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CYTOCHEMICAL LOCALIZATION OF GLUTAMATE DEHYDROGENASES DURING CARPOPHORE DEVELOPMENT IN *COPRINUS CINEREUS*

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SUMMARY

The NAD- and NADP-linked glutamate dehydrogenases were detected in frozen sections of carpophore tissues by means of tetrazolium salt. Material at all stages of development was examined. From the earliest stages NAD-glutamate dehydrogenase was uniformly distributed in the basidial cells of the hymenium, and as the carpophore developed an increasing proportion of cells of the subhymenium came to express enzyme activity. The NADP-glutamate dehydrogenase (NADP-GDH) was initially limited to basidial cells in isolated patches of the hymenium. As development proceeded the proportion of basidia exhibiting NADP-GDH activity increased, as did the proportion of subhymenial cells showing this enzyme activity. No NADP-GDH activity was detectable in sections of stipe tissue.

INTRODUCTION

In catalyzing the reversible reductive amination of 2-oxoglutarate, glutamate dehydrogenase enzymes play a central role in metabolism. They provide a major route for ammonia assimilation and also act at crucial steps in the intermediary metabolism of both carbon and nitrogen. In common with many other fungi (Casselton, 1969), *Coprinus cinereus* (= *C. lagopus sensu* Lewis) possesses two species of glutamate dehydrogenase (GDH), one linked to the co-enzyme NAD (NAD-GDH), and the other linked to NADP (NADP-GDH) (Fawole and Casselton, 1972; Stewart and Moore, 1974). As far as can be judged from its regulatory (Stewart and Moore, 1974) and kinetic properties (Al-Gharawi and Moore, 1977), NAD-GDH is able to aminate or deaminate as metabolic conditions require and is found at moderate to high levels of activity in all types of mycelia whatever the nature of the medium used to support growth (Fawole and Casselton, 1972; Stewart and Moore, 1974). In contrast, the NADP-GDH is judged to be specialized for amination (Al-Gharawi and Moore, 1977), is encountered only in the earliest stages of mycelial growth on complex media, and otherwise appears only in response to growth on particular synthetic media (Fawole and Casselton, 1972; Stewart and Moore, 1974). The role of NADP-GDH is thus obscure. A continuing need for the amination of 2-oxoglutarate is implied by the observation that in *C. cinereus* 2-oxoglutarate dehydrogenase is inoperative and the Krebs cycle bypasses this step by means of the glutamate decarboxylation loop (Moore and Ewaze, 1976). However, so rare is its occurrence in the enzyme spectrum of the vegetative mycelium that it is unlikely that NADP-GDH makes much contribution to so fundamental a pathway. Instead, it is believed that NADP-GDH

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serves a specific function related to morphogenesis. This opinion is based on the observation that during development of the mushroom carpophore NADP-GDH activity increased only in the cap, remaining at an extremely low level of activity in the stipe. This was in contrast to NAD-GDH, which increased in activity in both parts of the carpophore (Stewart and Moore, 1974). Dissection of carpophore caps prior to preparation of cell-free homogenates suggested that the NADP-GDH activity was associated with the gill tissue rather than the spores (Stewart and Moore, 1974). The investigation reported here was undertaken to provide more detailed information on the localization of GDH activity by use of enzyme-specific cytochemical methods appropriate to the light microscope, in the hope that this approach might show whether or not the GDH enzyme activities were homogeneously distributed in the tissues usually employed for spectrophotometric analyses.

Materials and Methods

Organism

The BC9/6,6 × H1 dikaryon of *Coprinus cinereus* (Schaeff. ex Fr.) S. F. Gray was used throughout for the production of carpophores. This is the same mycelium used in earlier studies (Stewart and Moore, 1974) under the name *C. lagopus*. Carpophores were produced by growing the dikaryotic mycelium on sterilized horse dung using incubation and illumination regimes described previously (Moore and Ewaze, 1976).

Tissue preparation

Cubes of approximately 5 mm were excised from the caps and stipes of carpophores at five different stages of development (Stewart and Moore, 1974). To ensure comparability the tissue segments were always removed from the part of the cap close to its free edge and from the immediately adjacent portion of the stipe. The excised tissue was embedded in water agar (1·5%, w/v), rapidly frozen in liquid nitrogen and sectioned at −15 °C using a Slee Cryostat. Sections (usually 20 μm thick) were mounted on pre-cleaned slides directly from the knife and, if necessary, were stored within the microtome.

