MULTIPLE ORNITHINE DECARBOXYLASE FORMS IN *PHYSARUM POLYCEPHALUM*: INTERCONVERSION INDUCED BY CYCLOHEXIMIDE

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1. Introduction

The activity of the enzyme, ornithine decarboxylase (EC 4.1.1.17) which is rate limiting in the synthesis of the polyamines, putrescine, spermidine and spermine, is rapidly stimulated by conditions which promote growth, and declines quickly when growth is inhibited [1,2]. Since cycloheximide and actinomycin D block this stimulation, and frequently cause a rapid loss in existing activity, it is thought that the mechanism of control of this apparently labile enzyme is through the regulation of its synthesis and degradation [3–6]. Recently, however, Mitchell and Sedory [7] presented evidence suggesting that the rapid fluctuations in the ornithine decarboxylase activity of *Physarum*, which had been exposed to cycloheximide, were in a large part due to variations in the ability of this enzyme to be activated by its coenzyme, pyridoxal 5′-phosphate (PLP). Regulation by such an enzyme modification is in agreement with the accumulating evidence for at least partial control of this enzyme by post-transcriptional mechanisms [8–11]. In this report we present evidence for the existence of multiple ornithine decarboxylase forms in *Physarum* which are involved in the regulation of this enzyme's activity.

2. Materials and methods

2.1. *Chemicals*

Pyridoxal 5′-phosphate, L-ornithine, blue dextran and cycloheximide were purchased from Sigma Chemical Co.; Sephadex G-200 (40–120 μl) from Pharmacia Fine Chemicals Inc.; and DL-[1-^{14}C]ornithine · HCl (29 Ci/mole) from Amersham/Searle Corporation.

2.2. *Culture techniques*

Cultures of *P. polycephalum* were maintained and sampled as described earlier [3].

2.3. *Preparation of enzyme extracts*

Frozen tissue pellets containing about 3–5 mg of protein were suspended in 3 ml of ice-cold 0.05 M borate buffer (pH 7.8), containing 0.5 mM dithiothreitol and 0.2 mM EDTA, disrupted by sonicating for 30 sec and assayed immediately.

2.4. *Ornithine decarboxylase assay*

Enzyme activity was determined by measuring the liberation of $^{14}CO_2$ from DL-[1-^{14}C]ornithine as detailed earlier [3], except hyamine hydroxide was used to absorb the $^{14}CO_2$ which was counted in a toluene-based scintillation fluid. The incubation mixtures are described in the figure legends.

2.5. *Sephadex G-200 column chromatography*

Enzyme extracts were prepared for chromatography by sonicating the frozen pellets in 0.05 M borate buffer (pH 7.8), containing 1.0 mM dithiothreitol, 0.5 mM EDTA and 2 μM PLP. One ml of this extract was mixed with 2 mg blue dextran, applied to a 40 × 1.6 cm Pharacia column containing Sephadex G-200 which had been pre-equilibrated with this buffer, and eluted with the same buffer at 4.5 ml/h. Estimation of mol. wts were made according to the techniques of Andrews [12]. All procedures performed at 4°C.

3. Results

3.1. *Cycloheximide-induced variations in ornithine decarboxylase activity*

The lower curve in fig.1 illustrates the very rapid initial increase and eventual loss of ornithine decarboxylase activity in *Physarum* exposed to cycloheximide. However, when the same enzyme preparations were assayed in the presence of an unphysiologically high coenzyme level, 200 μM PLP, the variations in activity were greatly reduced (upper curve, fig.1).

3.2. *Variable coenzyme activation of cycloheximide treated samples*

The disagreement between the estimation of enzyme stability by assays at high and low PLP levels was further investigated by assaying these enzyme fractions at PLP concentrations increasing from 0.2 to 500 μM PLP. When this data was graphed as a double reciprocal plot, it became obvious that this enzyme did not demonstrate normal Michaelis–Menten saturation kinetics throughout the entire coenzyme range (fig.2). At low coenzyme levels (0.2–1.0 μM PLP) all time points extrapolated to about the same K_M for PLP, 0.46 μM. At higher PLP levels (1.0–200 μM PLP), however, all the curves bent down sharply to approach about the same maximum velocity at saturating PLP levels. Such reciprocal plots, concave from below, have been reported previously in association with ornithine decarboxylase activity at high coenzyme concentrations [13,14].

