THYMIDINE KINASE ENZYME VARIANTS IN *PHYSARUM POLYCEPHALUM*; CHANGE OF PATTERN DURING THE SYNCHRONOUS MITOTIC CYCLE

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1. Introduction

Thymidine kinase activity (EC 2.7.1.75) exhibits periodic changes in macroplasmodia of *Physarum polycephalum* correlated with the synchronous nuclear division cycle [1,2]. Studies with inhibitors of RNA and protein synthesis suggest that production of this enzyme is mainly restricted to a small segment (1 h) of the 10 h nuclear cycle starting shortly prior to the onset of the synchronous mitosis [3]. Preliminary attempts to isolate the enzyme from plasmodial extracts have shown that thymidine kinase activity appears in several fractions behaving differently during purification procedures. This communication reports on the separation of at least three enzyme variants by isoelectric focusing. The absolute and relative quantities of these variants change characteristically during the synchronous mitotic cycle. The results suggest that during the induction period a single enzyme species is produced which undergoes post-transcriptional modifications giving rise to multiple enzyme variants.

2. Methods

2.1. *Preparation of plasmodial extracts and assay of enzyme activity*

Disc shaped macroplasmodia of *Physarum polycephalum* (strain M3b) with a diameter of 2–5 cm were cultivated on a sterile liquid medium as described [4,5]. Entire plasmodia were harvested at selected stages of the mitotic cycle, frozen in dry ice together with 2–5 ml 50 mM TGM buffer (50 mM Tris–HCl, pH 7.5 at 25°C, 25% w/v glycerol, 5 mM 2-mercaptoethanol) and stored at −25°C. Frozen samples were quickly thawed, disrupted with a Potter-Elvehjem homogeniser and centrifuged for 1 h at 150 000 × *g*. Thymidine kinase activity and protein were determined in the supernatant. The enzyme assay mixture (total volume 70 μl) contained 86 mM Tris–HCl pH 7.5, 8.7 mM ATP-Na, 8.7 mM $MgCl_2$, 2.6 mM EDTA-Na, 3.6 mM mercaptoethanol, 2.5 mg/ml bovine serum albumin, 18% w/v glycerol, 3.6 μM [^{14}C] thymidine (60 mCi/mmol, 0.215 μCi/ml), and plasmodial extract (0.5–1.0 mg protein/ml); aliquots (20 μl) were pipetted onto pieces of anion exchange paper (Whatman DE 81; 1.6 × 2.0 cm) after 0, 15, and 30 min incubation at 26°C and processed as described [1,2]. The amount of [^{14}C] thymidine phosphorylated and retained on the paper was measured by liquid scintillation counting.

2.2. *Isoelectric focusing*

5% acrylamide gels (0.5 × 7.0 or 0.5 × 8.0 cm) containing 10% glycerol, 1.5% Ampholine (pH 3.5–10.0) 1.5% Ampholine (pH 5.0–8.0), and 0.2% *N,N'*-methylenebisacrylamide were polymerised for 20 min in glass tubes after the addition of 0.067% *N,N,N',N'*-tetramethylethylenediamine and 0.067% ammonium persulfate. Isoelectric focusing was performed for 6 h at 4°C in an apparatus essentially as described by Righetti and Drysdale [6], using 10 mM H_3PO_4 as anolyte (bottom) and 20 mM NaOH as catholyte (top). In order to remove excess persulfate the gels were pretreated for 45 min at 1 mA/tube. 20 μl of plasmodial extract were applied to each gel, mixed with 5 μl Ampholine (15%, pH 3.5–10.0) and

covered with 50 μl of a mixture of 10% w/v glycerol and 3% Ampholine (pH 3.5–10.0). Isoelectric focusing was started with 1 mA/tube; the voltage increased up to 500 V within 1 h and was kept constant at this level until the end of the run. Gels were frozen in dry ice for 1 h and sliced with a razor blade cutter into 2 mm sections. The slices were incubated for 15 h at 26°C in small plastic vials containing 100 μl of thymidine kinase assay mixture (0.13 M Tris–HCl, pH 7.5; 12.4 mM ATP-Na, 12.4 mM $MgCl_2$, 5 μM [^{14}C]thymidine 60 mCi/mmol, 3.6 mM EDTA-Na, 3 mM mercaptoethanol, 6.5 mg/ml bovine serum albumin, and 15% w/v glycerol). The reaction was terminated by pipetting aliquots of 20 μl onto pieces of Whatman DE-81 paper which were processed as described above. The pH gradient was determined in a control gel with a glass electrode after incubating groups of 2 slices in 1 ml distilled water for 15 h.

