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An isotopic assay of dUTPase activity based on coupling with thymidylate synthase[o]

Barbara Gołos and Wojciech Rode[✉]

Nencki Institute of Experimental Biology, Polish Academy of Sciences, L. Pasteura 3, 02-093 Warszawa, Poland

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A new rapid, sensitive and convenient procedure is presented allowing determination of dUTPase activity. With [5-^{3}H]dUTP used as the substrate, dUTPase, converts it to the corresponding monophosphate and is coupled with thymidylate synthase-catalyzed reaction, resulting in tritium release from [5-^{3}H]dUMP. Following charcoal absorption of the labeled nuleotides, radioactivity of tritiated water is determined. The new assay was tested to show comparable results with a previously described assay, based on measuring dUTPase-catalyzed [5-^{3}H]dUMP production.

dUTPase (deoxyuridine triphosphatase, deoxyuridine diphosphohydrolase, EC 3.6.1.23) catalyzes the hydrolysis of dUTP to dUMP and inorganic pyrophosphate. The reaction prevents frequent incorporation of dUTP, a good DNA polymerase substrate, into DNA. Its product, dUMP, is a substrate [1] for thymidylate synthase (EC 2.1.1.45), catalyzing $N^{5,10}$-methylenetetrahydrofolate-dependent methylation, leading to dTMP dihydrofolate formation [2].

Previously described assays of dUTPase activity were based on (i) determination of monophosphate produced from the released pyrophosphate in the inorganic pyrophosphatase-catalyzed reaction coupled with that of dUTPase [3], (ii) measurement of radioactively labeled reaction product (dUMP), separated by thin-layer chromatography [4], (iii) estimation of radioactively labeled reaction substrate (dUTP) consumption, with [5-^{3}H]dUTP separated from [5-^{3}H]dUMP on DE-81 filter discs (allowing elution of the monophosphate) [5] and (iv) spectrophotometric monitoring of dihydrofolate production (assumed to be equivalent to dUTPase-catalyzed dUMP formation) in the reaction catalyzed by thymidylate synthase, coupled with that of dUTPase [6]. All those assays are either time-consuming or suffer from limited sensitivity.

Looking for an assay combining sensitivity with speed, we took advantage of the thymidylate synthase reaction, coupled to that of

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[✉]Corresponding author: Wojciech Rode; Fax: (4822) 822 5342; e-mail address: rode@nencki.gov.pl

dUTPase, in order to monitor tritium released by thymidylate synthase from [5-^{3}H]dUMP, produced by dUTPase from [5-^{3}H]dUTP. Separation of tritium (in the form of tritiated water) was achieved by charcoal adsorption of the labeled nucleotides [7]. We present here a comparison of results obtained when dUTPase activity was determined in regenerating rat liver extracts either by measuring the reaction product, [5-^{3}H]dUMP, separated with the use of thin-layer chromatography or by monitoring tritium released from [5-^{3}H]dUTP, converted by dUTPase to [5-^{3}H]dUMP, in the coupled thymidylate synthase-catalyzed reaction.

MATERIALS AND METODS

Reagents. [5-^{3}H]dUTP (20 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, U.S.A.), dUTP sodium salt, dTTP sodium salt, dCTP sodium salt and (6*RS*)-tetrahydrofolic acid from Sigma (St. Louis, MO, U.S.A.), Cellulose F plates from Merck (Darmstadt, Germany) and Norit A from Serva (Heidelberg, Germany). Preparation of electrophoretically homogeneous regenerating rat liver thymidylate synthase was as reported earlier [8]. All other reagents were of analytical grade.

Preparation of regenerating rat liver dUTPase. Regenerating rat liver tissue, obtained as previously described [8], was homogenized in a Potter-Elvehjem homogenizer with 3–5 volumes of ice-cold 0.05 M phosphate buffer (pH 7.5), containing 0.1 M KCl and 0.01 M 2-mercaptoethanol. The homogenate was centrifuged at 20000 × *g* for 30 min at 4°C and the supernatant fractionated with streptomycin sulfate and ammonium sulfate as described earlier [9]. The 70% ammonium sulfate precipitate was dissolved in 0.01 M phosphate buffer (pH 7.5) containing 0.01 M 2-mercaptoethanol, 0.02 M dUMP and 0.1% Triton X-100. The resulting solution was passed through an affinity column (equilibrated with the same buffer), containing 10-formyl-5,8-dideazafolate, capable of specific dUMP-dependent thymidylate synthase binding [10]. The effluent from the column, further referred to as dUTPase, containing no detectable thymidylate synthase activity, was frozen and could be stored at –20°C for years.

dUTPase assay monitoring [5-^{3}H]dUMP production. The reaction mixture in a total volume of 40 μl contained 50 mM Tris/HCl buffer (pH 7.5), 6.25 mM Na/K phosphate buffer (pH 7.5), 6.25 mM NaCl, 1 mM $MgCl_2$, 5 mM mercaptoethanol, 50 μM [5-^{3}H]dUTP (50 nCi/sample) and the dUTPase preparation corresponding to 5–50 μg protein. Samples were incubated for 20 min at 37°C. The reaction was stopped by addition of 5 μl 72% trichloroacetic acid. After 30 min, samples were centrifuged, and 30 μl sample of each supernatant was neutralized with 4 μl 5 M NaOH. Following addition of 6 μl of a solution containing 5 mM dUMP and 5 mM dUTP (nonradioactive carriers), a 20 μl sample was developed on a Cellulose F plate with a mixture of n-butanol/aceton/85% acetic acid/ 5% $NH4OH/H_2O$ (3.5:2.5:1.5:1.5:1, by vol.). dUMP and dUTP spots were localized under UV lamp. They were scraped off, mixed with a toluene-based scintillator and radioactivity was determined in a liquid scintillation counter allowing quenching callibration.