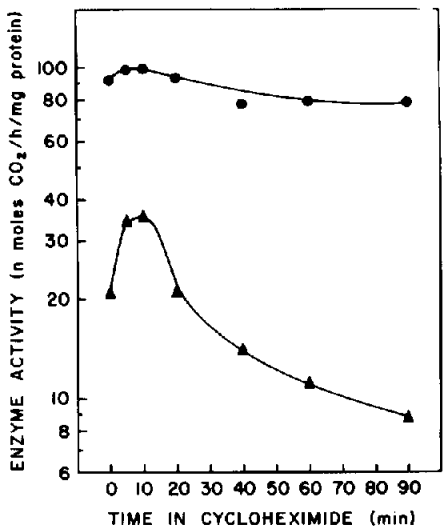

Fig.1. Decay in ornithine decarboxylase activity in microplasmodia exposed to 25 μg/ml of cycloheximide. Inhibitor was added to a shake-flask culture of Physarum microplasmodia at time 0 and subsequently culture aliquots were extracted, quick-frozen in liquid nitrogen and assayed as described in Materials and methods. Assay mix contained 100 μl of enzyme extract and 1.9 ml of 0.05 M borate buffer (pH 7.8), 0.5 mM dithiothreitol, 0.2 mM EDTA, 0.10 mM L-ornithine (0.10 μCi) and either 1 (▲) or 200 (●) M PLP.

One interpretation of such a complex coenzyme activation pattern is that we are observing two or more simultaneous reactions, perhaps involving distinct enzymes, which may be separated and identified by their relative affinity for coenzyme. Therefore, small aliquots of crude enzyme were applied to Sephadex G-200 columns equilibrated with borate buffer, and the resulting fractions were assayed at both low (1.0 μM) and high (200 μM) PLP levels. As seen in fig.3, all the enzyme activity stimulated by 1.0 μM PLP eluted in a peak which corresponded to an approximate molecular weight of 160 000 daltons. The use of 200 μM coenzyme in the assays not only increased the activity in the 160 000 mol. wt. peak (peak II) but also stimulated previously unobserved ornithine decarboxylase activity in fractions very close to the void volume (peak I). Occasionally a small peak of activity was also observed at about mol. wt. 80 000 (peak III). No activity was observed above boiled enzyme controls when fractions were assayed without any added coenzyme, thus the activation of peak II by 1 μM PLP must be the expression of enzyme with a low K_M for the coenzyme. This enzyme form may therefore represent the active state of this enzyme in the low PLP environment found in vivo. The decarboxylation of ornithine in peaks I and III, stimulated only by very high coenzyme levels, then may indicate either inactive forms of this enzyme, or other enzymes capable of releasing the carboxyl group from ornithine under these saturating coenzyme conditions.

3.3. *Cycloheximide-induced interconversion of enzyme forms*

Repeated separations were made, on identical Sephadex G 200 columns, of samples taken at various times after cycloheximide addition (fig.4). Although the total activity measured in the presence of 200 μM