3. Results and discussion

Analysis of crude plasmodial extracts by isoelectric focusing on polyacrylamide gels demonstrates the existence of several fractions with thymidine kinase activity (fig.1). At least three peaks are separated with

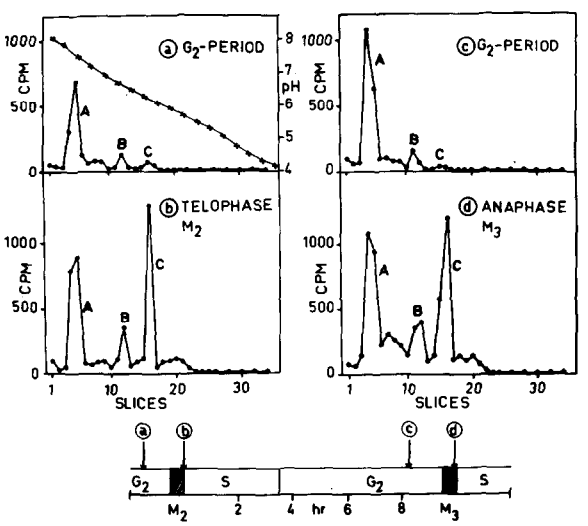

Fig.1. Isoelectric focusing in 5% polyacrylamide gels. Individual plasmodia were harvested at time points indicated by arrows (a,b,c,d), homogenised with 2 ml (a,b) or 3 ml (c,d) 50 mM TGM buffer, and processed as described under 'Methods'. 20 μl samples (20–30 μg protein) were analysed by isoelectric focusing (7 cm gels).

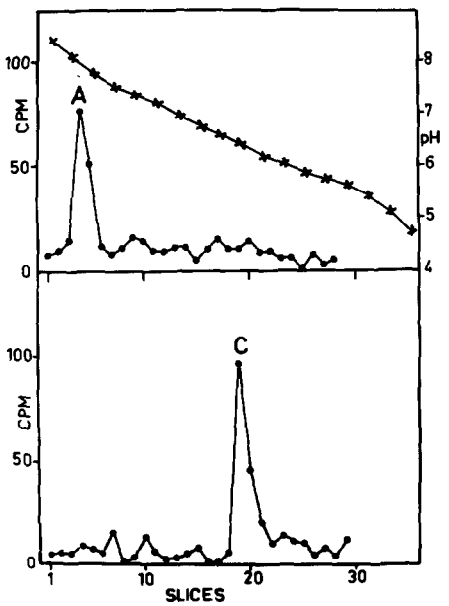

Fig.2. Repeated isoelectric focusing of peak fractions (A) and (C). 20 μl of a plasmodial extract (telophase) were processed as in fig.1. Individual gel slices were incubated with 75 μl 50 mM TGM buffer at 0°C for 6 h and analysed for thymidine kinase activity using 25 μl aliquots. 30 μl of the peak fractions (A) and (C) were rerun on 2 separate gels (8 cm) under the same conditions as the original whole extract. The distribution of enzyme activity in these gels, as well as the pH gradient in a control gel, were determined as in fig.1.

isoelectric points at pH 7.8 ± 0.15 (A), pH 6.7 ± 0.1 (B), and pH 6.3 ± 0.1 (C). Small shoulders appear consistently at pH 7.2 and pH 5.9. The main fractions (A) and (C) behave as single peaks when eluted from the gel and analysed again by isoelectric focusing (fig.2). This indicates that (A) and (C) represent individual enzyme species with different physicochemical properties rather than artefacts generated by the separation procedure.

The absolute and relative amounts of thymidine kinase variants change drastically during the mitotic cycle (fig.1,3). Fraction (C) increases sharply during mitosis reaching a distinct maximum at telophase. Fractions (A) and (B) increase with a significant delay relative to (C) and continue to increase during S-phase concomitantly with a rapid drop of (C). Fraction (A) becomes the dominant species later on in the cycle whereas (C) virtually disappears in G_2-period. (B) comprises 10–15% of the total activity throughout the cycle.

