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ISOLATION OF A HOMOGENEOUS PREPARATION OF HUMAN THYMIDYLATE SYNTHETASE FROM HeLa CELLS

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Abstract—Thymidylate synthetase from HeLa cells was purified to electrophoretical homogenity as a result of affinity chromatography on a 10-formyl-5,8-dideazafolate-ethyl-Sepharose column. Electrophoretical analysis of the formation of the enzyme–5-fluorodeoxyuridylate–5,10-methylenetetrahydrofolate complex shows the presence of two binding sites for 5-fluorodeoxyuridylate on the enzyme molecule. The molecular weight of the enzyme subunit was 36,000. The apparent Michaelis constants for dUMP and (±)-L-5,10-methylenetetrahydrofolate were 2.0 and 31 μM, respectively. The enzyme exhibited a temperature-dependent conformational change with a transitional temperature of 35°. Activation energies above and below this temperature were 8.1 and 20.3 kcal/mole, respectively.

Thymidylate synthetase (methylenetetrahydrofolate: deoxyuridine-5′-monophosphate C-methyltransferase; EC 2.1.1.45) catalyses the conversion of deoxyuridylate to thymidylate [1] and is the only source of thymidine nucleotides synthesized *de novo* in a cell. Since this enzyme is one of the targets of cancer chemotherapy [2], properties of human tumor thymidylate synthetase(s) are of particular interest. However, studies on mammalian thymidylate synthetase(s) have been hampered by its relatively low concentration in tissues and its instability. A recently described affinity chromatography system, based on dUMP-dependent binding of the enzyme to an immobilized analog of 5,10-methylenetetrahydrofolate, tetrahydroamethopterin [3, 4], enabled purification of thymidylate synthetase from poor sources with yields sufficient to allow further studies. Application of the same system for the purification of the enzyme from cultured CCRF-CEM human lymphoblastic leukemia cells resulted in a nearly homogeneous preparation [5]. Since tetrahydroamethopterin columns are unstable, an attempt was made to use amethopterin immobilized on aminohexyl-Sepharose for the purification of thymidylate synthetase from blast cells of patients with acute myelocytic leukemia [6]. Chromatography on this adsorbent, involving both biospecific and hydrophobic interactions, allowed for high purification of the enzyme from its prepurified preparation. Due to the small amount of the final enzyme preparation obtained, no criterion of purity could be applied.

We report here the successful isolation and some properties of homogeneous thymidylate synthetase from HeLa cells. The purification procedure takes advantage of the dUMP-dependent adsorption of thymidylate synthetase on a stable affinity adsorbent, 10-formyl-5,8-dideazafolate [7], coupled to Sepharose via ethylenediamine (Fig. 1).

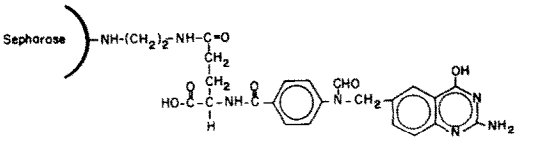

Fig. 1. Structure of the affinity adsorbent, 10-formyl-5,8-dideazafolate ethyl-Sepharose.

MATERIALS AND METHODS

Cell culture. HeLa cells were routinely maintained in Joklik's medium containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY). Cells were harvested in the logarithmic phase of culture growth by centrifugation at 600 *g* for 5 min, washed once with cold saline solution, and stored as a pellet at −20° until use.

Enzyme assay. A modification of the isotopic method of Roberts [8] was used. The standard reaction mixture in a total volume of 40 μl contained: 2.0 nmoles of [5-^{3}H]dUMP (*ca.* 5 × 10^7 cpm/μmole), 18 nmole (±)-L-tetrahydrofolate, 36 nmoles formaldehyde, 0.4 μmole 2-mercaptoethanol, 2 μmoles NaF, 2 μmoles phosphate buffer, pH 7.5 (unless otherwise indicated), and the enzyme (≤0.2 unit). In controls the enzyme was substituted with buffer. The reaction was started by addition of the enzyme and was terminated after a 1-hr incubation at 37° by addition of 200 μl of a suspension of charcoal (Norit, 100 mg/ml) in 2% trichloroacetic acid (unless otherwise indicated). After centrifugation, 100 μl of the supernatant fluid were added to 3 ml of aqueous counting scintillant (Amersham/Searle Co., Arlington Heights, IL) and counted in a liquid scintillation counter. Thymidylate synthetase activity is expressed in units defined as the amount required to release 1 nmole of tritium per hr under conditions of the assay.

