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ACTIVITY, ISOENZYME PATTERN, AND SYNTHESIS OF UDPGLUCOSE 4-EPIMERASE DURING DIFFERTIATION OF *PHYSARIUM POLYCEPHALUM*

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Summary

1. The specific activity of UDPglucose 4-epimerase (EC 5.1.3.2) increases by about 50% during the first 24 h of starvation-induced differentiation (sphe-rulation) of *Physarum polycephalum*.

2. At all stages during differentiation, the enzyme activity is very sensitive to actinomycin-C and cycloheximide, inhibitors of transcription and translation, with a half life against cycloheximide of about 20 min (if added 12 h after the induction of differentiation).

3. The isoenzyme pattern, as revealed by isoelectric focusing in sucrose gradients, does not change during spherulation. One main band with a p*I* of 6.7, with a shoulder (p*I* 7.6) and a minor band (p*I* 6.0) was observed in extracts both from growing and differentiating cultures.

4. Density labelling experiments using deuterated amino acids with subsequent analysis by equilibrium density gradient sedimentation in 15—35% (w/w) metrizamide gradients revealed a rather slow rate of enzyme synthesis, which is in contrast to the observed high sensitivity against actinomycin-C and cycloheximide.

Introduction

The transfer of log-phase growing microplasmodia of the true slime mold *Physarum polycephalum* to a nutrient-free salts medium [1] triggers a series of events at the cytological and biochemical level which finally leads to the formation of thick-walled sclerotia (called spherules) (for recent reviews see refs 2 and 3). This differentiation is characterised by a pronounced shift in the N-free carbohydrate metabolism: glycogen, the main carbohydrate present during growth [4] is rapidly degraded whereas a slime is secreted, beginning after about 12 h of starvation [5]. This slime is a polymer with a mean degree of

polymerization of 128 [6], composed of galactose monomers only with few sulfate and phosphate groups attached. In order to get some information about the regulatory events responsible for its synthesis during the starvation-induced differentiation, we studied the first enzyme in the specific pathway of slime synthesis: UDPglucose 4-epimerase (EC 5.1.3.2) which converts UDPG to UDPGal.

Our findings, reported in this paper, indicate that this enzyme is not governed by a developmental program during differentiation, and that its activity is not responsible for the regulation of the metabolic change we are interested in learning about.

Materials and Methods

Materials

The biochemicals were obtained from Boehringer-Mannheim GmbH (Mannheim-Waldhof, G.F.R.). Cycloheximide came from Calbiochem, ampholines from LKB (Bromma, Sweden), and metrizamide from Nygaard (Oslo, Norway). The deuterated amino acids and ^2H_2O were purchased from Sharp und Dohme GmbH (München, Germany). We wish to thank the Pharma Abteilung, Farbwerke Bayer (Leverkusen, W.-Germany) for a generous gift of actinomycin-C, and the SA Melle-Bezons (92 Neuilly s.S., France) for a sample of the nonionic detergent Cemulsol NPT-12. Tryptone and yeast extract for the culture media came from Difco (Detroit, U.S.A.). All other chemicals were reagent grade and bought from E. Merck AG (Darmstadt, W. Germany).

Cultures

The strain M_3 CIV was used in this study of *P. polycephalum*. Our methods of growing this organism and the induction of spherulation have been described earlier [7].

Preparation of enzyme extracts and determination of UDPglucose 4-epimerase activity

The procedure of harvesting the plasmodia and preparing the enzyme extract have been described earlier [8]. UDPglucose 4-epimerase activity was determined according to the method of Wilson and Hogness [9]: the assay mixture contained 0.3 μmol UDPGal, 3 μmol NAD, 10 μg UDPglucose dehydrogenase (6 mU), 100 μmol Tris/acetate, pH 8.0, and 10—50 μl of the enzyme extract in a final volume of 1 ml. The assays were usually carried out within less than an hour after the preparation of the extracts.

The extracts were stored on ice before assaying. The reaction was started by the addition of the enzyme extract and the increase in A_{340nm} was monitored at 30° C in a Zeiss PMQ II spectrophotometer. One enzyme unit (U) catalysed the conversion of 1 μmol substrate per min at this temperature.

Isoelectric focusing

Isoelectric focusing was done using the standard methods [10] and the LKB-equipment at the following conditions: 5 ml enzyme extract containing about 30 mg of total protein were loaded on a 110 ml column containing 1%

ampholines pH 3—10 and 10 mM dithioerythrit. At a voltage of 1000 V (initial current 2.4 mA) the focusing was run for 38 h at 2° C. Fractions of 25 drops each were collected and aliquots taken for enzyme assays. The pH was measured in every fifth fraction at 0° C, the value being corrected to 20° C.

