Purification and Properties of NAD-dependent Glutamate Dehydrogenase from *Phycomyces* Spores

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The NAD-dependent glutamate dehydrogenase from *Phycomyces* spores was purified more than 300-fold. Estimation of M_r by gel filtration gave a value of 98 000 whereas after SDS-PAGE one major band of M_r 54 000 was found, suggesting that the enzyme is a dimer. The enzyme was virtually dependent on the presence of AMP for activity and showed half-maximal activation at 9.5 and 43 μ M-AMP in the direction of amination and deamination respectively. ADP was nearly as effective at 20-fold higher concentrations. Other nucleotide monophosphates were ineffective and nucleoside triphosphates were slightly inhibitory. Hyperbolic kinetics were found for all substrates yielding K_m values of about 10 mM for ammonium, 1 mM for 2-oxoglutarate and 0.1 mM for NADH in the direction of amination, and 10 mM for glutamate and 0.7 mM for NAD in the direction of deamination.

INTRODUCTION

The sporangiospores of *Phycomyces blakesleeanus* are constitutively dormant and germinate only after a treatment of, e.g. 5 min at 50 °C (Sommer & Halbsguth, 1957) or 15 min in 0.1 M-ammonium acetate (Borchert, 1962). One of the main features of early germination is a temporary increase in cyclic AMP content (Van Mulders & Van Laere, 1984) correlated with a substantial conversion of reserve trehalose to glycerol (Van Schaftingen & Van Laere, 1985) which greatly affects the water status of the spores (Van Laere *et al.*, 1987; Van Laere & Hulsmans, 1987). The rapid breakdown of the glutamine reserve of the spores (Furch, 1981), is possibly as important, although quantitatively less dramatic. A key enzyme in glutamine breakdown, NAD-dependent glutamate dehydrogenase, is generally believed to be responsible for oxidative deamination of glutamate *in vivo*. According to Lejohn (1971) the enzyme from *Phycomyces* and most Mucorales is not regulated, although the enzyme from *Absidia* (also belonging to the Mucorales) is activated by AMP, Ca^{2+} , and Mn^{2+} and inhibited by a variety of effectors. The NAD-glutamate dehydrogenase from *Candida* (Hemmings, 1980) and *Saccharomyces* (Uno *et al.*, 1984), on the other hand, is regulated by phosphorylation and dephosphorylation.

In order to gain some insight into the regulation of glutamate breakdown in *Phycomyces* spores, the glutamate dehydrogenase from dormant spores was purified and some of its properties were investigated.

METHODS

Organism and preparation of spores. Sporangiospores from *Phycomyces blakesleeanus* Burgeff (ATCC 56533) were obtained by growing the fungus for 2 weeks at 18 °C on moistened, sterilized brown bread. Spores were harvested by cutting off the sporangiophores above the substrate. The sporangia were shaken in sterilized distilled water to liberate the spores. The suspension obtained was poured through a thick layer of glass-wool to remove sporangiophore debris. The spores were collected by centrifugation, washed several times with water, filtered and dried with a stream of dry air. When dry, they were stored at 4 °C for no longer than 2 months.

Enzyme purification. Dry spores (1 g) were homogenized with glass beads in a Braun microdismembrator with 4 ml 50 mM-Tris/HCl, 1 mM-PMSF (phenylmethylsulphonyl fluoride), pH 8 (extraction buffer). The 30 min 100000 g supernatant was applied to a TSK-DEAE column and eluted with a linear gradient of 0 to 1 M-NaCl in extraction buffer. The fractions from the single peak of activity were pooled, dialysed against extraction buffer and subjected to affinity chromatography on an Affigel-blue column eluted with a 0 to 1 M-NaCl gradient in extraction buffer. Pooled fractions containing enzymic activity were then dialysed against half-strength extraction buffer. Final purification was done on a Pharmacia FPLC system using a Mono-Q column and a 0 to 1 M-NaCl gradient in 20 mM-histidine/HCl, pH 6, followed by gel filtration on a Superose-12 column using 25 mM-Tris/HCl, 0.15 M-NaCl, pH 8 as solvent. A sample of the purified enzyme was subjected to SDS-PAGE using a discontinuous system on 12.5 to 25% acrylamide gradient gels (Laemmli, 1970).

Enzyme assay. Unless stated otherwise, the enzyme was assayed in the direction of reductive amination with 25 mM-Tris/HCl, 3 mM-2-oxoglutarate, 50 mM-NH₄Cl and 0.2 mM-NADH, pH 8 (final volume 1 ml). Oxidative deamination was measured in 25 mM-glycine/NaOH, pH 9 with 10 mM-glutamate and 1 mM-NAD as substrates. In most assays 0.3 mM-AMP was included since hardly any activity could be detected in purified extracts in the absence of AMP. The decrease or increase in absorbance at 340 nm was monitored spectrophotometrically at 25 °C. Blanks (2-oxoglutarate or glutamate omitted) were subtracted from the readings. These blanks became negligible after the first purification step. [Enzyme activity is given as units, where 1 unit is equivalent to 1 μ mol NAD(H) oxidized or reduced min⁻¹.] For the kinetic constants *SE* values (*n* = 3) are included. *K_m* values were determined from plots of *s/v* against *s*.

