PURIFICATION OF THYMIDYLATE SYNTHETASE FROM L. CASEI BY AFFINITY CHROMATOGRAPHY[1]

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Summary: An affinity column has been constructed by coupling 2'-deoxyuridylic acid through the 5'-phosphate group to Sepharose via a 6-p-aminobenzamidohexyl chain. This material adsorbs thymidylate synthetase quantitatively from a crude extract of Lactobacillus casei, allowing purification to homogeneity in a single step.

Thymidylate synthetase, the target enzyme for the potent tumor-inhibitory drug 5-fluorouracil (1-3), and perhaps the rate-limiting enzyme in DNA biosynthesis (4), has been the object of considerable research in this laboratory (2,3,5,6) and others. Until now, the purification of this enzyme has been carried out by sequences of laborious conventional procedures (5,7-11). Especially from Ehrlich ascites carcinoma cells, thymidylate synthetase is quite labile and in very low content (6). In order to facilitate our studies with this enzyme, we sought to develop a purification procedure based on affinity chromatography (12). Excellent purifications of other enzymes have been obtained using recently developed affinity systems based on an agarose matrix (13).

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The success of affinity chromatography depends in many cases on attaching a substrate or competitive inhibitor far enough from the matrix to minimize steric interference with the enzyme (13). Using this principle, we have coupled the substrate 2'-deoxyuridylic acid (dUrd-5'-P) to Sepharose through a p-aminobenzamidohexyl chain. When used in column chromatography, this material has the ability selectively to adsorb thymidylate synthetase. The enzyme from an amethopterin-resistant Lactobacillus casei was purified to apparent homogeneity in one step from the 30-70% ammonium sulfate precipitate of the crude lysate.

Materials and Methods

6-Aminocaproic acid, p-nitrobenzoyl chloride, and cyanogen bromide were obtained from Aldrich Chemical Company, Milwaukee, Wis. Sepharose 4B-200 was from Sigma Chemical Co., St. Louis, Mo. An amethopterin-resistant strain of Lactobacillus casei was kindly provided by Dr. F. M. Huennekens and was grown according to Dunlap et al. (12). IR spectra were determined on a Beckman IR10, UV spectra on a Beckman DB-G, and NMR spectra on a Perkin-Elmer Model R12. The elemental analysis was done by Spang Microanalytical Laboratories, Ann Arbor, Mich. Paper chromatography was carried out using Whatman No. 40 paper, in n-butanol-acetic acid-water (5:2:3). Analytical TLC was performed on Eastman chromatogram sheets using the system chloroform-methanol (4:1). All other reagents were the best grade available.

6-p-Nitrobenzamidohexan-1-ol (I). 6-Aminocaproic acid (6.6 g; 50 mMoles) was silylated and reduced with lithium aluminum hydride according to Venkateswaran and Bardos (14) to give 6-aminohexan-1-ol in 80% yield. Without further purification, the amino alcohol was taken up in 50 ml of tetrahydrofuran and treated with 7.4 g (40 mMoles) of p-nitrobenzoyl chloride and 4.0 g (40 mMoles) of triethylamine. After one hour at room temperature, the solvent was evaporated and the residue taken up in ethyl acetate. The solution was washed successively with 1 N hydrochloric acid, 1 N sodium hydroxide, and water. The ethyl acetate layer was dried over magnesium sulfate, and the residue obtained after evaporation of the ethyl acetate was crystallized from chloroform-benzene to give 8.6 g (90%) of 6-p-nitrobenzamidohexan-1-ol, m.p. 81-83°; IR (KBr), 1640 cm^{-1} (amide C = O stretch); NMR ($DCCl_3$), τ 1.90 (quartet, 4H, aromatic). Anal. ($C_{13}H_{18}N_2O_4$)
Calcd.: C, 58.70; H, 6.77; N, 10.50.
Found: C, 59.05; H, 6.61; N, 10.69.

