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Cytochemical localisation of NADP-linked glutamate dehydrogenase newly induced in nitrogen-starved mycelia of *Coprinus cinereus*

(*Coprinus*; glutamate dehydrogenase; regulation; cellular localisation)

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1. SUMMARY

Electron microscopic cytochemistry shows that following transfer of mycelium from nitrogen-rich to nitrogen-free medium, the NADP-linked glutamate dehydrogenase activity, which is known to be derepressed by such treatment, is localised in the vicinity of the cell membrane and the nucleus. Detection of similar cytochemical reaction products in material subjected to lengthened incubation in the original medium, rather than to transfer (which showed no significant change in the level of spectrophotometrically-detectable enzyme activity) implies that regulation of the enzyme may include an activation process which is mimicked by the preparation methods employed for electron microscopy.

2. INTRODUCTION

Maturation of the fruit body cap of *Coprinus cinereus* is accompanied by a specific pattern of changes in enzyme activities and metabolite levels [1,2]. At least some of these changes can be induced in a particular monokaryotic mycelium, code number BC9/6,6, when it is transferred to a nitrogen-free medium. This sort of experiment has been employed to determine the nature of the regulatory signals involved [3]. Because of its ease of assay, particular attention has been given to the NADP-linked glutamate dehydrogenase (EC 1.4.1.4). This enzyme shows a steadily increasing level of activity in the cap as the latter develops, though it is absent from the fruit body stipe [4]. Furthermore, the enzyme is derepressed in mycelium of strain BC9/6,6 on transfer to nitrogen-free medium [5]. In both fruit body cap and nitrogen-starved BC9/6,6 mycelium, increase in NADP-linked glutamate dehydrogenase shows a strong positive correlation with increase in activity of glutamine synthetase [6], and it has been suggested that these two enzymes contribute to an ammonium-scavenging system [2].

We have previously used light microscope cytochemistry to locate NADP-linked glutamate dehydrogenase in sections of the fruit body cap [7]. In this paper we report the results of attempts to localise the enzyme in mycelial samples examined by electron microscopy.

3. MATERIALS AND METHODS

The monokaryon strain BC9/6,6 (ATCC42725) of *Coprinus cinereus* (Schaeff.: Fr.) S.F. Gray was used throughout. For the medium-transfer experiment, the mycelium was first grown in a medium containing 1% Bacto Casamino Acids (hydrolysed casein), 10 mM glucose, 30 mM NH_4Cl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 2 mM Na_2SO_4, 0.5 mM $MgSO_4$ and 3 μM thiamine hydrochloride. Incubation for 4–5 days on an orbital shaker operating at 120 strokes/min at 37°C was sufficient to produce a useful yield of mycelium.

For the transfer, mycelium was harvested by centrifugation, washed, recentrifuged and then resuspended for further incubation in the nitrogen-starvation medium. This consisted of a basal solution containing 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 2 mM Na_2SO_4, 0.5 mM $MgSO_4$ and 3 μM thiamine hydrochloride, to which was added 100 mM sodium pyruvate. After transfer to the nitrogen-starvation medium, the culture was re-incubated on the orbital shaker for 18 h at 37°C. Mycelia incubated in the original medium without transfer for a further 18 h were used as a control.

All mycelia were finally harvested by filtration through Whatman GF/A filters and washed in deionised water. Samples for enzyme cytochemistry were taken and fixed immediately. The residue was weighed and stored at −40°C for spectrophotometric analysis of enzyme activity.

After tests of a number of techniques, a copper ferrocyanide method was adopted as a routine method to visualise dehydrogenase activity. This was a modification of the method devised by Ogawa et al. [8] and Kerpel-Fronius and Hajos [9] and used by Hanker et al. [10] and by Jones [11] for the demonstration of dehydrogenases. In this method, ferricyanide is used as an electron acceptor, and is converted on reduction into insoluble electron-opaque cupric ferrocyanide.

