ACTION OF 5'-FLUORODEOXYURIDINE ON SYNCHRONOUS NUCLEAR DIVISION AND THYMIDYLATE SYNTHETASE ACTIVITY OF *PHYSARUM POLYCEPHALUM*

P. GRÖBNER and P. LOIDL

Institut für Medizinische Chemie und Biochemie der Universität, Fritz-Preglstr. 3, 6020 Innsbruck, Austria

Received 9 February 1982

1. Introduction

Macroplasmodia of the myxomycete *Physarum polycephalum* exhibit natural synchrony of nuclear division [1] and therefore serve as a model system for studies of mitotic cycle-dependent events. Two specific enzymes, thymidylate synthetase (EC 2.1.1.45) and thymidine kinase (EC 2.7.1.21) are responsible for the formation of dTMP, a precursor of DNA synthesis. Thymidine kinase has been extensively studied in *Physarum* in connection with the cell cycle-dependent enzyme activity pattern [2,3], the interconversion of enzyme variants [4,5] and the differentiation stages in the life cycle [6].

Attempts to detect thymidylate synthetase in *Physarum polycephalum* have thus far been unsuccessful, although data on thymidylate synthetase are clearly needed for studying dTMP synthesis [7]. Thymidine kinase-deficient mutants of *Physarum* (which do not show differences in growth under laboratory culture conditions as compared to wild-type) and FdUrd-experiments have made it necessary to determine the thymidylate synthetase activity in this organism [7,8]. FdUrd is intracellulary phosphorylated by thymidine kinase and FdUMP leads to a strong inhibition of thymidylate synthetase by formation of an enzyme · FdUMP · 5,10-CH_2-H_4folate · complex [9–13].

Here, we correlate the effect of FdUrd on the mitotic cycle with thymidylate synthetase activity, since we established an assay to measure this enzyme in *Physarum*. Mitosis is strongly delayed after application of FdUrd in *Physarum* wild-type M_3b but not in the thymidine kinase-deficient mutant TU63. This fact is reflected in thymidylate synthetase activity, which is strongly reduced in wild-type M_3b but not in the thymidine kinase-deficient mutant TU63. The delay of mitosis as well as the inhibition of thymidylate synthetase in FdUrd-treated plasmodia of wild-type is cancelled after application of exogenous dThd.

2. Methods

2.1. *Preparation of plasmodial extracts*

Plasmodial strains used were the Tromsö University mutant TU63 and the wild-type M_3b (Wis 1 isolate). Microplasmodia of both strains were maintained in submersed culture in semi-defined nutrient medium [14], supplemented with 0.013% hemoglobin instead of hematin. Macroplasmodia were prepared by fusion of microplasmodia [16]. The naturally synchronous nuclear divisions, which occur every 8–10 h in untreated macroplasmodia, were determined in ethanol-fixed smears under phase contrast. For investigations of FdUrd-effects in vivo and subsequent enzyme analysis FdUrd, Urd or dThd were added to the growth medium.

For determination of enzyme activities macroplasmodia were harvested 2 or 7 h after mitosis, frozen in dry ice together with 1 ml 0.1 M PGM-buffer (0.1 M sodium phosphate, pH 7.0 at 25°C, 10% (w/v) glycerol, 5 mM 2-mercaptoethanol) and stored at −28°C. Frozen samples were thawed, disrupted by sonication (MSE Ltd., Crawly, Sussex; low power, amplitude 1; 3 times each for 3 s at 0°C) and centrifuged for 30 min at 50 000 × *g* at 0°C. Thymidylate synthetase and thymidine kinase activities were determined in the supernatant. Protein analysis was done as in [15].

2.2. *Thymidine kinase assay*

This was as in [4] in extracts diluted 1:4 with

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

50 mM TGM-buffer (50 mM Tris–HCl, pH 7.5 at 25°C, 25% (w/v) glycerol, 5 mM 2-mercaptoethanol).

2.3. *Thymidylate synthetase assay*

Thymidylate synthetase was measured by a modification of the method in [17]. The assay is based on the release of ^3H as ^3H_2O, from dUMP ^3H-labelled in position 5 of the pyrimidine ring system, when dTMP is formed; the remaining substrate is absorbed onto charcoal and the supernatant fluid measured by liquid scintillation counting. After we had determined the K_m for dUMP (7×10^{-6} M) and 5,10-CH_2-H_4folate (2×10^{-4} M) as substrates and the pH-optimum (7.0) we established the following standard assay:

Solution (A): The stock solution of 4 mM H_4folate, 90 mM 2-mercaptoethanol, 40 mM formaldehyde, 20 mg bovine serum albumin/ml and 0.2 M sodium phosphate buffer (pH 7.0) was stored frozen and prepared freshly every 2 weeks.