Affinity adsorbent. 10-formyl-5,8-dideazafolate

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(supplied by Dr. John Hynes of the Medical University of South Carolina, Charleston) was coupled to aminoethyl-Sepharose by a procedure similar to that described for tetrahydroamethopterin [3], except that no hydrogen atmosphere was necessary.

Ammonium sulphate fraction of HeLa cell extract. The cell pellets were thawed with 3 vol. of 0.01 M phosphate buffer, pH 7.5, containing 0.1 M KCl and 0.01 M 2-mercaptoethanol, sonicated (Bronwill 'Biosonic' 5 × 10 sec at a setting of 50) and centrifuged at 20,000 *g* for 30 min at 4°. The supernatant fluid was fractionated with ammonium sulphate to obtain a fraction precipitated between 30 and 70% saturation, which was then dissolved in several volumes of 0.01 M phosphate buffer, pH 7.5, containing 0.01 M 2-mercaptoethanol and 0.1% Triton X-100 (buffer A). To the solution dUMP was added to a final concentration of 20 μM.

Affinity chromatography. Isolation of thymidylate synthetase starting with a 30–70% ammonium sulphate fraction of the cellular extract was performed at 4° and involved a sequence of two affinity chromatography steps followed by concentration of the enzyme on DEAE-cellulose. The 30–70% ammonium sulfate fraction (corresponding to about 10,000 units of enzyme activity) was dissolved in buffer A containing 20 μM dUMP and was passed slowly (*ca.* 0.5 ml/min) through a 10-formyl-5,8-dideazafolate-ethyl-Sepharose column (2.5 × 7 cm) previously saturated with the same buffer. The column was then washed with 3000 ml of 0.2 M phosphate buffer, pH 7.5, containing 0.01 M 2-mercaptoethanol, 0.1% Triton X-100 (buffer B) and 20 μM dUMP, and the enzyme was eluted with buffer B without dUMP. To the pooled fractions, containing thymidylate synthetase activity, 20 μM dUMP was added and affinity chromatography repeated as described above. The pooled active fractions resulting from the second affinity chromatography were diluted with 0.005 M phosphate buffer containing 0.01 M 2-mercaptoethanol and 0.1% Triton X-100 so as to reach a final phosphate concentration lower than 0.05 M and passed through a DEAE-cellulose column (1.5 × 5 cm) equilibrated with buffer A. The enzyme, adsorbed on DEAE-cellulose, was eluted with buffer B containing 20% sucrose.

Electrophoretical analysis. The thymidylate synthetase preparations were tested for homogeneity by polyacrylamide gel electrophoresis at 4° in a system similar to that described by Davis [9] except that separation gels (7.5%) contained 0.1% Triton X-100 and spacer gels were absent. Gels were stained for protein as described by Weber and Osborn [10].

The molecular weights of denatured proteins were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS), as described by Weber and Osborn [10]. Phosphorylase B (mol. wt = 94,000), bovine serum albumin (mol. wt = 68,000), ovalbumin (mol. wt = 43,000), carbonic anhydrase (mol. wt = 23,000), soybean trypsin inhibitor (mol. wt = 21,000) and lysozyme (mol. wt = 14,300) were used as molecular weight standards.

Protein determination. The procedure of Sedmak and Grossberg [11] was used, with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The high biological specificity of the chromatographic system used results from the fact that thymidylate synthetase binds to immobilized 10-formyl-5,8-dideazafolate, a potent inhibitor of this enzyme, similarly as to immobilized tetrahydroamethopterin [3–5], only in the presence of dUMP. The enzyme is then eluted solely due to the absence of dUMP in the buffer (see Material and Methods). In order to achieve such a biospecificity for isolation of HeLa thymidylate synthetase, a high concentration (≥0.2 M) of the eluting buffer was necessary, since in a low ionic strength buffer, binding of the enzyme to the affinity column was not entirely dUMP-dependent, probably due to hydrophobic interactions [3].

Results of the affinity chromatography of the 30–70% ammonium sulfate fraction of the cell extract are presented in Table 1. A single affinity chromatography step did not result in a homogeneous preparation of the enzyme. Final purification, with 80 per cent yield, was achieved by repeating the same procedure (see Material and Methods). Since thymidylate synthetase was eluted from the affinity column in a rather large buffer volume (Table 1), the

Table 1. Purification of thymidylate synthetase from HeLa cells

Purification stage	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification	Yield (%)
30–70% $(NH_4)_2SO_4$ fraction	86.5	8440	6400	1.3	1	100
Pooled fractions after first affinity chromatography	674	6300	10.2	620	477	75
Pooled fractions after second affinity chromatography	790	6850	0.36*	19,100*	14,692	81

* Assayed after concentration of the pooled fractions on DEAE-cellulose column.

