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Purification of mouse liver thymidylate synthetase by affinity chromatography using 10-methyl-5,8-dideazafolate as the affinant[1]

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Summary. Thymidylate synthetase (methylenetetrahydrofolate: 2′-deoxyuridine-5′-monophosphate C-methyltransferase; EC 2.1.1.45) from neonatal mouse liver has been purified 714-fold by affinity chromatography on aminohexylsepharose bound 10-methyl-5,8-dideazafolate.

Thymidylate synthetase catalyzes the 5,10-methylenetetrahydrofolate-dependent conversion of deoxyuridinemonophosphate (dUMP) to thymidylate. The properties of this enzyme in mammalian systems are of particular interest because the reaction catalyzed is the lone de novo source of the thymidylate required for DNA synthesis. Therefore, the enzyme is the target for a number of cancer chemotherapeutic agents and has been the subject of extensive investigations[2].

Attempts to purify the enzyme from various sources by conventional means have been hampered by the innate instability and very low concentrations of the enzyme present in most tissues. However, limited amounts of purified enzyme have been secured using traditional multistep procedures[3–15]. Recently, affinity chromatography has been successfully utilized for the purification of thymidylate synthetase from several sources. The enzyme from *Lactobacillus casei* has been purified to apparent homogeneity with 2′-deoxyuridylate[16], 5-fluoro-2′-deoxyuridylate (FdUMP)[17], and N^{α}-[pteroyltetra-(γ-glutamyl)]-lysine[18] as affinity ligands. Successful application of these affinity matrices to the purification of the mammalian enzyme has not been reported. Affinity columns based on dUMP-dependent binding to immobilized tetrahydromethotrexate have been used to purify both the bacterial and the mammalian enzyme[19,20]. The major difficulty with this biospecific adsorbent is the instability of the reduced folate analog. The adsorbent cannot be stored for long periods and columns are typically used only once. Dolnick and Cheng have avoided this problem by using methotrexate as the affinity ligand[21]. Although the purification achieved on the methotrexate column appeared to involve both specific and hydrophobic interactions, the utilization of a stable affinant represents a significant advantage over previously reported methods.

Quinazoline analogs of folic acid are quite stable and have been found to be exceptionally effective inhibitors of thymidylate synthetase from a number of sources.[22–25]. Recently, the successful application of 10-formyl-5,8-dideazafolate-aminoethyl-sepharose to the purification of thymidylate synthetase from L1210 leukemia cells has been reported[26]. We report here the immobilization of a somewhat more potent inhibitor of both the L1210 enzyme ($I_{50} = 0.5 \times 10^{-7}$ M) and the enzyme from *L. casei* ($I_{50} = 4 \times 10^{-7}$ M)[25] and its application to the purification of thymidylate synthetase from neonatal mouse liver. Binding to the 10-methyl-5,8-dideazafolate column is dUMP-dependent and presumably is the result of reversible ternary complex formation.

Materials and methods. [6-^{3}H] FdUMP (17 Ci/mmole) and [5-^{3}H] dUMP (18 Ci/mmole) were purchased from Moravek Biochemicals. Tetrahydrofolate was prepared according to the method of Davis[27] and converted to the methylene derivative by addition of formaldehyde for storage at −70 °C. The 10-methyl-5,8-dideazafolate was synthesized as previously described[28] and was coupled to the amine group of a spacer on Sepharose 4B (Pharmacia) using 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide as

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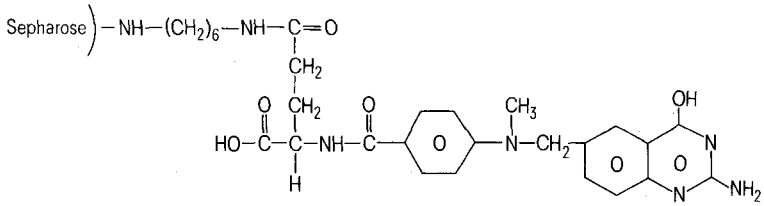
Purification stage	Protein (mg)	Volume (ml)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
Crude homogenate	1450	98	1.4×10^{-5}	1.0	100
Streptomycin sulfate (1%)	1040	103	2.2×10^{-5}	1.6	100
Ammonium sulfate (30–55%)	365	9.5	3.4×10^{-5}	2.4	59
DEAE-cellulose	75.6	27	1.9×10^{-4}	13.4	34
Affinity column	0.56	8	1.0×10^{-2}	714	20