Density labelling and analysis in metrizamide/^2H_2O gradients

According to the procedure described earlier [11] about 3 ml of packed plasmodia were suspended in 15 ml of the starvation medium supplemented with 10 mg/ml deuterated amino acids and incubated for the time intervals indicated in the Results section. The cultures were harvested and the extracts analysed by equilibrium density gradient sedimentation in a 15—35% (w/w) metrizamide/^2H_2O gradient with β-galactosidase from *Escherichia coli* as an internal density marker [12]. Conditions of the runs: 350 000 × *g*, 17 h, 4° C in a 5-ml tube in a swinging bucket rotor. For an evaluation of the slope of the gradient, the refractive index of every tenth fraction was determined.

The other fractions were analysed for the activities of the two enzymes.

Protein determination

Protein was determined by the Folin method [13], using albumin as a standard.

Results

Properties of the enzyme in crude extracts

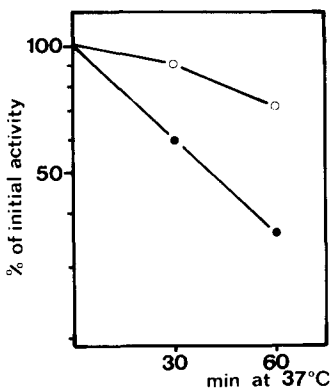
The activity was linearly proportional to the amount of enzyme extract present in the assay, with linear time kinetics. It had an equal rate of reaction in Tris/acetate, Bicine/NaOH, and Tricine/NaOH buffer with a pH optimum at 8.0 in all three buffer systems tested. The apparent K_m for UDPGal as a substrate was $1.1 \cdot 10^{-4}$. The specificity of our assay is indicated in Table I.

Both extracts from growing and differentiating plasmodia were stable in their epimerase activity at 30° C for at least 1 h. At 37° C the stability differed, with the activity in extracts from growing cultures being more resistant to heat inactivation (Fig. 1). Thus, the enzyme appeared to be much more stable than the one described for the cellular slime mold *Dictyostelium discoideum*, where

TABLE I

COMPONENTS REQUIRED IN THE MIXTURE FOR ASSAYING EPIMERASE ACTIVITY

Omission	Observed activity (mU) in extract from growing plasmodia
None	12
UDPGal	0.05
NAD	0.05
UDPG dehydrogenase	0.03
Slime mold extract	None observed



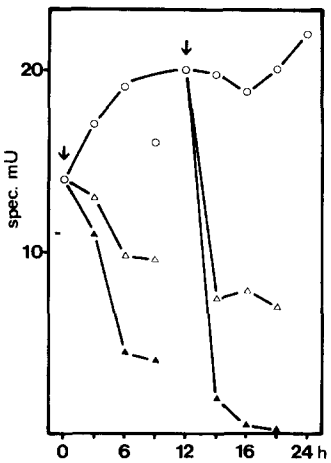


Fig. 1. Differential heat-stability of UDPglucose 4-epimerase in extracts from growing and 24 h-starved plasmodia from *P. polycephalum*. ○———○, extracts from growing plasmodia; ●———● extracts from 24 h-starved plasmodia incubated at 37° C.

Fig. 2. Specific activity of UDPglucose 4-epimerase and the influence of actinomycin-C and cycloheximide on it during spherulation of *P. polycephalum*. ○———○, specific activity in the controls; △———△, specific activity after the addition of actinomycin-C; ▲———▲, specific activity after the addition of cycloheximide. The arrows indicate the time of addition of the inhibitors.

the enzymic activity disappeared while standing on ice within 30 min and had to be stabilized with glycerol [14].

The influence of the following nucleotides on the reaction rate and the apparent K_m of the epimerase activity was tested: AMP, cyclic AMP, ADP, ATP, UMP, UTP, CMP, CTP, and ITP. In all cases, no change in the activity or the apparent K_m was observed. Unlike the UDPglucose 4-epimerase from rat liver [15], the *Physarum* enzyme does not seem to be controlled by nucleotides.

Activity during differentiation

The specific activity increases by about 50% during the first 24 h after the induction of spherulation by transferring growing plasmodia to the nutrient-free salts medium (Fig. 2). Since after this time period the first thick-walled spherules appear, the experiment was always terminated to avoid the problem of breaking the spherules in the presence of the plasmodia. The UDPglucose 4-epimerase activity was very sensitive against the inhibitors of transcription and translation, actinomycin-C and cycloheximide. The addition of both inhibitors also resulted in an immediate sharp decrease in enzymatic activity at all stages of differentiation, examples of which are given in Fig. 2.

