Short Communication

Thymidylate Synthase Activity from Chlamydomonas Cells and Cultured Tissues of Nicotiana, Pinus, and Daucus

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ABSTRACT

Prior preparation of thymidylate synthase from Chlamydomonas reinhardtii resulted in an improved assay for thymidylate synthase. Nevertheless, preparations from tobacco seedlings and cotton root tips (0.25 centimeter) were inconsistent with respect to enzyme activity, extracts from actively growing cell cultures of Chlamydomonas, Nicotiana hybrid callus, Pinus callus, and Daucus proembryonic cells contained significant levels of thymidylate synthase (12.3 to 23.8 nanomoles of thymidylate formed per milligram of protein per hour).

We report here a modified and more sensitive assay system for thymidylate synthase and its preliminary application using seedling, plant tissue culture, and cell extracts.

MATERIALS AND METHODS

Cell and Tissue Cultures. The minus strain of Chlamydomonas reinhardtii Dengeard (Indiana culture no. 89) was synchronously grown (13). Nicotiana callus was derived from a cross of N. glauca Graham × N. langsdorffii Weimann grown in nipple flasks on Murashige and Skoog basal medium (18) with 2.0 µg/g 2,4-D and 0.25 µg/g kinetin. Pine (Pinus taeda L.) callus was cultured on agar containing Brown and Lawrence medium (4) supplemented with 0.05 µg/g kinetin and 0.5 µg/g 2,4-D. Carrot (Daucus carota L.) tissue was derived from hypocotyl sections which had been excised from seedlings and minced. The small white proembryonic globules (22) were grown in nipple flasks with 2.0 µg/g naphthalamide acetate and 1% coconut milk (18).

Extract Preparation. Chlamydomonas cells were harvested by centrifugation, washed twice with 50 mM Tris-HCl (pH 7.4) containing 1.0 mM DTT and 0.1 mM EDTA, and then ruptured in 10 to 20 ml of the same medium by at least two successive passages through a French pressure cell. Extracts were clarified by centrifugation at 10,000g for 1 h, and the resulting supernatant fractions were served as crude enzyme sources.

All tissue culture samples were homogenized in Tris buffer (10 ml/g fresh weight) using a Potter-Elvehjem tissue grinder after initial disruption with a mortar and pestle. The extracts were centrifuged as before and the supernatant solutions were used as enzyme sources.

Preparation of N14,N15-Methylenetetrahydrofolate. During the course of preliminary experimentation, we were unable to determine with consistency the presence of thymidylate synthase in plant extracts even though we employed a number of assay approaches (14, 23–25) that have apparently proved adequate for microbial and animal studies. For the most part, such assays rely on the spontaneous formation (2, 23) of N14,N15-methylenetetrahydrofolate from formaldehyde and tetrahydrofolate during the course of incubation with enzyme. Thus, it was previously assumed that the formation of N14,N15-methylenetetrahydrofolate was not rate-limiting relative to TMP synthesis.

We were successful in assaying for thymidylate synthase only if the N14,N15-methylenetetrahydrofolate solution was carefully prepared in advance, under nitrogen, according to the following steps (modified from a procedure suggested by R. J. Harvey, personal communication). The DL-tetrahydrofolate (final concentration of 10 mM) was added to a 50 mM 2-mercaptoethanol solution made with boiled, deionized water. KOH (10 mM) was added to help solubilize the tetrahydrofolate as well as to adjust the pH to between 5.0 and 6.0 (12). After the addition of [14C]formaldehyde (to yield 1.0 µCi/assay) and unlabeled formaldehyde (10 mM final...
concentration), the resulting mixture was stored under N2 at 0 to 5°C for 36 h prior to use. Although 2-mercaptoethanol is potentially inhibitory to thymidylate synthase activity, it is required for stabilization of Dl-tetrahydrofolic acid (12). The efficacy of each batch was tested by NADPH, NADP, and NAD in methylating dUMP using crude enzyme extracts from log phase Escherichia coli (K-12mr) cells, which had been prepared as described above for Chlamydomonas. Introduction of 02 (20) during the incubation period invariably led to altered spectral characteristics (12, 23) and nominal TMP formation. Batches of NADPH, NADP, and NAD in methylating dUMP using crude enzyme extracts from log phase E. coli cells and did not detect any significant differences among the various sources.

Thymidylate Synthase Assay. This assay was a radiometric adaptation of Witschi's spectrophotometric method (25). The reaction mixture consisted of: 5 μmol Tris-HCl (pH 7.4) containing 20 μmol 2-mercaptoethanol; 3 μmol dUMP; 1.0 μmol (100 μl) of the [14C]NADPH, NADP, and NAD in methylating dUMP solution (1 μCi/μmol) described above; crude enzyme extract and, if necessary, enough deionized water to give a final assay volume of 50 μl.