dUTPase assay monitoring thymidylate synthase-dependent tritium release. The reaction mixture in a total volume of 70 μl contained 50 mM Tris/HCl buffer (pH 7.5), 30 mM Na/K phosphate buffer (pH 7.5), 3.5 mM NaCl, 1 mM $MgCl_2$, 30 μM [5-^{3}H]dUTP (50 nCi/sample), 60 μM cold dTTP or dCTP (added in order to decrease the influence of a contaminating non-specific dNTP-hydrolyzing activity), 1 mM (6*RS*)-tetrahydrofolate, 2 mM formaldehyde, 3% saccharose and 10 mM mercaptoethanol. After 10 min preincubation at 37°C with homogeneous thymidylate synthase preparation (minimum activity of 0.2 nmol/min), the reaction was started by addition of the dUTPase preparation containing

5–50 μg protein (maximum dUTPase activity 5 pmol/min) and terminated, after 20 (unless otherwise indicated) min at 37°C, by addition of 200 μl of charcoal suspension (Norit A, 100 mg/ml) in 2% trichloroactic acid. The mixture was centrifuged at 10000 × ***g*** for 5 min, 100 μl sample of each supernatant added to a dioxane-based scintillator and counted in a liquid scintillation counter. Control samples were incubated with thymidylate synthase for 30 min and the dUTPase preparation was added after addition of charcoal suspension.

Protein contents. The procedure of Spector [11] was used, with bovine serum albumin as a standard.

Statistically evaluated results. These are presented as mean ± S.E.M. (N = number of experiments).

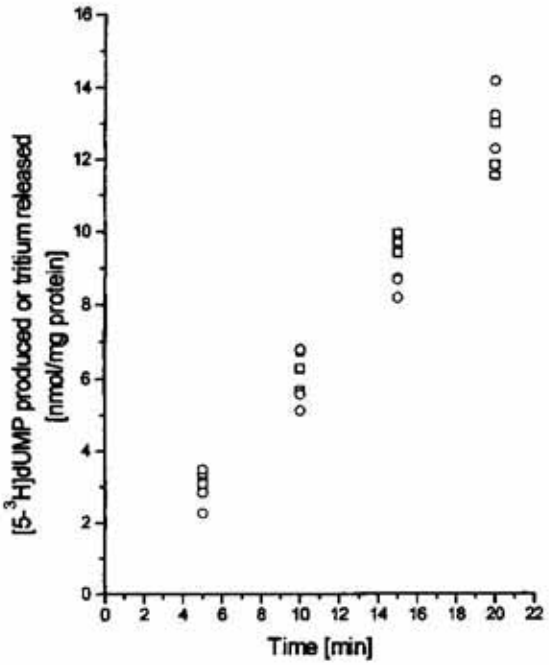

Figure 1. Dependencies of dUTPase activity assayed by measuring [5-^{3}H]dUMP production (squares) or monitoring thymidylate synthase-dependent tritium release (circles) on time.

RESULTS

The specific activity of the dUTPase preparations was 37.6 ± 0.5 nmol/h per mg protein (n = 4) and 40.0 ± 1.3 nmol/h per mg protein (n = 4) when determined by the assays monitoring [5-^{3}H]dUMP production and thymidylate synthase-dependent tritium release, respectively. The depedence of activity estimated with both assays on the reaction time was similar (Fig. 1). Thus, under conditions of the new assay the dUTPase reaction was apparently tightly coupled with the thymidylate synthase reaction.

Under the assay conditions (Materials and Methods) the release of tritium was proportional to time and protein content of the dUTPase preparation (not shown). The same was always true when consumption of the substrate in the course of the reaction was not higher than 5% and total thymidylate synthase activity in a sample was minimum 20-fold higher (the presence of higher activity did not influence the results of the assay) than dUTPase activity (not shown).

Small amounts of a non-specific dNTP-hydrolyzing activity were detected in the dUTPase preparations. Addition of equimolar with dUTP or higher concentrations of dTTP or dCTP (apparently competing with dUTP for the activity) to the reaction mixture increased measured dUTPase specific activity by some 10% (with the tritium release monitoring assay, addition of equimolar concentration of dTTP and dCTP resulted in the determined activity increase from 34.1 nmol/h per mg protein without additions to 40.0 nmol/h per mg protein and 36.2 nmol/h per mg protein, respectively).

DISCUSSION

A rapid, sensitive and simple procedure is presented allowing determination of dUTPase activity. It does not suffer from interference of inorganic pyrophosphatase whereas interference of non-specific dNTP-hydrolyzing activity may be overcome by addition of a pyrimidine deoxynucleoside triphosphate.

The assay is also very sensitive, at least 10^2-fold more sensitive than the corresponding spectrophotometric method [6], allowing determination of dUTPase activity in the range of 1–10 pmol/min. A limitation of the new assay is requirement for highly purified thymidylate synthase. The commercially available preparation of the enzyme from methotrexate-resistant *Lactobacillus casei* (New England Enzyme Center, Tufts University, Boston, MA, U.S.A.) was found to contain dUTPase activity.

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