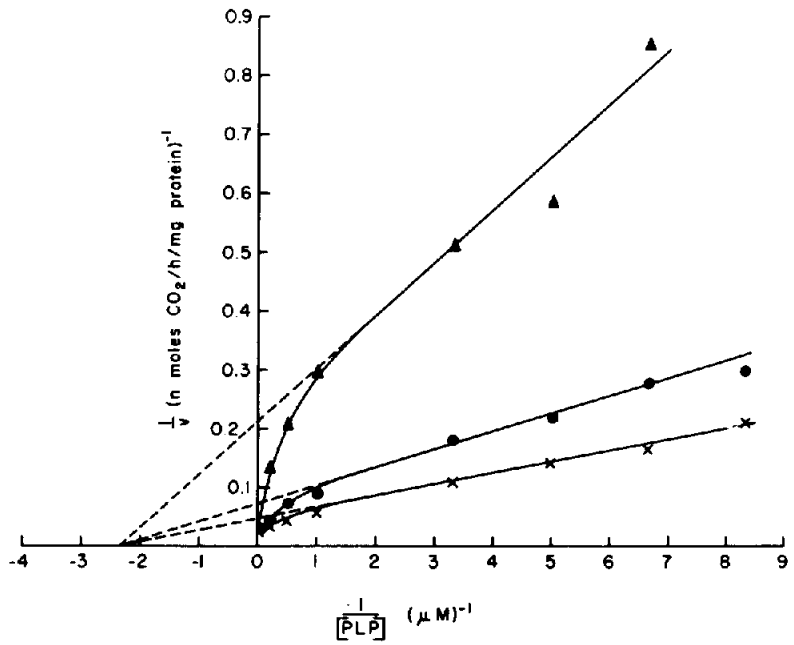

Fig.2. PLP activation of ornithine decarboxylase following cycloheximide inhibition of protein synthesis. Aliquots were extracted and assayed, as described in the legend to fig.1, 0 (•) 5 (X) and 100 (▲) minutes after the addition of cycloheximide, using the various coenzyme levels indicated.

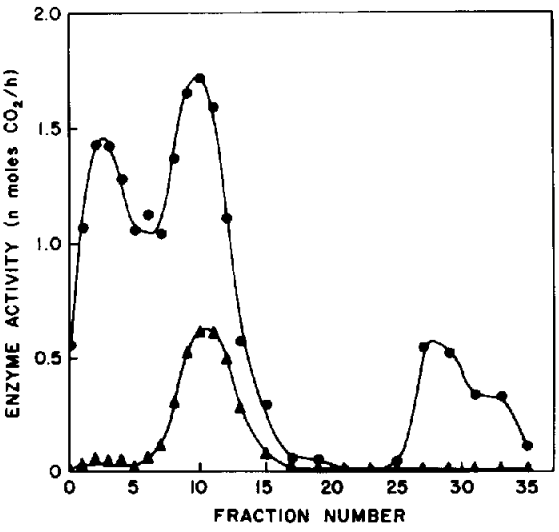

Fig.3. Multiple peaks of ornithine decarboxylase activity eluted from a 40 X 1.6 cm Sephadex G-200 column. A sample of microplasmodia were sonified in column buffer and 1 ml aliquots fractionated on Sephadex G-200 as described in Materials and methods. Fractions (0.8 ml) following the blue dextran peak were collected, and 0.1 ml aliquots assayed, as in the legend for fig.1, using either 1 (▲) or 200 (•) μM PLP.

PLP varied only slightly during this cycloheximide inhibition period (fig.1), the distribution of this activity in the column eluant changed considerably. In all cases the recovery off these columns was between 90 and 100% of the activity applied. Activity stimulated by 1 μM PLP did not shift from the peak II position but rather increased and decreased in direct proportion to the height of the same peak when measured in the presence of 200 μM PLP.

Within 5 min of cycloheximide addition almost all the activity in peak I, detected using 200 μM PLP, was shifted to peak II, the low K_M peak. After 10 min, low K_M activity was progressively lost with a simultaneous increase in peak I, the higher mol. wt. unit, until, after 90 min, more activity was in peak I than II. This interconvertibility between these peaks of activity, along with the kinetic support for two such reactions in crude enzyme preparations, suggested that these may be two states of the same enzyme, i.e. active and inactive, with different affinities for coenzyme, rather than two separate enzymes.