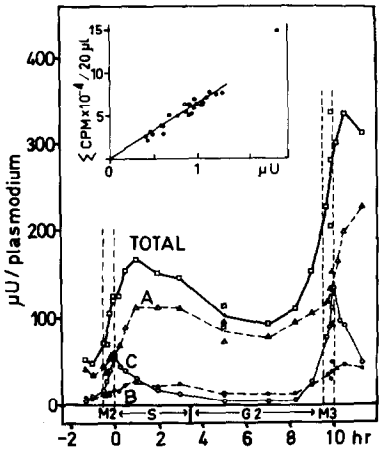

Fig.3. Changes of total thymidine kinase activity and of enzyme variants during the synchronous nuclear division cycle. 24 replicate macroplasmodia were prepared. Entire plasmodia were harvested at various time intervals, starting 1.5 h prior to the expected second synchronous mitosis. 20 μl samples (20–60 μg protein) of the high speed supernatants were immediately taken for isoelectric focusing; 50 μl aliquots were analysed for total thymidine kinase activity and protein respectively. The distribution of enzyme activity in the gels was determined and plotted as in fig.1. The contents of enzyme variants (A), (B), and (C) per plasmodium were computed from the areas under the corresponding peaks.
Inset: Recovery of enzyme activity from gels after isoelectric focusing. Abscissa: enzyme activity (μU) of whole plasmodial extracts (20 μl) applied to each gel. Ordinate: relative amount of enzyme product formed by incubating gel slices for 15 h with radioactive substrate; each point represents the radioactivity (cpm) measured in 20 μl aliquots/slice and summed for all slices of each gel.

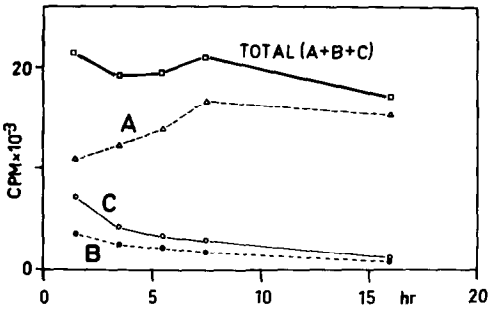

Fig.4. In vitro changes of thymidine kinase enzyme variants. The high speed supernatant obtained from a single macroplasmodium at telophase 3 was stored at 0°C. Aliquots (20 μl, 40 μg protein) were analysed at various time intervals by isoelectric focusing and the distribution of enzyme activity in the gels was determined as in fig.1. The areas under the peaks (A), (B), and (C) are plotted as well as the total activity recovered from each gel.

The sequential appearence of thymidine kinase variants in the mitotic cycle suggests, that fraction (C) is produced 'de novo' during the induction period and functions as a precursor for variant (A) and possibly also for (B). This is supported by the observation that the pattern of enzyme variants in plasmodial extracts changes similarly in vitro as in the living plasmodium (fig.4): the relative content of fraction (C) in crude plasmodial extracts obtained from plasmodia at the time of mitosis decreases during storage in the cold, whereas (A) increases concomitantly. Most likely this indicates a spontaneous transformation of enzyme variant (C) into variant (A). Preliminary observations suggest that this process depends on a factor (enzyme ?) present in the crude plasmodial extract, since no transformation is observed during storage of a partially purified fraction (C) [Gröbner and Sachsenmaier, in preparation].

Multiple forms of thymidine kinase have been observed in several systems. In mammalian cells different isoenzymes were found in the cytosol and in mitochondria [7,8]. New species of thymidine kinase were identified after viral infection; certain *Herpes* viruses appear to code for a new virus specific thymidine kinase, whereas other viruses may derepress cellular genes (SV40, human adenoviruses) [9,10]. Different thymidine kinase isoenzyme patterns have also been linked to different developmental stages, malignant transformation, and low or high proliferative activity [11–16]. These differences have been discussed in terms of different genetic expression, i.e. introduction of viral genes or activation and repression of pre-existing cellular genes. By contrast, the observations reported here strongly suggest that at least in some cases variants of thymidine kinase may arise from post-transcriptional modification of an unstable primary enzyme species. The cycle dependent variation of the isoenzyme pattern in *Physarum* appears as a direct consequence of the discontinuous production of a 'precursor'-enzyme. A similar situation may also exist in mammalian cells. The cycle dependent thymidine kinase pattern observed in partially synchronised human kidney cells by Adler and McAuslan [17] would agree with the assumption that a single

thymidine kinase gene is activated discontinuously at the onset of the S-period followed by post-transcriptional modifications of the enzyme during later stages of the cycle.

Acknowledgements

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