RESULTS

Purification

During all purification steps no evidence was found for a second NAD-dependent or an NADP-dependent glutamate dehydrogenase in the spores. Table 1 shows a typical result of a procedure yielding a 318-fold purification with a yield of about 11.5% by a combination of ion-exchange chromatography on TSK-DEAE, affinity chromatography on Affigel-blue and high performance (FPLC) ion-exchange and gel filtration chromatography on a Mono-Q column and a Superose-12 column. Losses of yield mainly occurred during the dialysis steps between the different columns. An estimation of the *M_r* of the enzyme on the Superose-12 column yielded a value of 98000 using bovine liver catalase (240000), rabbit muscle aldolase (158000), bovine serum albumin (68000), bovine pancreas chymotrypsinogen (25000) and horse heart cytochrome *c* (12500) as standards. After SDS-PAGE one major band with an estimated *M_r* of 54000 was found; some minor bands indicated the presence of some other contaminating proteins (Fig. 1). These data suggest that the native enzyme is a dimer.

pH profile

The purified enzyme had a pH optimum between pH 8 and 8.5 in Tris buffer for reaction in the reductive direction and at pH 9 in the oxidative direction (Fig. 2). Reductive activity was slightly higher (about 20%) in phosphate buffer but otherwise gave the same profile (not shown). In the absence of AMP, the enzymic activity of the purified enzyme was about 5% of the activity in the presence of saturating concentrations of AMP.

Kinetic constants

In the presence of 0.3 mM-AMP, the enzyme showed hyperbolic kinetics towards all three of its substrates for reductive amination, the *K_m* values being 10.8 ± 0.4 mM for ammonium,

Table 1. Purification of NAD-dependent glutamate dehydrogenase from *Phycomyces* spores

Stage	Specific activity [units (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Crude extract (10 ⁵ g supernatant)	0.2079	—	100
TSK-DEAE	0.3885	1.86	79.8
Affigel-blue	2.978	14.32	31.9
Mono-Q	14.327	68.90	12.2
Superose-12	66.169	318.3	11.5

Fig. 1

Fig. 2

Fig. 1. SDS-PAGE of purified *P. blakesleeanus* glutamate dehydrogenase on a 12.5 to 25% gradient gel (lane S) together with *M*_r markers (lane R) from top to bottom: phosphorylase b (92500), bovine serum albumin (68000), ovalbumin (45000), carbonic anhydrase (30000), soybean trypsin inhibitor (21500) and lysozyme (14400). Coomassie brilliant blue R-250 was used for staining.

Fig. 2. Effect of pH on reductive amination (■, □) or oxidative deamination (●, ○) by glutamate dehydrogenase from *P. blakesleeanus* in the presence (■, ●) or absence (□, ○) of 0.3 mM-AMP.

Fig. 3

Fig. 4

Fig. 3. Effect of 2-oxoglutarate concentration (●) (reductive amination) and glutamate concentration (■) (oxidative deamination) on the activity of glutamate dehydrogenase from *P. blakesleeanus*.

Fig. 4. Effect of AMP (■) and ADP (□) concentrations on the reductive amination, and of AMP (●) on oxidative deamination, by glutamate dehydrogenase from *P. blakesleeanus*.

1.15 ± 0.15 mM for 2-oxoglutarate (Fig. 3) and 0.105 ± 0.012 mM for NADH. NH_4Cl concentrations in excess of 25 mM inhibited the enzyme. Oxidative deamination yielded a K_m of 10.7 ± 0.6 mM for glutamate (Fig. 3) and 0.71 ± 0.09 mM for NAD.

Table 2. *Effect of different compounds on glutamate dehydrogenase activity (reductive amination) in the presence of 0.3 mM-AMP*

Except where indicated, compounds were present at 1 mM. Results are given as a percentage of the control $\pm$ SE ($n = 3$). Activities in the absence of effectors (100%) were 58.3 and 18.5 units (mg protein)⁻¹ for amination and deamination, respectively.