Isoenzyme pattern

The epimerase activity was stable enough to permit preparative isoelectric focusing in sucrose gradients in the presence of 10 mM dithioerythrit. Fig. 3 shows the isoenzyme pattern from extracts made from growing cultures, which was identical to the one observed from extracts from cultures starved for 24 h:

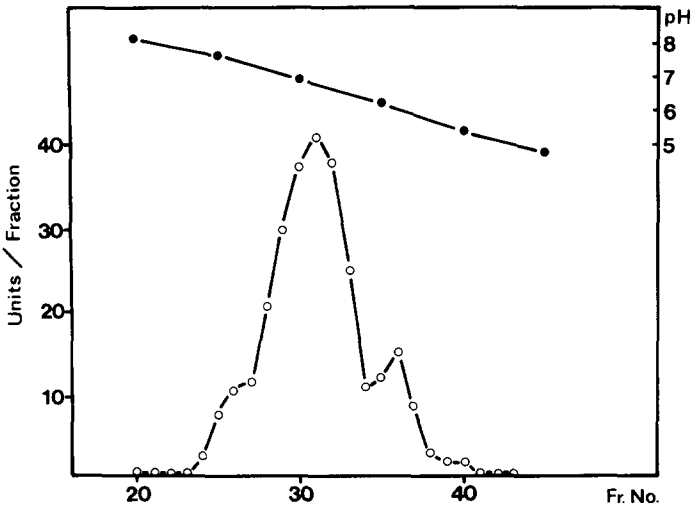


Fig. 3. Isoelectric focusing of UDPglucose 4-epimerase from extracts from growing plasmodia.

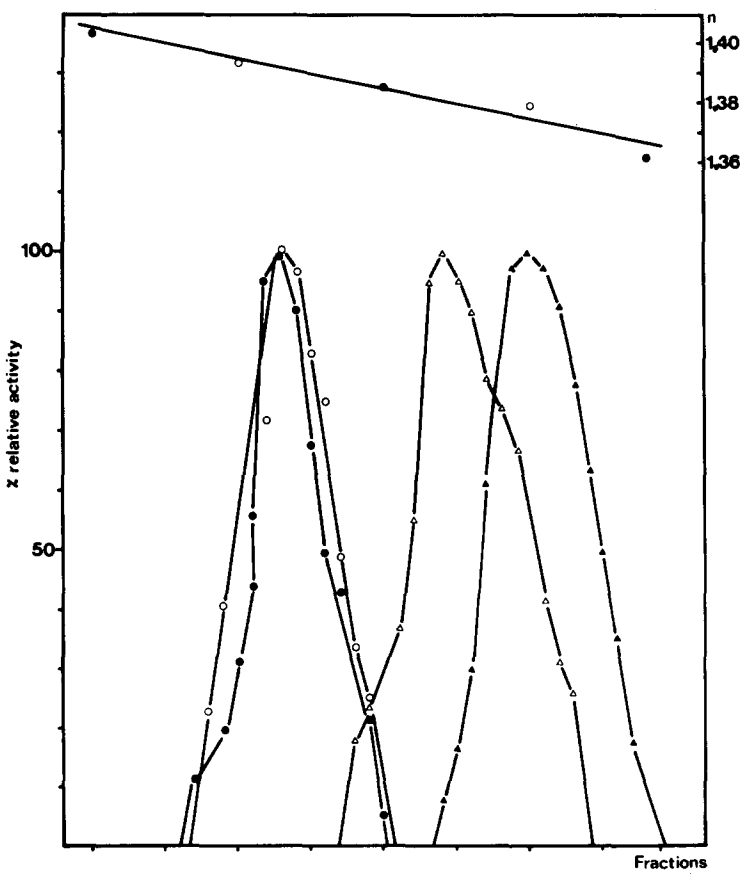


Fig. 4. Equilibrium density gradient centrifugation of UDPglucose 4-epimerase from extracts made from 24 h-starved cultures either in normal starved cultures or in cultures induced in the presence of deuterated amino acids. In two parallel experiments, *P. polycephalum* was induced to spherulate either in the normal starvation medium or in the presence of 10 mg/ml deuterated amino acids. The extracts from both types of plasmodia were analysed by equilibrium density gradient sedimentation in metrizamide gradients with β-galactosidase from *E. coli* added as an internal density marker. The fractions obtained after the run were divided and assayed for β-galactosidase activity and UDPglucose 4-epimerase activity. The two different gradients are superimposed in this figure at the position of the internal marker β-galactosidase. The upper curve indicates the density slope in the gradient via the refractive indices in every tenth fraction of the gradients. The open symbols refer to the gradients from deuterated extracts, the closed symbols the gradients from unlabelled extract. ○———○, β-galactosidase; △———△, UDPglucose 4-epimerase.

a main band with a p*I* of 6.8, a shoulder at pH 7.6, and a second small band with a p*I* of 6.0.

