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FACTORS AFFECTING THE AMOUNT AND THE ACTIVITY OF THE GLUTAMATE DEHYDROGENASES OF *COPRINUS CINEREUS*

A. AL-GHARAWI and D. MOORE

Department of Botany, The University, Manchester M13 9PL (U.K.)

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Summary

Kinetic analyses done with cell-free extracts of this basidiomycete fungus showed that the NADP-linked glutamate dehydrogenase exhibited positively co-operative interactions with the substrates 2-oxoglutarate and NADPH, negatively co-operative kinetics with $NADP^+$ and was extremely sensitive to inhibition of deamination activity by ammonium and/or ammonia. The NAD-linked enzyme showed positive co-operativity with NADH, Michaelis-Menten kinetics with all other substrates and was subject only to mild inhibitions by the reaction products. Considered together with the values of the Michaelis constants, these results indicate that the former enzyme is primarily concerned with the amination of 2-oxoglutarate when the concentration of this substrate exceeds about 4 mM, while the NAD-linked enzyme is able to aminate or deaminate as metabolic conditions require. Synthesis of both enzymes was repressed by addition of carbamyl phosphate or *N*-acetyl-glutamate to mycelial cultures growing in media containing glucose and ammonium as carbon and nitrogen sources. Growth in media containing urea results in repression of the NADP-linked glutamate dehydrogenase and derepression of the NAD-linked enzyme. Such results indicate a connexion between the glutamate dehydrogenases and the urea cycle. It is suggested that under normal conditions of growth on complex media nitrogen is assimilated in the form of amino acids and that the glutamate dehydrogenases act in support of transaminases to allow this process to continue, and in support of the urea cycle to allow the disposal of excess nitrogen.

Introduction

There are two glutamate dehydrogenases in the basidiomycete *Coprinus cinereus*; one linked to NAD (EC 1.4.1.2) and the other to NADP (EC 1.4.1.4) [1,2]. During mycelial growth and vegetative maturation the NAD-linked

enzyme shows the highest activities and during sporophore growth the specific activity increases about three-fold in both cap and stipe. In contrast, NADP-glutamate dehydrogenase shows very little activity in mycelium and sporophore stipe, but gradually increases in activity in the cap until the specific activity is 20—50 times increased over the basal mycelial level [2]. Cap and stipe tissues are intimately connected and are composed of isogenic cells, so this continued repression of NADP-glutamate dehydrogenase in the stipe alongside its derepression in the cap is an example of tissue-specific developmental gene regulation. Such a pattern of regulation in the sporophore is not shared by many agarics, and is not even common among *Coprinus* spp. [3].

Here we examine the response of the two enzymes in vitro to metabolites which may accompany them in vivo, in the hope that comparative analysis of the effects of systematic changes to the assay mixtures and growth media will allow identification of differences between the enzymes which have physiological significance.

Materials and Methods

The organism, media, culture conditions, enzyme extraction and assay methods were all described by Al-Gharawi and Moore [4]. Most of the kinetic analyses were carried out with cell-free extracts which had been desalted with a small column of Sephadex G-25. The main conclusions were subsequently confirmed on material which had been partially purified (3 to 5-fold) by gel filtration with Bio-Gel A1.5m (Bio-Rad Laboratories Ltd., Bromley, Kent) of proteins collected after fractional precipitation with ammonium sulphate. Throughout the paper enzyme specific activities are expressed as nmol NAD(P)H oxidized per min per mg protein for the reductive amination of 2-oxoglutarate or as nmol $NAD(P)^+$ reduced per min per mg protein for the oxidative deamination of L-glutamate.

Results and Discussion

The kinetic constants (Table I) can be taken to suggest that NADP-glutamate dehydrogenase is most likely to be involved in the amination of 2-oxoglutarate, whereas the NAD-linked enzyme shows no clearly preferred reaction direction. Support for these conclusions comes from experiments in which the products of the reactions were separately included in the assay mixtures. Generally speaking, all the reaction products mildly inhibited the activity of NAD-glutamate dehydrogenase, but there were two circumstances in which the response of the two enzymes greatly differed (Fig. 1). Firstly, the deamination activity of NADP-glutamate dehydrogenase proved to be much more sensitive to inhibition by ammonium chloride; and secondly, although in the amination reaction the initial presence of glutamate inhibited the activity of NAD-glutamate dehydrogenase, the aminating activity of the NADP-linked enzyme was increased by the initial presence of the product L-glutamate. The latter effect suggests some form of allosteric interaction and the plot of reaction velocity against 2-oxoglutarate concentration proved to be sigmoidal (Fig. 2a). The Hill plot (Fig. 2b) was biphasic with the point of inflection corresponding

TABLE I

KINETIC CONSTANTS FOR THE TWO GLUTAMATE DEHYDROGENASE ENZYMES OF *COPRINUS*

The enzymes were extracted from sporophore tissues. K_m values (mM) are shown as the mean and standard error of 2—4 separate estimations made from the results of 2—6 different experiments. Entries indicated with an asterisk are the substrate concentrations which gave half-maximum velocity in those reactions which did not follow Michaelis-Menten kinetics.