3.4. *Coenzyme-induced changes in enzyme size in vitro*

Since the inactive forms of this enzyme were

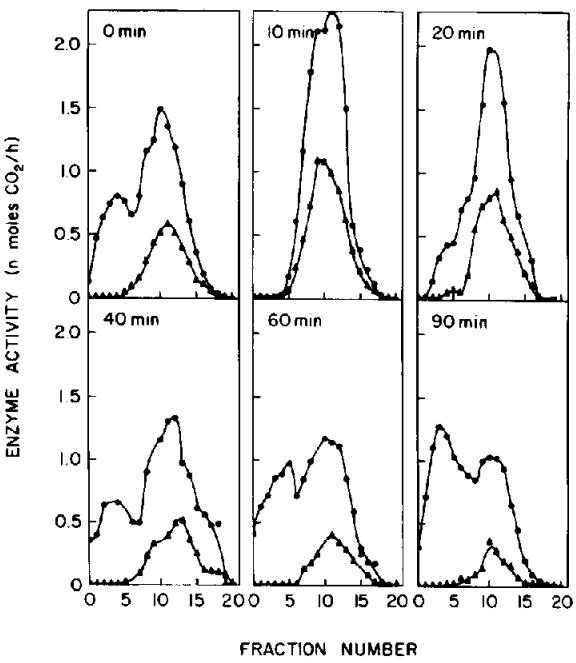

Fig.4. The interconversion of forms I and II following cycloheximide addition. Cultures were treated with cycloheximide and samples extracted as described in the legend to fig.1. Using the same column on consecutive days, separations were made as described in the legend to fig.3, of enzyme extracted at various times after the addition of cycloheximide. Coenzyme levels and graph symbols are as described in the legend for fig.3.

either much larger or smaller than the active 160 000 dalton form it was of interest to know whether activation was merely determined by a certain degree of polymerization of inactive enzyme subunits, or if it involved a more complex alteration or addition to the basic enzyme protein. The former mechanism was suggested by the observation that the peak I enzyme form, resulting from treatment with cycloheximide for 90 min, could be completely converted to the peak II form when enzyme preparation and column separation were performed in buffer containing 200 μM PLP. However, an identical sample, dialyzed first with high PLP for 6 h, and subsequently dialyzed against the normal, low PLP buffer for 12 h showed both peak I and II forms when separated on a column containing low PLP buffer. Peak II enzyme, resulting from 5 min exposure to cycloheximide, did not show any such reversion to form I by similar treatment. Thus, unlike the in vitro activation of this enzyme by high PLP levels, the activation of this enzyme to produce form II in vivo must involve a modification which stabilizes this form.

4. Discussion

The interconversion of forms I and II in vitro (in response to coenzyme level changes) and in vivo (in response to cycloheximide treatment) is quite similar to the variations found in the multiple form patterns of rat liver tyrosine aminotransferase, another pyridoxal enzyme [15–19]. In that enzyme it is believed that the multiple forms represent post-translational modifications associated with enzyme activation, hormonal response, and eventual degradation [17]. The interconversion of the *Physarum* ornithine decarboxylase forms, which also appears to involve a post-translational modification, allows the regulation of form II, and since this form has the greatest affinity for coenzyme, enzyme activity. Thus the activity fluctuations reported previously [7] in response to cycloheximide, were due to this interconversion between the low K_M (active) and high K_M (inactive) enzyme forms, which produced a dramatic variation in the overall K_M for PLP when measured at intermediate coenzyme levels.

The mol. wt. of the peak III form is approx. 80 000, or about one half that of peak II, suggesting that the latter may be a dimer of the peak III enzyme form. This relationship is similar to that found in the pyridoxal enzymes, L-serine dehydratase [20] and aspartate β-decarboxylase [21]. While simple dissociation may account for the reversible conversion of form I to II by high PLP in vitro, the in vivo conversion is more stable and therefore probably involves a change in enzyme protein which stabilizes this particular size unit. The activation and inactivation of mammalian ornithine decarboxylase by monomeric and dimeric form conversion was initially suggested by Jänne and Williams-Ashman in 1970 [22], however until now there has been no evidence to substantiate the existence of such interconverting states in vivo. The efficiency and sensitivity of post-translational enzyme modification make this an attractive mode of activity regulation which would allow extremely rapid fluctuations in activity, which are characteristic of this enzyme, without the necessity for an abnormally short protein half-life.

Acknowledgements

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