Synthesis during differentiation

We measured the synthesis of UDPglucose 4-epimerase during the differentiation by labelling the plasmodia with deuterated amino acids and subsequent analysis by equilibrium density gradient sedimentation [16] in 15—35% (w/w) metrizamide/^2H_2O gradients [12]. The addition of the amino acids does not affect the time course of spherulation [11] and had no effect on epimerase activity. Any synthesis occurring during the time in which the deuterated amino acids are present should result in a higher buoyant density of the epimerase activity in the fractions of the gradient.

The label was applied during three different time periods: 0—6 h after the transfer (when the highest increase in specific activity was observed), 18—24 h after the transfer and a continuous label for the whole 24 h period.

When the label was applied for only 6 h, no density labelling could be detected. The enzyme had the same density as the one from extracts cultivated on light isotopes. Only in extracts from cultures which had been labelled for the whole 24 h period, an incorporation of the heavy isotope into UDPglucose 4-epimerase was observed (Fig. 4). But even then no homogenous peak was obtained. The peak from the deuterated extracts was considerably broader than the control peak and exhibited a shoulder in the position of the unlabelled enzyme.

Discussion

In growing plasmodia of *P. polycephalum*, a specific activity of about 12—15 mU of UDPglucose 4-epimerase was found (Fig. 2, Table I). The rate of total slime synthesis during the time of maximal slime secretion during differentiation was found to be 2.1 mU [17]. Thus, the epimerase activity in growing plasmodia is about six times higher than the maximal rate of slime production during differentiation and would be high enough to cope with this synthesis rate under the conditions of cell metabolism. This observation is in good agreement with the findings of McCormick et al. [5], who found that after the inhibition of protein synthesis by cycloheximide the same amount of slime was formed under conditions of starvation as in the untreated controls. The switch from glycogen synthesis during growth to slime synthesis during spherulation is not triggered by the activity of epimerase and perhaps not by an event related to protein synthesis considering the findings of McCormick et al. [5].

Our data on the activity pattern of the epimerase during differentiation are similar to the pattern obtained for the activity of the enzyme UDPgalactosamine 4-epimerase in the same system [18]. No developmental program can be ascribed to the activity changes of both enzymes during this differentiation like the ones described for UDPglucose 4-epimerase and other enzymes during the differentiation of *D. discoideum* [14,19].

The very high sensitivity of the epimerase activity against actinomycin-C and cycloheximide (cf. Fig. 2) could lead to the interpretation that this enzyme has a high turnover rate with a half life after 12 h of starvation of about 20 min.

This has not been confirmed by our density labelling experiments. Only after a continuous label period of 24 h was any incorporation of the heavy isotope detectable in this enzyme. But even then about 30% of the enzyme present at the beginning of the label period was still remaining in the plasmodium, as could be seen from the "light" shoulder of this peak. With label periods of 6 h only, no incorporation was observed at all, although we utilized the very sensitive assay system with an internal density marker as a reference. Even a small incorporation of the heavy isotope should be then detectable in shift to a higher density compared to the marker.

The results on the discrepancy between the inhibitor data and density-labelling experiments confirm our earlier findings on the enzyme glucose-6-phosphate dehydrogenase. The specific activity of this enzyme decreases during the starvation-induced spherulation of *P. polycephalum* [20]. This decline in activity was significantly enhanced by the addition of actinomycin-D and cycloheximide. This was interpreted by us as the result of an unbalanced relationship between degradation and synthesis of this enzyme occurring during differentiation. Using the more direct method of density labelling, however, we have been unable to detect any synthesis of this enzyme during spherulation [21]. This proved our first interpretation to be wrong. In view of these results we have to assume that part of the action of the antibiotics actinomycin-C and cycloheximide in the plasmodia must be related to side effects of these drugs and not to the inhibition of RNA- and protein synthesis. The nature of these artifacts has yet to be elucidated in our system.

Similar observations on discrepancies between the observed influence of actinomycin-D and cycloheximide on enzyme activity and the actual rate of protein synthesis have been made in the mouse liver system [22—24] by Yagil and coworkers. They studied the effect of a change in the diet on the enzyme glucose-6-phosphate dehydrogenase and found an inhibition of the induction of this enzyme by both inhibitors. Using immunological methods, however, they could prove that the change in activity of the enzyme did not envolve the synthesis of a new enzyme protein, as was suggested by the inhibitor studies.

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