Substrate	NAD-linked glutamate dehydrogenase	NADP-linked glutamate dehydrogenase
Amination reaction		
2-Oxoglutarate	2.02 ± 0.1	5.50*
Ammonium	18.80 ± 1.3	2.00 ± 0.04
NAD(P)H	0.073*	0.05*
Deamination reaction		
L-Glutamate	3.97 ± 0.2	36.90 ± 1.7
$NAD(P)^+$	0.23 ± 0.02	0.06*

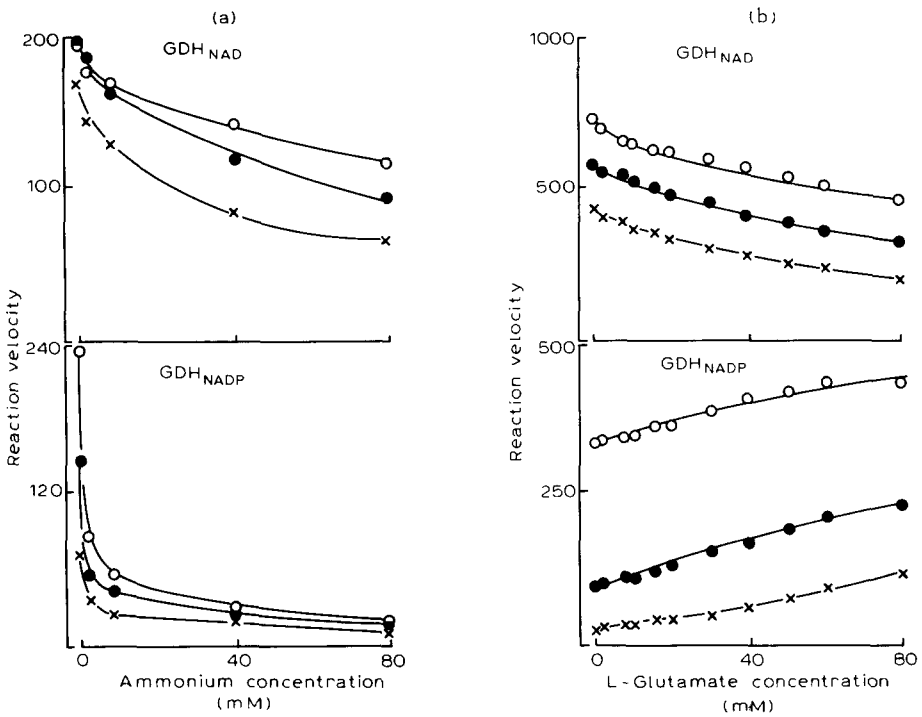

Fig. 1. Comparison of the effects of some reaction products on the activities of the glutamate dehydrogenases of *Coprinus*. (a) Effect of ammonium on the deamination reaction at three concentrations of the substrate L-glutamate, 120 mM (○), 40 mM (●), and 10 mM (X). All assay mixtures were adjusted to the optimum pH (9.6 for the NAD-linked glutamate dehydrogenase and 9.0 for the NADP-linked enzyme) before initiation of the reactions by addition of enzyme. (b) Effect of L-glutamate on the amination reaction at three concentrations of the substrate 2-oxoglutarate, 9.6 mM (○), 4.8 mM (●), and 2.4 mM (X). Reaction velocity in each case is the specific activity expressed in nmol coenzyme oxidized or reduced/min per mg protein.