Tissue was fixed in buffered formaldehyde solution 'D' [12] for 20–25 min at 4°C, washed in 0.1 M phosphate buffer, pH 7.2 with sucrose, and incubated in copper ferricyanide medium for 90 min at 37°C. After several washes in buffer with sucrose, the materials were post-fixed in buffered 2% osmium tetroxide for 1 h at 40°C, dehydrated in ethanol and embedded in epoxy resin [13]. The standard copper ferricyanide mixture contained 6.0 ml sodium potassium tartrate (0.5 M), 1.6 ml phosphate buffer at pH 7.2 (0.1 M) and 0.7 ml $CuSO_4$ (0.3 M) added dropwise, and the pH of the mixture was adjusted to 6.6–6.8; 0.3 ml of potassium ferricyanide (0.05 M) was then added dropwise. Enzyme substrate and coenzyme were included in the final concentrations as follows: L-glutamic acid (monosodium salt, 0.125 M) and 10 mg NADP. Sucrose (0.02 M) was added to all incubation media. All tests included controls lacking the substrate and an unincubated blank.

Identification of the electron-dense end-product of the cytochemical tests was confirmed by X-ray microanalysis, copper and iron being used as markers of the end-product of cupric ferrocyanide. Thick sections (200–250 μm) were cut from the same blocks used for cytochemical tests and mounted on 400 mesh formvar-carbon-coated aluminium grids which provided minimum interference with the copper and iron emission peaks. The sections were coated with a second, stabilising, layer of carbon and examined in an AEI CORA analytical transmission electron microscope using a beam current within the range of $1–2 \times 10^7$ A on 60 kV accelerating voltage, a 250–400 nm spot size and a count time of 100 s.

For spectrophotometric assays of enzyme activity in tissue homogenates the amination assays described by Al-Gharawi and Moore [14] were used.

4. RESULTS AND DISCUSSION

Specific activities of NADP-linked glutamate dehydrogenase as assayed in cell-free homogenates are shown in Table 1. These data show that the material used for electron microscopy followed the pattern observed many times before, in which mycelium transferred to the pyruvate nitrogen-free medium exhibits derepression of NADP-glutamate dehydrogenase. Material left in the original medium for a further 18 h showed no detectable change in this enzyme level.

Cytochemical examination of mycelia grown for 4 days in the original (amino acid-rich) medium