Solution (B): Prepared fresh just prior to use, containing 0.208 mM d[5-^{3}H]UMP (385 mCi/mmol, 80 μCi/ml) and 8 mM NADPH.

Procedure: Aliquots (50 μl) of extracts were incubated at 25°C with 25 μl solution (A) and 25 μl solution (B), aliquots (25 μl) were pipetted into 50 μl 12.5% (w/v) trichloroacetic acid after 0, 30 and 60 min incubation to terminate the reaction. Charcoal suspension (175 μl, 100 mg/ml) was added and the vigorously mixed samples were left at 25°C for 15 min. Clear supernatants were obtained by centrifugation for 10 min at 10 000 × *g* and 100 μl were counted in 10 ml Instagel (Packard Instrument Co., Downers Grove IL) in a Packard Tricarb 2425 liquid scintillation spectrophotometer. Counts in blanks from which enzyme was omitted or 0 min-values ranged from 600–1000 cpm. Samples were assayed with sufficient amounts of extract to record 5000–50 000 cpm. Duplicate samples agreed within ± 5%.

3. Results and discussion

Now that it has become possible to measure thymidylate synthetase activity in *Physarum*, we have studied the effect of FdUrd + Urd (Urd is added to prevent incorporation of FUrd into RNA [18]) on the nuclear division cycle and on the dTMP synthesizing enzymes. The mechanism of FdUrd-induced inhibition has been described in *E. coli*: (1) FdUrd is phosphorylated by thymidine kinase; (2) FdUMP inactivates thymidylate synthetase [9].

In wild-type M_3b FdUrd + Urd induced a 400% delay of mitosis as compared to untreated control plasmodia (table 1). In the thymidine kinase-deficient mutant TU63, which has wild-type thymidylate synthetase activity, FdUrd can not be phosphorylated and therefore it could only slightly influence the length of the intermitotic time (130% as compared to the control cycle). These observed effects are in line with earlier findings [7].

The effects of FdUrd + Urd on the mitotic cycle are in strong correlation with the measured thymidylate synthetase activity (table 1). After the addition of FdUrd + Urd to wild-type M_3b plasmodia in vivo 1 h before mitosis 2, we found only 10% of the control thymidylate synthetase activity in plasmodial extracts harvested 2 h after mitosis 2. Nearly all activity was restored when the plasmodial extract was dialysed to remove FdUMP. However, after the addition of FdUrd in vitro to the thymidylate synthetase assay mixture of an untreated control extract, the measured thymidylate synthetase activity decreased to ~20% of the untreated extract; obviously this inhibition is caused by the phosphorylation of FdUrd by thymidine kinase (with ATP as phosphate donor). In the thymidine kinase-deficient mutant FdUrd could influence the thymidylate synthetase activity neither in vivo nor in vitro.

FdUMP forms a stable covalent thymidylate synthetase · FdUMP · CH_2-H_4folate · complex [9–13]. However, in Ehrlich Ascites tumor cells thymidylate synthetase, pre-exposed to FdUMP and CH_2-H_4folate, regained full activity after dialysis [19], which is in line with our results. In [10], dissociation of the complex of *L. casei* thymidylate synthetase was shown to be a first order process; the complex is only stable when the enzyme has been denatured, which supports the assumption that the bonds involved are not intrinsically labile, but the native enzyme itself seems to catalyze the breakdown of the complex to regenerate free thymidylate synthetase [10,12].

To further examine the stability of the thymidylate synthetase · FdUMP · cofactor · complex, we performed the following in vivo experiments in wild-type M_3b: After addition of FdUrd + Urd to macroplasmodia 1 h before mitosis 2 we have found residual enzyme activities of ~10% 2 h after mitosis 2 and of 20% 7 h

Table 1
Effect of FdUrd + Urd on measured thymidylate synthetase (TS) and thymidine kinase (TK) activities and on the length of the intermitotic period (M2–M3) of wild-type M_3b and TK-deficient mutant TU63

Strain	Treatment	Enzyme spec. act. (μunits/mg protein)				Intermitotic period (M2–M3) (h)
		TS		TK		
		M2 + 2 h	M2 + 7 h	M2 + 2 h	M2 + 7 h	
M_3b	In vivo					
	Control[a]	37.1	28.7	82.7	56.2	8.5
	FdUrd + Urd[b]	3.8	6.1	95.4	76.1	43.6
	FdUrd + Urd until M2 + 2 h, dThd was added at M2 + 2 h[c]	3.8	22.9	95.4	71.9	12.9
	FdUrd + Urd + dThd[d]	18.1	18.3	78.7	54.5	10.5
	In vitro					
	Control, FdUrd + Mg^{2+} + ATP were added to the TS-assay[e]	6.7	–	–	–	–
	FdUrd + Urd, TS-assay after dialysis[f]	23.5	–	76.3	–	–
TU63	In vivo					
	Control[a]	32.7	25.6	0.8	0.7	9.2
	FdUrd + Urd[b]	30.6	22.7	0.8	0.7	11.9
	In vitro					
	Control, FdUrd + Mg^{2+} + ATP were added to the TS-assay[e]	29.3	–	–	–	–
	FdUrd + Urd, TS-assay after dialysis[f]	24.7	–	0.6	–	–