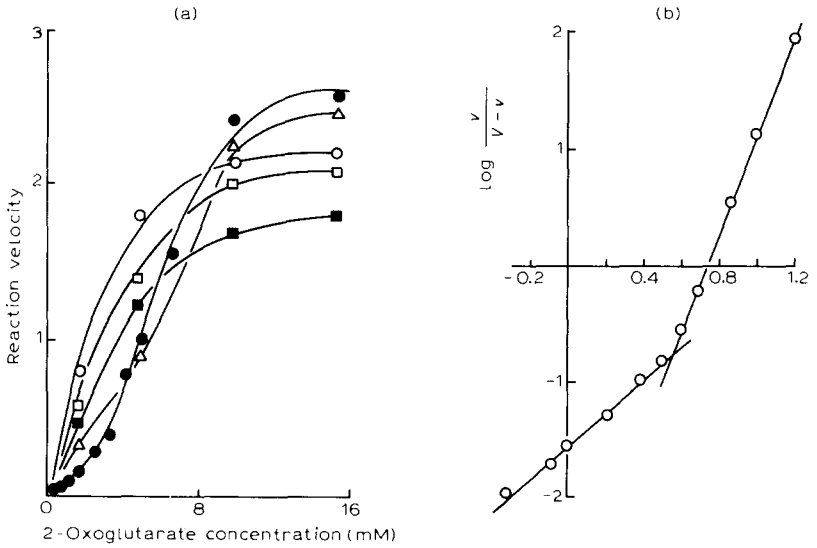

Fig. 2. Kinetics of the amination reaction promoted by the NADP-linked glutamate dehydrogenase. (a) Plots of reaction velocity (in arbitrary units) against substrate concentration for the normal assay mixture (●). Also drawn are plots for assay mixtures to which were added (to 15 mM) the activators glutarate (○), D-glutamate (□), L-2-hydroxyglutarate (■), and *n*-valerate (△). (b) Hill plot for the normal assay mixture.

to a substrate concentration of 3.6 mM. Hill numbers were calculated from this plot and also by means of the procedure of Fajszi and Endrenyi [5]. At low substrate concentrations the Hill number was 1.1—1.4, while at concentrations in excess of about 4 mM it was 3.6—4.1; suggesting that as the substrate concentration increases above the threshold level of about 4 mM at least four co-operating binding sites come into effect.

The analogue D-glutamate has been identified as an allosteric activator of NADP-linked glutamate dehydrogenase [4]. Investigation of a range of other structural analogues of 2-oxoglutarate and L-glutamate for ability to activate revealed that only glutarate, L-2-hydroxyglutarate, D-glutamate and *n*-valerate were able to effect considerable activation when present at 15 mM concentration (Fig. 2a). We also surveyed a range of amino acids (including alanine, aspartate, 4-aminobutyrate, 2-aminobutyrate and glutamine), glycolytic intermediates (including fructose 1,6-diphosphate, glucose 1-phosphate and pyruvate), Krebs and ornithine cycle intermediates (including carbamyl phosphate, isocitrate, oxaloacetate, *N*-acetyl-L-glutamate, succinate and urea) and purine nucleotides (including AMP, ATP and IMP) for the ability to affect the progress of the amination reaction conducted by this enzyme. Apart from an occasional slight inhibition (up to 10%) none had any significant effect on enzyme activity. A notable exception to this generalization was glyoxylate, which caused 60—90% inhibition of activity depending on substrate concentration.

Comparison of the structures of the analogues tested with their effects on amination activity leads to the conclusions that the most effective allosteric

activators are 5-carbon chains (2-aminobutyrate and 4-aminobutyrate were not effective) which have two carboxylic acid groups (*n*-valerate was much less effective than glutarate) and little or no substitution, particularly in the L-orientation, at the 2-carbon position (glutarate > D-glutamate > L-2-hydroxyglutarate > L-glutamate). In the normal metabolic environment of glutamate dehydrogenase we would anticipate that 2-oxoglutarate would best satisfy these requirements. Thus we interpret the allosteric interaction as an example of positive substrate co-operativity being used as a switch mechanism which allows substrate to accumulate to a threshold level of about 4 mM before maximum enzyme activity is released.

The NAD-linked glutamate dehydrogenase showed 'normal' Michaelis-Menten kinetics with the substrates 2-oxoglutarate, ammonium and L-glutamate. The NADP-linked enzyme exhibited Michaelis-Menten kinetics with glutamate and ammonium. In both cases relationships with the coenzymes were complex (Fig. 3). Both enzymes gave a sigmoidal response in plots of reaction velocity against concentration of reduced coenzyme. The Hill number for