Enzyme cytochemistry

Glutamate dehydrogenase activity was demonstrated by use of tetrazolium salt in a method based on that of Reiss (1967). The optimum composition of the reaction mixture was determined by experiment and shown, particularly in regard to pH and substrate concentration, to be similar to the optimal reaction mixtures for cell-free extracts (Al-Gharawi and Moore, 1974). The reaction mixtures contained L-glutamic acid, monosodium salt, 42 mg; NAD^+, 0·5 mg *or* $NADP^+$, sodium salt, 1·0 mg; Nitro Blue Tetrazolium, 0·25 mg; 0·2 M glycine/NaOH buffer, 0·25 ml; distilled water, 0·75 ml. The glycine/NaOH buffer was adjusted to pH 9 for the NADP-linked enzyme and to pH 9·6 for the NAD-GDH. Suitable sections were immersed in reaction mixture and incubated at 37 °C for 1 h. They were then washed with distilled water and mounted in 30% (v/v) glycerol for microscopic examination. All of the chemicals used in the reaction mixture were obtained from the Sigma Chemical Company.

Results and Discussion

Segments of stipe tissue revealed exactly the pattern of enzyme differentiation to be expected from previous enzymological observations (Stewart and Moore, 1974): a positive and, as development proceeded, an increasing reaction was obtained with incubation mixtures containing NAD^+, although no lasting activity of NADP-GDH could be detected (Plate 1, Nos 1 and 2). The increase in NAD-GDH activity in the stipe was achieved by uniform increase in activity in the component cells. At all stages, stain deposition was homogeneously distributed in all stipe cells (Plate 1, No. 1). This was not true for the cap.

Spectrophotometric data give rise to the expectation that NAD-GDH activities would be moderate in the youngest primordia and would increase as development proceeded; this was observed cytochemically. In sections cut from primordia of Stage 1 (2 to 6 mm tall, prekaryogamy) the NAD-reaction mixture gave rise to stain deposition in a continuous line which corresponded with the hymenium layers of the gills (Plate 1, No. 3), and this localization was maintained through Stage 2 (6 to 9 mm tall, karyogamy and meiosis) and Stage 3 (over 10 mm tall, postmeiotic, volva free) (Plate 1, No. 4). However, it was clear that considerable quantities of stain were deposited in the subhymenial gill tissues, although particularly thin sections revealed that the basidia were the most heavily stained (Plate 1, No. 5). This tendency for stain deposition to become more homogeneously distributed through the gill tissues was continued into the later stages of development leading up to spore discharge. Although the formation of spore-wall pigmentation prevents assessment of enzyme activity in the later stages, it is clear that young unpigmented spores give a positive response to the NAD-reaction mixture (Plate 1, No. 5). These cytochemical observations correspond quite well to the spectrophotometric data obtained earlier (Stewart and Moore, 1974). Although the intensity of staining cannot be reliably related to the specific activity, it is clearly reasonable to suppose that the tendency towards a more homogeneous distribution of enzyme-specific staining as the carpophore develops is evidence that an increasing proportion of the cells come to possess NAD-GDH activity. Even if the pre-existing activities in individual cells do not increase, this derepression on an ever increasing scale can account readily for the steady increase in NAD-GDH specific activity observed in cell-free homogenates (Stewart and Moore, 1974).

Such correspondence between cytochemical results and data obtained by spectrophotometric analysis of homogenates did not obtain when attention was turned to NADP-GDH. Although only very low specific activities were recorded for primordia (Stewart and Moore, 1974) sections of Stage 1 and Stage 2 primordia responded positively to the NADP-reaction mixture. However, stain deposition was highly localized. The outer surface layer of the cap stained fairly uniformly (Plate 2, No. 5), to give a pattern similar to that recorded for phosphatases in *Coprinus kimurae* (Komagata and Okunishi, 1969). However, in *C. cinereus* the bulk of the NADP-GDH staining occurred in very isolated patches in the hymenium layer of the gills (Plate 2, Nos 2 and 3). As development proceeded stain deposition in sections became increasingly homogeneous (Plate 2, No. 4). The situation seems very like that recorded for the NAD-linked enzyme; i.e. as development proceeds an increasing proportion of the cap cells come to possess enzyme activity. In the case of NADP-GDH, however, the cytochemical and spectrophotometric data may appear to be at variance,

but the difference is not as great as its seems. Since so few primordium cells possess NADP-GDH activity the level of this activity will be enormously reduced by dilution on preparation of a cell-free homogenate. Consequently, the fact that a minority of the cells exhibit high NADP-GDH activity will be obscured by the generalization inherent in spectrophotometric analysis.