2'-Deoxyuridine-5'-(6-p-nitrobenzamido)hexylphosphate (II). The disodium salt of 2'-deoxyuridine-5'-phosphate (dUrd-5'-P) (0.5 g; 1.43 mMoles) was passed through Dowex 50 (H^+) resin in water to convert it to the acid form. The water was removed by evaporation and 3 x 5 ml portions of dry pyridine were re-evaporated from the residue. It was then taken up in 50 ml of dry pyridine, and treated with 2.0 g (7.5 mMoles) of I, along with 10 g of dicyclohexylcarbodiimide. After 48 hr at ambient temperature, a paper chromatographic check showed no dUrd-5'-P remaining, but instead a new product at R_f 0.8 (dUrd-5'-P had an R_f of 0.3). The precipitated dicyclohexylurea was removed by filtration and the pyridine

evaporated *in vacuo*. The residue was washed with chloroform to remove excess I, leaving a gummy residue, which was converted to a precipitate by dissolution in methanol and addition of ether. This material had an R_f of 0.0 by TLC (compared to 0.6 for I), and showed one spot at R_f 0.8 by paper chromatography. The new spot was positive to a molybdate spray test for phosphorus. NMR [(CD_3OD), τ 2.20 (quartet, 4H, aromatic), 2.75 (doublet, 1H, H-6), 4.80 (doublet, 1H, H-5), 7.15 (broad singlet, 4H, CH_2), 9.1 (broad singlet, 8H, CH_2)] showed that uridinyl, *p*-nitrobenzoyl, and aminohexyl fragments were present in equal ratios. Phosphorus analysis showed a base-phosphorus ratio of 1:1.

Coupling to Sepharose. II (1.2 mMoles) was dissolved in methanol and hydrogenated at 40 psi in the presence of 10% palladium on charcoal. When hydrogen absorption ceased (1 hr), the catalyst was removed, and the product dissolved in 50% aqeous dimethylformamide. Activation of Sepharose (15 ml wet volume) and coupling with the ligand was done at pH 9.0 according to Cuatrecasas *et al.* (13). This resulted in the attachment of 200 μMoles of uridine per g of Sepharose (dry weight).

Results

The adsorption effectiveness of the 6-*p*-aminobenzamidohexyl-dUrd-5'-P Sepharose column was demonstrated by placing a quantity of the crude 30-70% ammonium sulfate precipitate of the lysed *L. casei* on a column (1 X 10 cm). At a buffer concentration of up to 0.05 M, pH 7.1, no enzyme activity came through the column, regardless of the volume with which the column was washed. However, when the buffer concentration was increased to 0.1 M, quantitative recovery of the enzyme activity was achieved in a single sharp peak with no tailing (Fig. 1). There was no adsorption of thymidylate synthetase activity to a control column made by treating Sepharose with cyanogen bromide, but deleting the ligand coupling step.

Sodium dodecyl sulfate disc gel electrophoresis of the peak of enzyme activity by the method of Laemmli (15) showed only one band (Fig. 2). That this band was indeed thymidylate synthetase was established by treating the enzyme with [^{14}C]-5-fluoro-2'-deoxyuridylic acid, which has recently been shown to bind covalently to the enzyme in the presence of methylenetetrahydrofolate (16,17). It was found that the peak of radioactivity on the gel corresponded to the observed single band.

