protease may be co-regulated.

METHODS

Culture conditions. A wild type strain of *Neurospora crassa* (FGSC 424) was grown on slants of solid minimal medium (Vogel, 1956). Conidia were collected from 7–10-d-old cultures and inoculated at a concentration of 10^7 cells ml⁻¹ into Vogel minimal medium containing 2% (w/v) sucrose. The culturing was performed as indicated in the legends to the figure and tables. Incubations were carried out at 30 °C in a rotatory shaker at 120 r.p.m. After a period of incubation to allow mycelial development, the mycelia were collected, washed and resuspended in Vogel salts supplemented with different carbon sources as indicated for each experiment, and incubated as before. For experiments on enzyme induction we used 15–20 mg (dry wt) mycelia per 25 ml medium.

Measurements of enzyme activities. NAD(P)ase activity was determined by the method of Kaplan *et al.* (1951). One enzyme unit hydrolyses 1 µmol NAD min⁻¹ at 37 °C. Specific activities, for soluble enzyme or cell extracts, are expressed as units per mg dry wt of cells.

Alkaline protease was assayed by spectrophotometric measurement of released tyrosine (McDonald & Chen, 1965) with the modifications introduced by Drucker (1972). One enzyme unit was defined as the amount of protease which releases the colour-equivalent of 1 µg tyrosine in 1 min (Hagihara *et al.*, 1958).

Chemicals. NAD, PMSF, casein, bovine serum albumin, ovalbumin and amino acids were obtained from Sigma. Gelatin and Casamino acids were obtained from Difco.

RESULTS

The NAD(P)ase activity of cultures of *N. crassa* wild type grown with shaking in minimal liquid medium supplemented with sucrose and transferred to other carbon sources was measured (Table 1). NAD(P)ase activity in the culture medium increased sharply when the carbon source was 0.5% (w/v) casein. This increase was about 65-fold when compared to conditions of complete starvation, and about 90-fold when compared to controls (sucrose or glucose). With glycerol as carbon source the enzyme increased only 4- to 6-fold as compared to the control or starved cultures. Other carbon sources, such as cellulose, starch and Casamino acids, were inefficient in stimulating NAD(P)ase production. On the other hand, traces of sucrose or glucose were sufficient to decrease by 11-fold the formation of NAD(P)ase when the fungus was grown on 0.5% (w/v) casein.

In view of these results, we tried other proteins as possible inducers of NAD(P)ase. We used the optimal concentrations used by other authors to induce alkaline protease from *N. crassa* (Cohen & Drucker, 1977). Ovalbumin, gelatin, bovine serum albumin and casein were capable

Table 1. *Effect of carbon source on NAD(P)ase production*

Conidia of *N. crassa* were inoculated into 500 ml Vogel minimal medium containing 2% (w/v) sucrose. After 18 h growth at 30 °C with shaking, the mycelia were harvested by filtration, divided and suspended in 25 ml Vogel salts with the indicated carbon source and incubated for 6 h as before. NAD(P)ase was assayed in culture filtrates. Specific activity is expressed as units per mg dry wt. The results are the means of three different cultures.

Carbon source	NAD(P)ase specific activity
None	0.011
2% Sucrose	0.008
2% Glucose	0.007
1% Cellulose	ND
1% Starch	0.008
1% Glycerol	0.045
0.5% Casein	0.721
0.5% Casein plus 0.1% glucose	0.062
0.5% Casein plus 0.1% sucrose	0.063
0.5% Casamino acids	0.007

ND, Not detected (less than 0.006 units).

Table 2. *Effect of different protein substrates on NAD(P)ase production*

Culture conditions were as indicated in the legend to Table 1, except that the mycelia were resuspended in Vogel salts plus the indicated protein. NAD(P)ase was assayed in culture filtrates. The results are expressed as means $\pm$ SD for at least three replicates.

Protein	NAD(P)ase specific activity
1% Ovalbumin	0.14 $\pm$ 0.02
0.25% Gelatin	0.55 $\pm$ 0.07
1% Bovine serum albumin	0.89 $\pm$ 0.13
0.5% Casein	1.09 $\pm$ 0.25

Table 3. *Effect of nitrogen, carbon and sulphur starvation on NAD(P)ase production*

Conidia of *N. crassa* were inoculated into Vogel minimal medium containing 2% (w/v) sucrose. After 18 h growth, the mycelia were collected by filtration, suspended in fresh Vogel salts with omission of the indicated nutrient salt and the addition of 1% (w/v) casein, and incubated for 6 h with shaking. NAD(P)ase was assayed in culture filtrates. The results are expressed as means $\pm$ SD for at least three replicates.