These cytochemical observations thus appear broadly to confirm the results of spectrophotometric analysis. Both GDH enzymes are located in the gills and both increase in activity as carpophore development proceeds. However, the regulatory distinction between cap (which contains both enzymes) and stipe (which contains NAD-GDH only) which was evident from the earlier data is made even more acute by these cytochemical studies for they make it abundantly clear that adjacent cells of the same tissue can differ drastically in enzyme content. Both enzymes, but particularly NADP-GDH, were at first restricted to the hymenium and specifically the basidia (see particularly Plate 1, No. 3, and Plate 2, No. 4), which implies that the expression of enzyme activity was inhibited or repressed in paraphyseal cells of the hymenium and in the subhymenium generally. The initial patchy distribution of NADP-GDH emphasizes this point further since it implies that adjacent basidia of the same developmental age and of closely similar environmental exposure may either have no detectable enzyme activity or a very high enzyme activity. The means by which the regulatory signals responsible for these differences in enzyme activity are kept compartmentalized is not clear, although the fact that with carpophore development more and more cells become able to express enzyme activity shows that communication between cells *is* possible and implies that the initial compartmentalization is a positive process.

These studies have thus emphasized the complexity of the regulatory mechanism to which the GDH enzymes respond. They also emphasize the fact that the regulatory responses can be constrained to the compass of single cells and therefore warn against a too ready application of conclusions drawn from experimentation with homogenates of bulk tissues. A need for enzyme cytochemistry at the electron microscope level is also demonstrated by our observation that considerable GDH activity was detectable in young unpigmented spores (Plate 1, No. 5) since earlier work has shown that mature basidiospores have very low specific activities of both GDH enzymes (Stewart and Moore, 1974). The implication that the GDH enzymes are inactivated and/or transported out of the developing spore can only be tested by electron microscopy because of the intense pigmentation of the spore wall. It is also our intention to determine whether these enzymes show particular patterns of intracellular localization.