Structure of 5,8-dideaza-10-methylfolate-aminohexyl-sepharose.

previously described for folate[29]. All other chemicals and materials were purchased from Sigma with the exception of DEAE-cellulose (DE-52, Whatman).

Livers from neonatal C57BL/6M mice were homogenized in 5 vol. of 0.05 M Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM 2-mercaptoethanol and 25% sucrose and centrifuged at 29,000 × g for 40 min. All procedures were carried out at 4 °C. The crude homogenate was subjected to streptomycin sulfate (1%) and ammonium sulfate (30–55% saturation) fractionation prior to application to a 1.0 × 7 cm DEAE-cellulose column in buffer A (0.05 M Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerin). The column was washed with at least 5 column volumes of buffer A after which the thymidylate synthetase was eluted with a linear gradient from zero to 0.5 M KCl in buffer A. The eluate was pumped at 0.63 ml/min and 4-ml fractions were collected. Fractions 24-30 were pooled for application onto the affinity column in buffer A containing 100 μM dUMP. The 0.5 × 6 cm column was pumped at 0.22 ml/min and eluate monitored at 280 nm until absorbency returned to baseline. Thymidylate synthetase was then eluted with buffer A containing 0.5 M KCl (withdrawal of dUMP).

Thymidylate synthetase activity was determined by the Roberts assay[30] and protein concentration was estimated by the method of Bradford[31] using bovine serum albumin as a standard. Preparation and electrophoresis of the [^{3}H]-FdUMP complexes in the native and SDS-denatured state were as previously described[32,33]

Results and discussion. The structure of the biospecific adsorbent is shown in the figure. When the enzyme was applied in the presence of dUMP and the column washed with a 0.5 M KCl buffer solution containing dUMP, a peak of activity occurred accounting for about 25% of the total recovered enzyme activity. Little purification of the enzyme in this peak was observed. Withdrawal of dUMP from the elution buffer, however, gave another very broad band of enzyme activity which was extensively purified as summarized in the table. The enzyme was much more stable in crude extracts than in purified preparations, but could be stored at −20 °C in 10% glycerin, 0.05 M Tris-HCl (pH 7.4) and 10 mM 2-mercaptoethanol for 30 days with a loss of approximately 50% activity. It is interesting to note that no dUMP-dependent binding of the mouse liver enzyme on 10-methyl-5,8-dideazafolate-aminoethyl-sepharose or methotrexate-aminohexyl-sepharose columns was observed in analogous attempts to utilize these affinity matrices. It appears that affinity chromatography of thymidylate synthetase from different sources is highly empirical and a unique set of conditions seems to be required for each separation. Nevertheless, the purification of mouse liver thymidylate synthetase reported here yielded enzyme of relatively high purity although it was not homogeneous by polyacrylamide gel electrophoresis.

Ternary complexes of the enzyme with methylenetetrahydrofolate and [^{3}H]-FdUMP were prepared and electrophoresed in the native and SDS-denatured states. 2 electrophoretically distinguishable complexes were obtained on native gels analogous to those first observed by Aull et al.[3] for the dimeric enzyme from *L. casei*. Only a single band was observed for both the *L. casei* and mouse liver enzymes when complexes are treated with SDS and electrophoresed in the presence of the detergent. The mouse liver enzyme traveled with an R_f of 0.85 and the *L. casei* enzyme an R_f of 0.73. Thus, the mouse liver enzyme appears to be dimeric with identical subunits which are somewhat smaller than the 35,000 daltons of the *L. casei* protomers.

1 Supported by grant CA22754, awarded by the National Cancer Institute, DHEW.

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