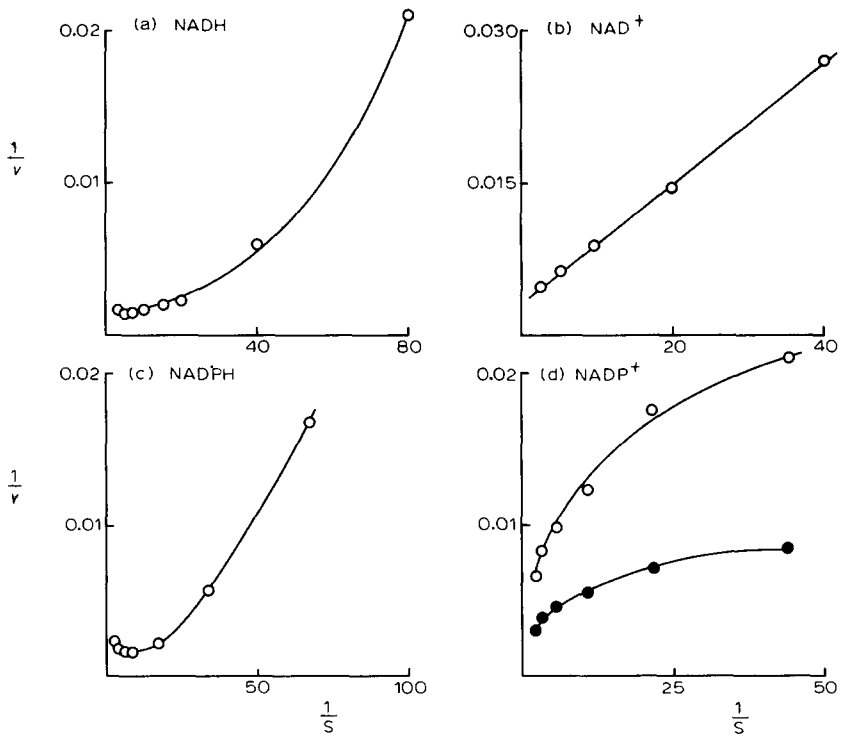

Fig. 3. Double reciprocal plots showing the relationships between enzyme activity and coenzyme concentration. (a) NAD-glutamate dehydrogenase in the amination reaction with 9.6 mM 2-oxoglutarate and 40 mM ammonium chloride. (b) NAD-glutamate dehydrogenase in the deamination reaction with 10 mM L-glutamate. (c) NADP-glutamate dehydrogenase in the amination reaction with 24 mM 2-oxoglutarate and 40 mM ammonium chloride. (d) NADP-glutamate dehydrogenase in the deamination reaction with two concentrations of L-glutamate, 20 mM (○), and 120 mM (●). In each case the abscissa is the reciprocal of the mM concentration of coenzyme.

NAD-glutamate dehydrogenase was 1.98, suggesting two binding sites for NADH. The NADP-linked glutamate dehydrogenase was more complex, the Hill plot being biphasic with the transition point at a concentration of 44 nM NADPH. Below this concentration the Hill number was 1.5; at higher concentrations it was 3.7. The relation between NAD-glutamate dehydrogenase and NAD^+ did not deviate from Michaelis-Menten kinetics, however, the NADP-linked enzyme was again exceptional. Lineweaver-Burk plots of reaction velocity against $NADP^+$ concentration were concave downwards (Fig. 3d) and the Hill number was 0.68, features which indicate negative co-operativity with respect to the substrate $NADP^+$ [6,7].

The activities and synthesis of the glutamate dehydrogenase enzymes of *Coprinus* are subject to complex control mechanisms. As in other filamentous fungi (e.g. *Aspergillus* [8,9] and *Neurospora* [10]) the two enzymes often show reciprocal responses to manipulation of the medium constituents (Fig. 4) and, generally speaking, the NAD-glutamate dehydrogeanse seems to be catabolite repressed while the NADP-linked enzyme is catabolite derepressed. Of numerous nitrogen sources examined for their effect on synthesis of these two enzymes only urea has been found to exert a dramatic influence, being