Starvation condition	NAD(P)ase specific activity
Carbon	0.73 $\pm$ 0.16
Nitrogen	0.02 $\pm$ 0.01
Sulphur	0.01 $\pm$ 0.005
Carbon and nitrogen	0.62 $\pm$ 0.23
Carbon and sulphur	0.83 $\pm$ 0.18
Nitrogen and sulphur	0.01 $\pm$ 0.003

of stimulating synthesis of the enzyme (Table 2). NAD(P)ase activity was practically the same in casein or bovine serum albumin, and these proteins were better inducers of NAD(P)ase than gelatin and ovalbumin. The latter protein was a very poor inducer.

The induction of NAD(P)ase by carbon starvation in the presence of protein as an alternative source is known to promote induction of protease in *N. crassa* (Drucker, 1972). Keeping this in mind, we assayed NAD(P)ase activity under other conditions that also increased the level of proteases, such as starvation for nitrogen or sulphur, or for combinations of nitrogen, sulphur and carbon (Cohen *et al.*, 1975; Cohen & Drucker, 1977). The only condition that promoted NAD(P)ase synthesis was carbon starvation (Table 3). Starvation for both carbon and nitrogen or both carbon and sulphur had no effect on NAD(P)ase level. Because of the marked change in the concentration of NAD(P)ase when *N. crassa* was grown on proteins as carbon source, we assayed the enzyme when the mycelia were incubated with PMSF, a inhibitor of alkaline protease activity (Cohen & Drucker, 1977). We incubated the mycelia for 6 h in Vogel salts plus 0.5% (w/v) casein and 1 mM-PMSF. When used *in vivo* the inhibitor reduced the mycelial NAD(P)ase level 40-fold and that of the soluble enzyme 35-fold in comparison with the control. This concentration of PMSF did not inhibit NAD(P)ase activity when assayed *in vitro* (data not shown).

The results with PMSF suggested that the production of NAD(P)ase might be somehow linked to that of proteases. This hypothesis was tested again by supplementing the carbon-deficient medium with various amino acids. Some of them are known to drastically repress protease biosynthesis (Cohen & Drucker 1977). The effects of some amino acids or a mixture of them (Casamino acids) on the production of NAD(P)ase and alkaline protease are given in Table 4. Methionine, cysteine, phenylalanine or tryptophan markedly repressed (by more than 75%) production of NAD(P)ase and alkaline protease; alanine and glycine repressed NAD(P)ase production by only about 25%. Glutamic acid and lysine had no effect on NAD(P)ase production. The effects of amino acids on alkaline protease levels were similar to those on NAD(P)ase, except for lysine, which repressed protease but not NAD(P)ase. Casamino acids strongly repressed the biosynthesis of both NAD(P)ase and alkaline protease.

Table 4. *Effects of amino acids on NAD(P)ase production*

Conidia of *N. crassa* were inoculated into Vogel minimal medium containing 2% (w/v) sucrose. After 18 h growth, the mycelia were harvested by filtration and suspended for 8 h in fresh Vogel salts with 0.5% casein plus the indicated amino acid (10 mM) or Casamino acids (0.1%). Enzymes were assayed in culture filtrates after dialysis against distilled water. The specific activity corresponding to 100% was 1.51 ± 0.21 units (mg dry wt)⁻¹ for alkaline phosphatase and 0.74 ± 0.12 units (mg dry wt)⁻¹ for NAD(P)ase. Three replicate experiments were done.

Amino acid(s)	NAD(P)ase specific activity (%)	Alkaline protease specific activity (%)
None (control)	100	100
Alanine	76	83
Glycine	79	75
Aspartic	76	75
Glutamic	106	88
Lysine	118	39
Methionine	24	17
Cysteine	7	10
Phenylalanine	9	0
Tryptophan	4	0
Casamino acids	26	9

Fig. 1. NAD(P)ase and alkaline protease production by *N. crassa* in the NAD(P)ase-inducing medium containing 1% (w/v) casein. Conidia were inoculated into 50 ml Vogel minimal medium containing 2% (w/v) sucrose. After 18 h growth, the mycelia were harvested by filtration, washed with sterile water and suspended in 100 ml fresh Vogel salts with 0.5% (w/v) casein as sole carbon source. The culture was reincubated with shaking at 30 °C. At 1 h intervals, 3 ml samples of culture were removed and the mycelium was separated from the culture medium by filtration. Culture filtrate was assayed for NAD(P)ase (○) and alkaline protease (●).