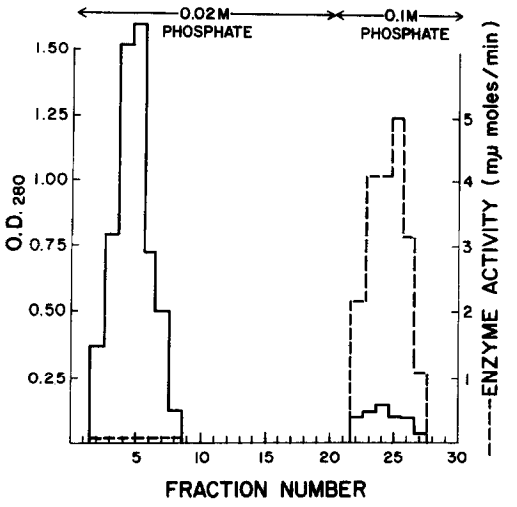

Fig. 1. Elution profile for thymidylate synthetase from L. casei on a 6-p-aminobenzamidohexyl-dUrd-5'-P-Sepharose column (1 x 10 cm). The 30-70% ammonium sulfate fraction (1.0 ml, corresponding to ~ 10 mg of protein) was placed on the column, and eluted with 0.02 M phosphate buffer, pH 7.1, containing 10 mM β-mercaptoethanol, until the A_{280} fell to zero. Then the column was eluted with 0.1 M phosphate buffer, pH 7.1, containing 10 mM β-mercaptoethanol, until all enzyme activity was removed. A_{280}: —— Enzyme activity: ----.

Fig. 2. Sodium dodecyl sulfate disc gel electrophoresis of sample obtained from tube no. 25 of Fig. 1.

Surprisingly, the enzyme was not removed from the column by dUrd-5'-P at concentrations up to 10^{-4}M. Possibly, in the absence of the cofactor methylenetetrahydrofolate, thymidylate synthetase may bind more strongly to the dUrd-5'-P column than to dUrd-5'-P itself.

To determine maximum binding capacity, the column was treated with the crude enzyme preparation until enzyme activity began to

seep through. Subsequent elution with 0.1 M phosphate showed that 2.0 μMoles/min of enzyme activity had remained bound. The specific activity obtained (2.4 μMoles/min/mg protein) compares favorably to the published value (2.5 μMoles/min/mg protein) for the purified enzyme (11). Work is in progress on the purification of thymidylate synthetase from Ehrlich ascites cells using this column.

References

1. Cohen, S.S., Flaks, J.G., Barner, H.D., Loeb, M.R., and Lichtenstein, J. Proc. Natl. Acad. Sci., U.S., 44, 1004 (1958).
2. Heidelberger, C., Kaldor, G., Mukherjee, K.L., and Danneberg, P.B., Cancer Res., 20, 903 (1960).
3. Reyes, P., and Heidelberger, C., Mol. Pharmacol., 1, 14 (1965).
4. Blakeley, R.L., In The Biochemistry of Folic Acid and Related Pteridines, John Wiley and Sons, Inc., New York (1969), pp. 219-266.
5. Fridland, A., Langenbach, R.J., and Heidelberger, C., J. Biol. Chem., 246, 7110 (1971).
6. Langenbach, R.J., Danenberg, P.V., Fridland, A., Cleland, W.W., and Heidelberger, C., Fed. Proc., 31, 419Abs (1972).
7. Mathews, C.K., and Cohen, S.S., J. Biol. Chem., 238, 367 (1963).
8. Friedkin, M., Crawford, E.J., Donovan, E., and Pastore, E.J., J. Biol. Chem., 237, 3811 (1962).
9. Blakley, R.L., J. Biol. Chem., 238, 2113 (1963).
10. Crusberg, T.C., Leary, R., and Kisliuk, R.L., J. Biol. Chem., 245, 5292 (1970).
11. Dunlap, R.B., Harding, N.G.L., and Huennekens, F.M., Biochemistry, 10, 88 (1971).
12. Cuatrecasas, P., Wilcheck, M., and Anfinsen, C.B., Proc. Natl. Acad. Sci., U.S., 61, 636 (1968).
13. Cuatrecasas, P., J. Biol. Chem., 245, 3059 (1970).
14. Venkateswaran, P.S., and Bardos, T.J., J. Org. Chem., 32, 1256 (1967).
15. Laemmli, U.K., Nature, 227, 680 (1970).
16. Langenbach, R.J., Danenberg, P.V., and Heidelberger, C., Biochem. Biophys. Res. Commun. In press.
17. Santi, D.V., and McHenry, C., Proc. Natl. Acad. Sci., U.S., 69, 1855 (1972).