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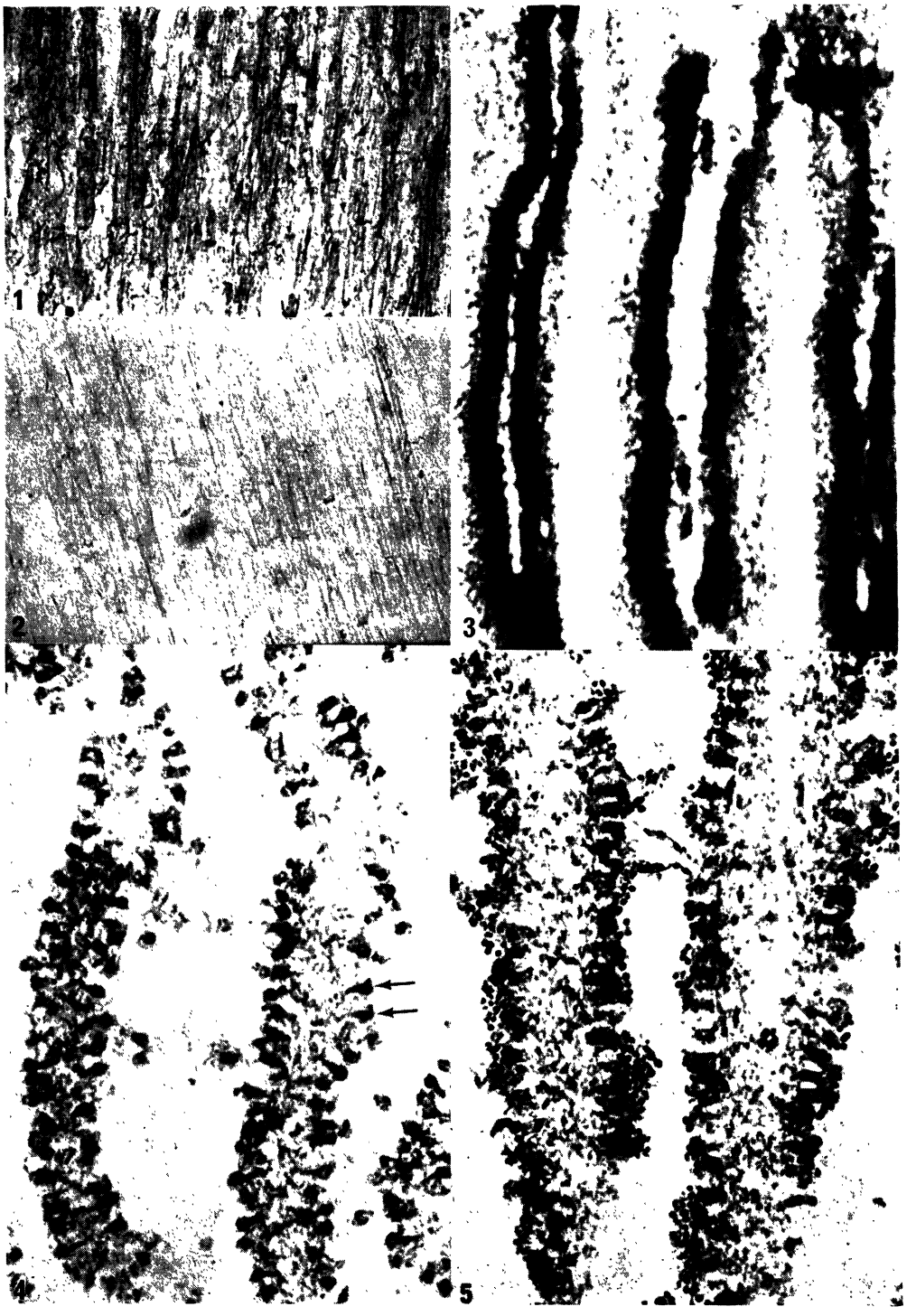

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EXPLANATION OF PLATES

PLATE 1

Glutamate dehydrogenases in carpophore sections of *Coprinus cinereus*.

No. 1. Stage 4 stipe, NAD-glutamate dehydrogenase test reaction. The reaction product is evenly distributed throughout the tissue. × 20.

No. 2. Stage 4 stipe, NADP-glutamate dehydrogenase test reaction. No reaction product is visible. × 20.

No. 3. Stage 1 gill lamellae, NAD-glutamate dehydrogenase test reaction. The intense positive reaction shows a continuous distribution in the hymenium region (compare Plate 2, No. 3). × 260.

No. 4. Late Stage 3 gill lamellae, NAD-glutamate dehydrogenase test reaction. Stain deposits occur in all regions, including basidia and developing spores (arrows). × 260.

No. 5. Early Stage 4 gill lamellae, NAD-glutamate dehydrogenase test reaction. Stain deposits occur in all regions of the lamellae and are clearly located in basidia and the still unpigmented spores (compare with the unstained tissue shown in Plate 2, No. 1). × 260.

PLATE 2

Glutamate dehydrogenase in carpophore sections of *Coprinus cinereus*.

No. 1. Early Stage 4 gill lamellae, unstained section. Note that the spores are still unpigmented. × 140.

No. 2. Stage 1 gill lamellae, NADP-glutamate dehydrogenase control reaction (using a reaction mixture lacking the substrate L-glutamate). Stain distribution here is similar to that in the test reaction (No. 3) in being patchy, but staining is much fainter and reveals the presence of endogenous substrate. × 140.

No. 3. Stage 1 gill lamellae, NADP-glutamate dehydrogenase test reaction. The staining is localized largely in isolated patches of the hymenium. × 260.

No. 4. Late Stage 3 gill lamellae, NADP-glutamate dehydrogenase test reaction. Note that the stain deposition is more homogeneous than in earlier stages (No. 3), occurring in all regions of the lamellae but being clearly localized in basidia. × 260.

No. 5. Stage 1, L.S. pileus, NADP-glutamate dehydrogenase test reaction. The reaction is localized into the outer surface of the pileus and the hymenium of the gills. × 260.