Our results with alkaline protease were quite similar to those obtained by Cohen & Drucker (1977). However, in our experiments, protease was induced without adding a low concentration of sugar to the induction medium. This was omitted because, as previously shown, 0.1% of glucose or sucrose was sufficient to cause NAD(P)ase repression. The mycelial mats which were used for the induction experiments had not been previously starved to deplete the endogenous sugar pool, as described by Drucker (1975); thus it is likely that these cells contained sufficient endogenous energy reserves to permit the initiation of protease synthesis.

To determine if NAD(P)ase appeared in the culture medium in synchrony with alkaline protease, mycelia grown in Vogel salts plus 2% (w/v) sucrose were harvested by filtration and suspended in fresh Vogel salts with 1% (w/v) casein as carbon source. At 1 h intervals samples were removed and assayed for extracellular NAD(P)ase and alkaline protease. The two enzymes began to appear in the medium in detectable amounts after 3 h incubation (Fig. 1). After this

time, the increase of NAD(P)ase paralleled that of alkaline protease, and it ceased after about 8 h of incubation.

DISCUSSION

This work shows, for the first time, that NAD(P)ase synthesis in *Neurospora crassa* is somehow related to carbon nutrition. NAD(P)ase production was induced by the presence of protein as the sole carbon source in the culture medium, and more efficient carbon sources such as sucrose and glucose were repressors of NAD(P)ase production (Table 1). It is interesting that NAD(P)ase was induced, in the presence of protein, even in shaken cultures, in which NAD(P)ase synthesis has previously been shown to be inhibited (Stine, 1968; Nelson *et al.*, 1976).

Microscopic examination of the induced shaken cultures revealed the absence of conidia or chains of proconidia (data not shown), which in surface cultures are normally responsible for NAD(P)ase production in *N. crassa* (Stine, 1968; Combpine & Turian, 1970; Urey, 1971; Nelson *et al.*, 1976).

Our results also suggested that NAD(P)ase synthesis was related to alkaline protease synthesis. Production of alkaline protease requires the presence of an extracellular protein as an alternative nutritional source during deprivation of carbon, nitrogen or sulphur (Drucker, 1972; Cohen & Drucker, 1977; Cohen *et al.*, 1975; Hanson & Marzluf, 1973, 1975; Metzenberg, 1979). The proteases secreted under the three different conditions were indistinguishable biochemically and immunologically (Cohen *et al.*, 1975; Hanson & Marzluf, 1975). Hanson & Marzluf (1975) isolated from nature a strain that had a protease with altered electrophoretic mobility and showed that the alteration was present in the proteases produced under nitrogen, sulphur or carbon starvation. These authors concluded that the structural gene for alkaline protease was under control of three different regulatory circuits.

The biological role of NAD(P)ase in *N. crassa* is still unknown. In our experiments NAD(P)ase and alkaline protease were simultaneously induced in the presence of protein (Fig. 1). The production of the two enzymes was inhibited by PMSF, and no activity could be detected either in the culture medium or in the crude mycelial extracts. The biochemical mechanism of the PMSF effect is not clear, but it was not a consequence of a general inhibition of protein synthesis or secretion because another extracellular enzyme, aryl- β -glucosidase, was both synthesized and secreted in a normal manner by conidia germinating in the presence of cellobiose as inducer, and 1 mM-PMSF (unpublished results). In any case, the parallel production of NAD(P)ase and protease appeared to indicate, at first glance, that NAD(P)ase production was dependent on that of protease. If this were correct, NAD(P)ase would be produced in any condition that promoted alkaline protease formation. However, starvation for either nitrogen or sulphur did not stimulate NAD(P)ase production (Table 3). Other results arguing against this hypothesis were obtained with *nada* (NAD(P)ase-deficient) and *cr-1* (crisp) mutants. In the former, proteolytic activity was comparable to that of the wild type strain (data not shown), and in the latter, the NAD(P)ase level was high (Jorge & Terenzi, 1980) but only traces of alkaline protease were secreted (E. S. Savtchenko and co-workers, unpublished).

The other possibility was that NAD(P)ase and alkaline protease synthesis were related at the control level. This was supported by the simultaneous occurrence of the enzymes in protein-supplemented medium (Fig. 1), and also by the fact that NAD(P)ase and protease were repressed in parallel by the same amino acids (Table 4). Thus, under conditions of carbon starvation NAD(P)ase and alkaline protease might be co-regulated by a common control gene. The existence of a control gene coordinating the expression of a family of enzymes which allows the organism to utilize alternative carbon sources was suggested by Hanson & Marzluf (1975), but has not been genetically demonstrated. It might be worthwhile to search for mutants deficient in the formation of NAD(P)ase and alkaline protease under carbon-limited conditions, as a way of investigating the mechanism controlling these two enzymes.

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