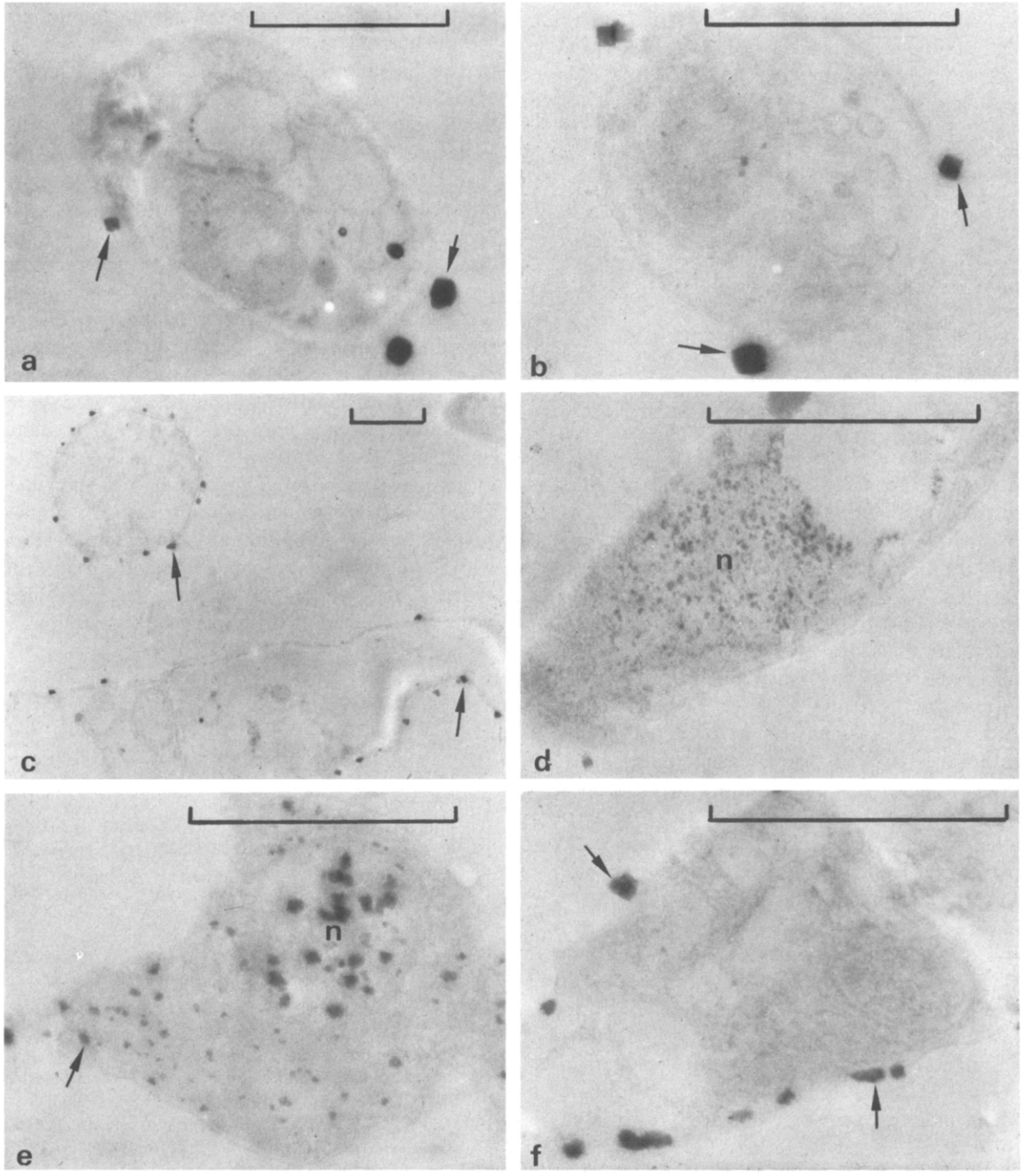

Fig. 1. Copper ferrocyanide tests for NADP-linked glutamate dehydrogenase in mycelium of *Coprinus cinereus* strain BC9/6,6 subjected to different conditions of cultivation. (a) and (b), Original (amino acid-rich) medium, the few reaction deposits (arrows) are largely associated with cell walls; (c) and (d), nitrogen-free transfer medium, extensive deposits are associated with cell walls (arrows) and nucleus (n); (e) and (f), original medium for a further 18 h incubation, deposits (arrows) are more abundant than in the original medium and more diffuse than in samples taken from the transfer medium. The scale bars corresponds to 1 μm.

Table 1

NADP-glutamate dehydrogenase activities in samples used for electron microscopic analysis compared with previous data

Enzyme specific activities are shown as nmol substrate used/min/mg protein. Previous data taken from Moore [3]; values in parentheses show number of replicates, other entries are the means of three determinations.

	Activities in samples used here	Previous data
Control, prior to transfer	45	43
Control, untransferred but incubated for a further 18 h	39	31 ± 28 (46)
Transferred to pyruvate medium	450	644 ± 329 (47)

showed only few reaction deposits, some of which were associated with the outside of cell walls (Fig. 1a, b), which is in accord with the low enzyme activity separately recorded for these samples (Table 1).

In mycelia incubated in the nitrogen-free pyruvate transfer medium, however, extensive reaction deposits were observed in association with the cell walls and the nucleus (Fig. 1c, d). This again is in accord with the enzyme activity level determined from homogenates of the same material. Control reactions demonstrated that these deposits were specific to the enzyme substrates used in the ferricyanide cytochemical reaction mixture and X-ray microanalysis confirmed the presence of both iron and copper in the deposits.

Mycelial samples incubated for a further 18 h in the original medium also showed increases in the level of deposits (Fig. 1e, f) and this does not accord with the data of Table 1. In general these deposits were rather more diffuse than those encountered in the transferred (nitrogen-starved) samples, but their appearance was substrate-dependent and they provided similar X-ray analytical profiles.

These observations suggest that when the enzyme NADP-linked glutamate dehydrogenase is derepressed on nitrogen starvation it comes to occupy a position close to the cell wall, having possibly originated in the nucleoplasm. A plasmalemma or periplasmic location would fit with the supposed function of the enzyme in an ammonium scavenging system.

The appearance of a similar reaction product in material subjected, not to transfer but to a lengthened incubation in original medium may indicate some complexity in the regulation of the enzyme. It has been shown that enzyme activity induced by the transfer to nitrogen-free medium is increased by de novo synthesis [5]. However, the time course of the increase in enzyme activity shows no lag period [3], so it remains possible that the overall elevation of enzyme activity is due to an initial activation of some preformed enzyme protein which is subsequently amplified by derepressed enzyme synthesis. Incubation for 5 days in the initial medium may precipitate events more characteristic of nitrogen starvation and lead to formation of an inactive precursor of the enzyme. This would not be detectable in cell-free homogenates, but the processing involved in preparation for the electron microscope, especially treatment with fixatives, could well activate this preformed molecule and lead to its cytochemical detection.

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