[a] Untreated control
[b] Addition of FdUrd (20 μM) + Urd (300 μM) 1 h before M2
[c] Addition of FdUrd + Urd 1 h before M2 as in [b]; at M2 + 2 h plasmodia were transferred to fresh nutrient medium + dThd (200 μM)
[d] Addition of FdUrd (20 μM) + Urd (300 μM) + dThd (200 μM) 1 h before M2
[e] FdUrd (100 μM), $MgCl_2$ (1 mM) and ATP (10 mM) were added to the TS-assay of control extracts of [a] (harvested at M2 + 2 h) 0.5 h before TS was measured
[f] Extracts of [b] were dialysed for 5 h against 250 ml 0.1 M PGM-buffer at 4°C before TS-activity was determined

Whole macroplasmodia were harvested 2 h or 7 h after mitosis 2 (M2) and processed as in section 2

after mitosis 2 as compared to untreated control plasmodia (table 1). When the same plasmodium was transferred to fresh nutrient medium, to which dThd has been added to dilute intracellular FdUrd, 2 h after mitosis 2, nearly all enzyme activity as compared to the control plasmodium was restored 5 h later. This correlated well with the observed intermitotic periods of 500% and 150%, respectively, in relation to the control cycle.

When dThd was added together with FdUrd + Urd before mitosis, we observed only a partial inhibition of thymidylate synthetase activity, as we expected, since thymidine kinase can phosphorylate only a small portion of the applied FdUrd; in this case mitosis was only slightly retarded as shown in table 1.

The differences between thymidylate synthetase as well as thymidine kinase activity measured 2 h after mitosis 2 and 7 h after mitosis 2 in control plasmodia

is due to the periodic fluctuation of specific enzyme activities with a maximum of activity 2 h after mitosis [20].

We think that this direct interaction between inhibitor and thymidylate synthetase explains the effect of FdUrd on the nuclear division cycle of *Physarum*.

References

[1] Howard, F. L. (1932) Ann. Bot. 46, 461–478.
[2] Sachsenmaier, W. and Ives, D. H. (1965) Biochem. Z. 343, 399–406.
[3] Gröbner, P. and Sachsenmaier, W. (1976) FEBS Lett. 71, 181–184.
[4] Gröbner, P. (1979) J. Biochem. 86, 1595–1605.
[5] Gröbner, P. (1979) J. Biochem. 86, 1607–1614.
[6] Gröbner, P. and Mohberg, J. (1980) Exp. Cell Res. 126, 137–142.
[7] Mohberg, J., Dworzak, E. and Sachsenmaier, W. (1980) Exp. Cell Res. 126, 351–357.
[8] Sachsenmaier, W. and Rusch, H. P. (1964) Exp. Cell Res. 36, 124–133.
[9] Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R. and Lichtenstein, J. (1958) Proc. Natl. Acad. Sci. USA 1004–1012.
[10] Santi, D. V., McHenry, C. S. and Sommer, H. (1974) Biochemistry 13, 471–481.
[11] Danenberg, P. V., Langenbach, R. J. and Heidelberger, C. (1974) Biochemistry 13, 926–933.
[12] Danenberg, P. V. (1977) Biochim. Biophys. Acta 473, 73–92.
[13] Lockshin, A. and Danenberg, P. V. (1980) Biochemistry 19, 4244–4251.
[14] Daniel, J. W. and Baldwin, H. H. (1964) in: Methods in Cell Physiology (Prescott, D. M. ed) vol. 1, pp. 9–41, Academic Press, New York.
[15] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[16] Guttes, E., Guttes, S. and Rusch, H. P. (1961) Dev. Biol. 3, 588–614.
[17] Roberts, D. (1966) Biochemistry 5, 3546–3548.
[18] Birnie, G. D., Kröger, H. and Heidelberger, C. (1963) Biochemistry 2, 566–572.
[19] Reyes, P. and Heidelberger, C. (1965) Mol. Pharmacol. 1, 14–30.
[20] Gröbner, P. and Loidl, P. (1982) Biochim. Biophys. Acta in press.