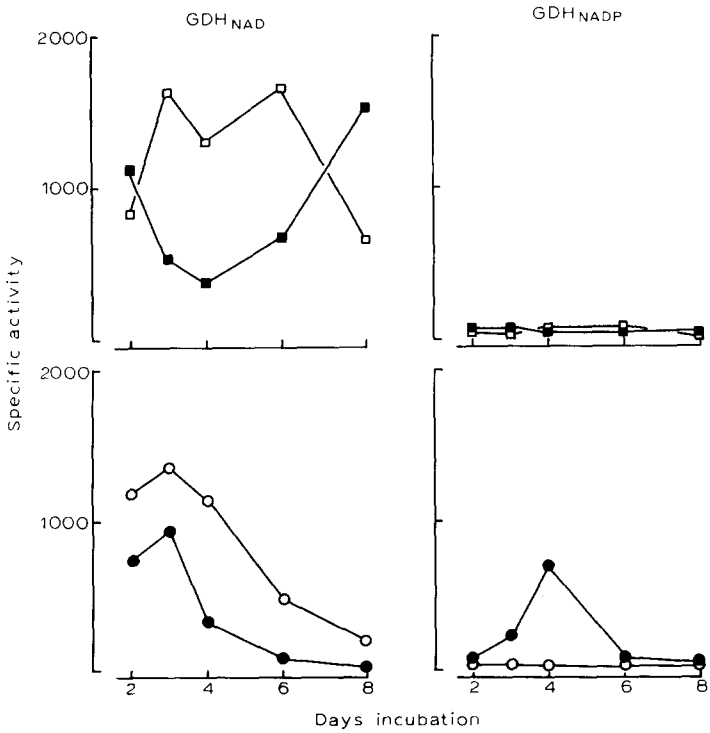

Fig. 4. Time courses showing the changes in specific activity of the two glutamate dehydrogenases in vegetative mycelia growing in four different media. The monokaryotic strain BC9/6,6 was grown at 37°C in an orbital shaker (120 strokes/min) in liquid media containing: 50 mM glucose + 50 mM ammonium tartrate (●), 50 mM sodium acetate + 50 mM ammonium tartrate (○), 50 mM glucose + 50 mM urea (■), or 50 mM sodium acetate + 50 mM urea (□).

able to repress NADP-glutamate dehydrogenase and derepress the NAD-linked enzyme irrespective of the nature of the carbon source [2]. In the present work we have added a variety of compounds (to 15 mM final concentration) to liquid cultures of monokaryotic mycelia in order to pursue the possibility of a connexion between glutamate dehydrogenase and the urea cycle and also to investigate the effects of activators of NADP-glutamate dehydrogenase activity on enzyme synthesis. Among the compounds which had no effect on either enzyme were L-alanine, 4-aminobutyrate, L-glutamate and 2-oxoglutarate. Glutarate caused 60—95% repression of the NAD-glutamate dehydrogenase of mycelia grown in medium containing 50 mM glucose + 50 mM ammonium tartrate, but had no effect on the NADP-linked enzyme. Carbamyl phosphate, *n*-valerate, and *N*-acetyl-L-glutamate all completely repressed synthesis of both glutamate dehydrogenase enzymes by mycelia grown in the glucose-ammonium medium. Little change was induced in the enzymes of mycelia grown in media containing 50 mM sodium acetate + 50 mM ammonium tartrate.

The repression caused by glutarate and *n*-valerate can probably best be understood by assuming that both compounds act as analogues of normal metabolites. The other two effectors seem to reinforce suggestions of a connexion between the glutamate dehydrogenases and the ornithine/urea cycle, although the effect of carbamyl phosphate is difficult to interpret in view of the instability of this compound [11—13]. At 37° C its half-life is less than one hour, so it is not clear how its influence can persist in an experiment which lasted 2—3 days. One or more of its decomposition products must be the active principle.

Conclusion

It is usually assumed that glutamate dehydrogenase is concerned with the assimilation of nitrogen in the form of ammonia, as must be the case in defined artificial media where ammonia is the sole nitrogen source. When *Coprinus* is grown on complex media large quantities of ammonia are excreted into the medium [2,14]. This suggests that on more 'natural' media growth involves assimilation of carbon and nitrogen in the form of amino compounds and that excess nitrogen is removed through the urea cycle. There may be a parallel between such mycelial growth and events occurring in the sporophore if the products of the autolytic digestion of cap tissues are re-used. The autolysate must be rich in amino sugars and amino acids; if the carbon skeletons of these are used in energy yielding metabolism then the disposal of excess nitrogen may be just as necessary in the sporophore as it seems to be in the mycelium. There is certainly evidence that the urea cycle operates in *Agaricus* and *Lycoperdon* [15—19].

It seems reasonable to speculate that in *Coprinus* the glutamate dehydrogenase enzymes act in support of transamination reactions which introduce amino nitrogen into metabolism, and also serve the urea cycle to allow the disposal of excess nitrogen. Whether the two glutamate dehydrogenases contribute equally to these functions cannot yet be decided. The kinetic analyses have revealed some very clear differences between the enzymes, so it is unlikely that they serve identical functions.

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