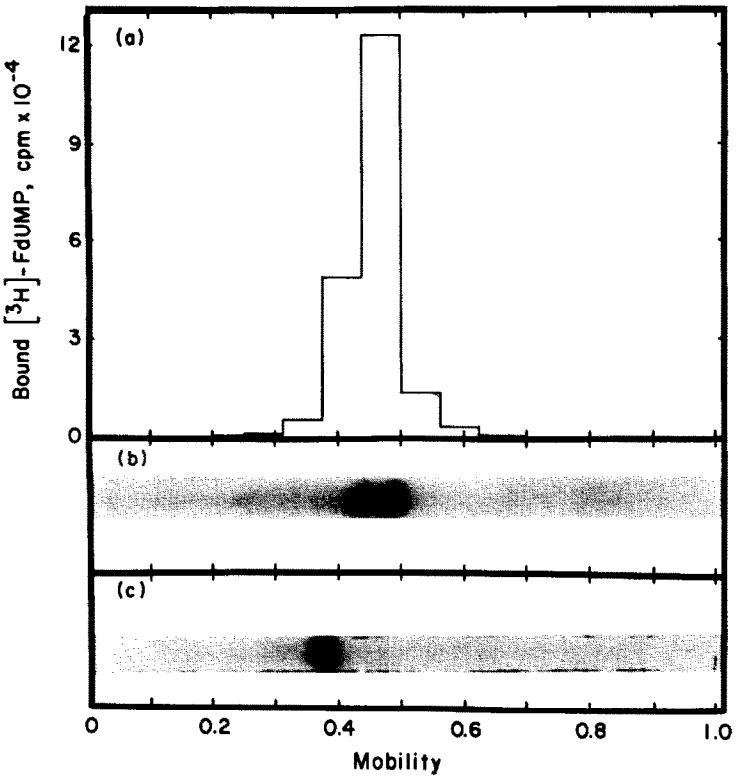

Fig. 2. Polyacrylamide gel electrophoresis of HeLa thymidylate synthetase after the second affinity chromatography (see Table 1). Each sample applied to a gel contained 5 μg protein. The samples were treated for 30 min at 37° with 0.5 μM [^{3}H]-5-fluorodeoxyuridylate and 100 μM 5,10-methylenetrahydrofolate before electrophoroesis. Gels were either sliced and assayed for bound label (a) or stained for protein (b). (c) Native purified enzyme.

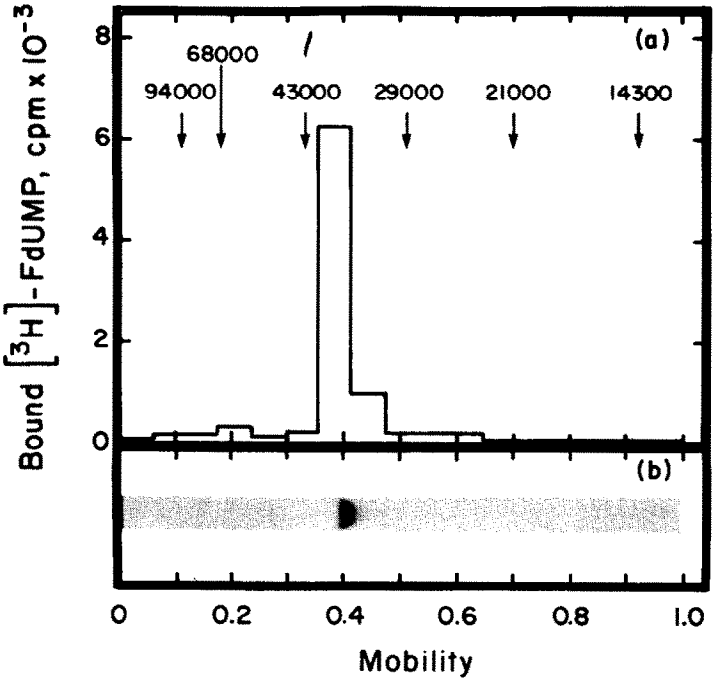

Fig. 3. SDS polyacrylamide gel electrophoresis of HeLa thymidylate synthetase after a second affinity chromatography (see Table 1). Before electrophoresis, samples (5 μg protein/sample) were treated for 30 min at 37° with 0.5 μM [^{3}H]-5-fluorodeoxyuridylate and 100 μM 5,10-methylenetetrahydrofolate and precipitated with trichloroacetic acid in the presence of deoxycholate [16]. The precipitates were dissolved in 0.1 ml of 0.01 M phosphate buffer, pH 7.0, containing 6 M urea, and, after addition of 5 μl of 14.4 M 2-mercaptoethanol, 3 μl of 0.05% bromphenol blue and 2 drops of glycerol, immersed for 5 min in a boiling water bath. (a) Bound [^{3}H]-5-fluorodeoxyuridylate was assayed in the sliced gel. Arrows indicate positions of molecular weight standards (see Materials and Methods). (b) Stained gel.