Addition	Relative activity	
	Amination	Deamination
None	100	100
ADP	90.8 $\pm$ 2.4	81.3 $\pm$ 0.9
ATP	88.5 $\pm$ 3.3	56.5 $\pm$ 4.0
GTP	78.1 $\pm$ 4.1	73.4 $\pm$ 3.5
UMP	92.7 $\pm$ 0.6	93.7 $\pm$ 1.3
GMP	94.2 $\pm$ 3.4	80.3 $\pm$ 2.2
Phosphoenolpyruvate	80.2 $\pm$ 5.1	78.5 $\pm$ 1.8
NADP ⁺	73.6 $\pm$ 3.7	78.1 $\pm$ 1.8
CaCl ₂	113.9 $\pm$ 6.9	102.1 $\pm$ 8.0
MgCl ₂	112.1 $\pm$ 3.7	105.3 $\pm$ 2.8
MnCl ₂	88.0 $\pm$ 3.8	54.4 $\pm$ 5.6
Sodium citrate	74.0 $\pm$ 3.8	86.6 $\pm$ 8.1
Fructose 1,6-bisphosphate (0.1 mM)	90.5 $\pm$ 1.5	83.2 $\pm$ 6.8
Fructose 2,6-bisphosphate (10 μ M)	99.3 $\pm$ 0.6	98.6 $\pm$ 9.5

Activators and inhibitors

The divalent cations Ca²⁺ and Mg²⁺ had only a marginal effect on the purified enzyme both in the presence and absence of AMP; Mn²⁺ was weakly inhibitory (Table 2). Addition of 1 mM-EDTA or up to 100 mM-KCl had no effect on the activity of the purified enzyme (not shown). AMP and ADP were very potent stimulators of the enzyme yielding half-maximal stimulation at 9.5 $\pm$ 1.0 μ M and 219 $\pm$ 10 μ M respectively when assayed in the direction of reductive amination (Fig. 4). During oxidative deamination half-maximal activation by AMP was found at 43 $\pm$ 3 μ M. Various putative stimulators or inhibitors were assayed but yielded only small effects. ATP at 1 mM inhibited the deamination reaction by some 40% (Table 2).

DISCUSSION

The NAD-dependent glutamate dehydrogenase is considerably different from other well-characterized fungal counterparts. The M_r of the enzyme (98000) is considerably smaller than the 225000 reported for some Oomycetes (Lejohn & Stevenson, 1970) and very much less than the values of at least 450000 or more for the NAD-glutamate dehydrogenases from the Ascomycetes *Candida* (Hemmings, 1980), *Saccharomyces* (Uno *et al.*, 1984) and *Neurospora* (Veronese *et al.*, 1974). The *Phycomyces* enzyme has maximal activity at pH 8 to 8.5 in the reductive and at pH 9 in the oxidative direction, like most other NAD-glutamate dehydrogenases (Smith *et al.*, 1975). In common with the enzymes from *Achlya* and *Pythium* (Lejohn & Stevenson, 1970) the enzyme from *Phycomyces* was inhibited by ammonium concentrations exceeding 50 mM, but no inhibition was apparent with 2-oxoglutarate at concentrations up to 15 mM. The K_m values for all substrates in both directions are similar to those reported for most other fungal glutamate dehydrogenases (Smith *et al.*, 1975).

Although several products had slight positive or negative effects on the activity, only AMP and ADP were significant activators and activation with AMP was obtained at physiologically significant concentrations. These properties and inhibition of the deamination reaction by ATP are consistent with a degradative rather than a biosynthetic role for the enzyme.

Our data on activation and inhibition of the enzyme differ from those of Lejohn (1971) who studied the enzyme isolated from mycelium of *Phycomyces*. Lejohn (1971) found no activators or inhibitors for the *Phycomyces* mycelial enzyme (type I). Moreover, the enzyme from *Phycomyces* spores does not fit into any of the phylogenetically related types as proposed by Lejohn (1971).

The enzyme of the Mucoralean *Absidia* (type II) is stimulated by AMP but is also inhibited by citrate, ATP, FDP, EDTA and GTP, which is not the case for *Phycomyces*. Also, stimulation by divalent cations is only marginal in *Phycomyces*. It cannot be excluded that the small but consistent activity in the absence of AMP is due to contamination by trace amounts of the type I glutamate dehydrogenase described by Lejohn (1971).

The NAD-dependent glutamate dehydrogenase from *Candida* and *Saccharomyces* is inhibited by phosphorylation (Hemmings, 1980; Uno *et al.*, 1984). No change in activity of the enzyme from *Phycomyces* occurred during incubation of crude extracts with cAMP and ATP, even in the presence of beef heart protein kinase. Under the same conditions the activity of trehalase is greatly enhanced (Van Laere & Hendrix, 1984). Phosphorylation-dependent regulation is also unlikely because no change in enzymic activity occurred during early germination (not shown), when endogenous cAMP concentration (and the activity of trehalase in the spores) increases dramatically (Van Mulders & Van Laere, 1984).

Peters & Sypherd (1979) found both an NADP- and an NAD-dependent enzyme in the related fungus *Mucor racemosus*. No NADP-dependent enzyme was found in *Phycomyces*, but one may be present in other developmental stages of the fungus although Lejohn (1971) claimed that no NADP-dependent glutamate dehydrogenases occur in the lower fungi, including the Mucorales.

The results of Lejohn (1971) may differ from our data because different developmental stages were investigated. The spore enzyme could be modified post-transcriptionally during germination or, more probably, during germination a new (AMP-insensitive) enzyme might be synthesized. Indeed, preliminary results show that stimulation by AMP in crude extracts gradually diminishes during germination.

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