The dUMP was omitted from control tubes; all reactions were incubated for 30 min at 22°C before termination by plunging the tubes in a boiling water bath for 5 min. After cooling, 1 ml of 10 μM Tris-HCl (pH 8.5) containing 30 μM MgCl2 and 0.5 mg of Crotilus adamantus venom was added to each tube to dephosphorylate TMP (25). Tubes were further incubated for 30 min at 37°C before the addition of 0.2 ml of 10 mM formaldehyde. After blending vigorously on a Vortex mixer, 0.4 ml of freshly prepared 40 mM dimeredone (5,5-dimethyl-1,3-cyclohexanediene) in 50% ethanol was added to each tube. The tubes were heated in a boiling water bath with intermittent shaking and then centrifuged at 12,000 g for 5 min. In order to separate the released thymidine from nucleotides, 1 ml of supernatant solution from each tube was placed on a double resin column composed of a top layer (2.5 x 0.8 cm) of Dowex 50 (H+ form, 200-400 mesh) and a bottom layer (2.5 x 0.8 cm) of Dowex 1-formate form, 200-400 mesh.

Columns were eluted with deionized H2O until 15 ml of eluate had been collected in five equal fractions. Radioactivity in a 1.0-ml aliquot from each fraction was determined after mixing with 10 ml of a modified Triton scintillation cocktail (1,998 ml toluene: 999 ml Triton X-100: 16.5 g PPO: 450 mg dimethyl POPPOP). Counts from control fractions (minus dUMP but otherwise identical and subjected to the foregoing postassay protocol) were subtracted from counts from respective complete fractions before calculating the nmol of TMP formed.

Protein Content. Protein was determined according to Lowry et al. (15). The levels of enzyme activities reported here were the means from between three and six separate determinations.

RESULTS AND DISCUSSION

In preliminary experiments we screened for thymidylate synthase activity in extracts from 96-h-germinated tobacco seedlings and the first 0.25 cm of root tips from 2- to 12-day-germinated cotton seedlings. At times there was apparent thymidylate synthase activity in such extracts but the majority of assays were negative. Previous findings in our laboratory (Birnbaum, unpublished data) also suggested that germinating tobacco seedlings contained thymidylate synthase activity. However, the specific activity was low, difficult to demonstrate repeatedly, and the radiosotope (originally as [14C]formaldehyde) could be detected in thymidine but not in TMP. Tobacco seedling extracts, inactive with regard to thymidylate synthase activity, were added to assay mixtures containing crude enzyme from E. coli cells but had no effect on the amount of TMP formed by the latter enzyme source. Thus, the inconsistent activity in tobacco seedlings was apparently not due to the presence of specific inhibitors of thymidylate synthase activity. The enzyme from seedlings may be labile in vitro (7) or unduly diluted by nondividing tissues during extraction (14).

As a result of these preliminary experiments, selected cell and tissue cultures were surveyed for thymidylate synthase activity under the assumption that repeatedly active extracts had to be prepared from materials containing a higher proportion of proliferating cells than would be present in tobacco seedlings or even cotton root apices. Results are shown in Table I. Extracts from synchronous cultures of Chlamydomonas cells, Daucus proembryonic tissue, and Pisum cells contained nearly the same level of thymidylate synthase (12.3-14.1 nmol TMP formed/mg protein-h). All of the tissue extracts were made from friable, actively growing cultures. Older cultures (less friable, variable but obvious "browning," reduced growth rates) were found to be totally devoid of extractable thymidylate synthase activity. The latter finding is consistent with results reported by Lorenson et al. (14) for nondividing chick embryo tissues. The levels of thymidylate synthase that we found (Table I) were as much as 2- to 4-fold greater than reports (in nmol TMP formed/mg protein-h) for pig thymus (6.2 nmol) (9), 7-day chick embryos (5.6 nmol) (14), and calf thymus (4.8 nmol) (11). However, meaningful comparison is difficult because of the differences in assaying for enzyme activity.

The phosphorylated derivative of 5-FUdR, 5-fluorodeoxyuridine-5'-monophosphate, is a well known, potent inhibitor of thymidylate synthase (8). Thymidylate synthase activity in Chlamydomonas extracts was reduced by 95% on a per cell basis when algal cells were cultured in the presence of 100 μM 5-FUdR for 48 h (data not shown). When the data were expressed on a protein basis, thymidylate synthase levels were reduced 87% by 5-FUdR treatment (25.7 and 3.3 nmol TMP formed/mg protein-h for control and 5-FUdR-treated cells, respectively).

Our findings of thymidylate synthase activities in selected cell and tissue culture extracts should allow future studies to focus on the purification and characterization of plant thymidylate synthase from such systems.

Table 1. Thymidylate Synthase Activity in Cell and Tissue Extracts

<table>
<thead>
<tr>
<th>Extract Source</th>
<th>Thymidylate Synthase Activity (nmol TMP formed/mg protein-h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydomonas</td>
<td>12.3</td>
</tr>
<tr>
<td>Daucus</td>
<td>13.7</td>
</tr>
<tr>
<td>Nicotiana</td>
<td>23.8</td>
</tr>
<tr>
<td>Pisum</td>
<td>14.1</td>
</tr>
</tbody>
</table>

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LITERATURE CITED


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22. Steward FC, Israel HW, Mott RL 1974 The isolation and culture of free cells from higher plants. Methods Enzymol 32: 723-732