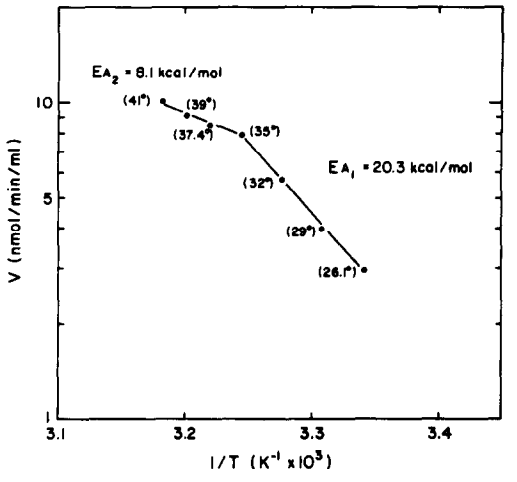

Fig. 4. Arrhenius plot. Samples were assayed at each of two enzyme concentrations (9.0 and 4.5 units in 0.04 ml) in duplicate for 5 and 10 min at the temperatures indicated. Assays were performed as described, except that the reaction mixtures contained 0.2 M Tris–HCl, pH 7.5, in place of phosphate and were 10% (v/v) in sucrose.

enzyme was concentrated before further analysis. The use of a small DEAE-cellulose column (see Materials and Methods) obviated the concomitant concentration of Triton X-100 present in all affinity chromatography buffers to stabilize the enzyme [12]. Lowering of the buffer ionic strength of the thymidylate synthetase preparation, for adsorption of the enzyme to DEAE-cellulose, had to be achieved by dilution rather than dialysis, since the latter procedure resulted in substantial loss of enzyme activity.

Concentrated HeLa thymidylate synthetase, obtained after a second affinity chromatography step, was found to be electrophoretically homogeneous (Fig. 2c). Based upon the ability of the system to detect impurities of 0.1 μg, we estimate the preparation to be greater than 98 per cent pure. The purified enzyme was stable for several weeks at −20°, and no loss of activity was observed at this temperature. Its specific activity, 19.1 μmoles/hr/mg protein (Table 1), is similar to that of pure L1210 (W. Rode *et al.*, unpublished results), Ehrlich ascites [13] and calf thymus [14] thymidylate synthetases (23.0, 28.2 and 21 μmoles/hr/mg protein respectively). On the other hand, it is much higher than the minimum specific activity of the enzyme from human leukemic blast cells (1.1 μmoles/hr/mg protein) [6] and substantially lower than the specific activity of CCRF-CEM enzyme (228 μmoles/hr/mg protein) [5]. Native HeLa thymidylate synthetase, similar to the *Lactobacillus casei* [15] and L1210 [12] enzymes, formed with 5-fluorodeoxyuridylate (FdUMP) and 5,10-methylenetetrahydrofolate two types of electrophoretically separable ternary complexes (Fig. 2a, b), suggesting the presence of two binding sites for FdUMP on the enzyme. The molecular weight of the enzyme subunit, estimated by SDS polyacrylamide gel electrophoresis, was 36,000 (Fig. 3), somewhat higher than that of CCRF-CEM enzyme (33,000) [5]. Traces of protein at the position corresponding to a molecular weight of 73,000 on the gel (Fig. 3) indicated that the native enzyme is composed of two equivalent-sized subunits. A quantitative comparison of FdUMP, bound to the enzyme after electrophoresis in the native or denatured state (Figs. 2a and 3a), reveals that the denatured complex of thymidylate synthetase with FdUMP and 5,10-methylenetetrahydrofolate dissociated after urea treatment, SDS electrophoresis, or both.

Initial velocity studies of the HeLa enzyme reaction showed apparent Michaelis constants for dUMP and (±)-L-5,10-methylenetetrahydrofolate to be 2.0 and 31 μM, respectively (not shown). An Arrhenius plot exhibited a biphasic curve with a transitional temperature at 35°, indicating a temperature-dependent conformational change (Fig. 4). Activation energies above and below 35° were 8.1 and 20.3 kcal/mole, respectively. Purified thymidylate synthetase from HeLa cells thus displays a biphasic Arrhenius plot with activation energies similar to the enzyme purified from human leukemia blast cells [6].

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