THE PREPARATION OF A MODIFIED GTP-SEPHAROSE DERIVATIVE AND ITS USE IN THE PURIFICATION OF DIHYDRONEOPTERIN TRIPHOSPHATE SYNTHETASE, THE FIRST ENZYME IN FOLATE BIOSYNTHESIS

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Summary: The conditions for coupling periodate oxidized GTP to a hydrazide Sepharose derivative are described. Approximately 1 μmole of the ligand was bound per milliliter of settled gel. Gel columns prepared from this material bind D-erythrodihydroneopterin triphosphate synthetase, the initial enzyme for folate biosynthesis in Lactobacillus plantarum. A yield of 28% and an overall enzyme purification of 765 fold were attained when the affinity technique was used with a conventional purification procedure.

INTRODUCTION

The use of affinity chromatography for the selective binding of specific proteins has proved extremely useful for the purification of a number of enzymes (1,2).

The studies of Robberson and Davidson (3) demonstrated that periodate oxidized RNA or UMP could be coupled to agarose. Their procedure involved the coupling of ε-aminocaproic acid methyl ester to a cyanogen bromide activated agarose followed by hydrazinoloysis. Periodate oxidized RNA or UMP was then reacted with the hydrazide agarose to form the respective hydrazone derivative.

We are currently investigating an enzyme (D-erythrodihydroneopterin triphosphate synthetase) from Lactobacillus plantarum which requires GTP as its substrate (4, 5, 8). GTP covalently coupled to an insoluble matrix to form an affinity chromatographic system would be valuable for the purification of this enzyme. This report describes the conditions of preparation and the application of such an affinity column for the purification of D-erythrodihydroneopterin triphosphate synthetase.

MATERIALS AND METHODS

Washed Sepharose 4B-(Pharmacia), 25 ml was suspended in water to a total volume of 100 ml. The suspension was stirred in an ice bath and 6 g of cyanogen bromide was added. The pH was maintained at 11 by the addition of 5N NaOH until proton release subsided. The gel was filtered on a Buchner funnel, washed with 10 volumes of ice cold 0.1 M $NaHCO_2$ buffer, pH 9.0 and resuspended in 40 ml of the same buffer. The gel was stirred at 4^0 and 2.5 g of ε-aminocaproic acid methyl ester (Cyclo Chem. Co.) in 10 ml of ice cold 0.1 M $NaHCO_3$ buffer (adjusted to pH 9.0 with NaOH) was added. The reaction mixture was stirred 16-18 hours at 4^0. The suspension was filtered, washed with 1 liter of ice cold water, resuspended to 15 ml with water and cooled to 4^0. Hydrazine hydrate, 70 ml, (98 - 100%), was added slowly in a well ventilated hood and the mixture heated at 70^0 for 15 min on a steam cone. After heating, the hydrazide gel was cooled to room temperature and washed with 1 liter of water.

Periodate oxidation and coupling of the oxidized nucleotide to the hydrazide Sepharose was accomplished as follows: 11.85 μmoles of GTP (E = 13,700 at 252 nm, pH 7.0) was dissolved in 2.5 ml of 0.1M citrate-phosphate buffer, pH 5.0 and to this solution [γ-^{32} P] GTP was added (final specific activity 4.65 x 10^5 dpm/μmole). Sodium periodate, 0.2 ml of a 0.1M solution was added and the reaction mixture incubated in the dark at room temperature for 30 min. To destroy excess periodate, 10 μl of a 40% solution of ethylene glycol was added and the mixture incubated an additional 10 min. Argon was then bubbled into the solution for 5 min to reduce the amount of formaldehyde formed in the reaction mixture. The volume of the solution was adjusted to 5 ml with citrate-phosphate buffer, pH 5.0, 5 ml of the hydrazide gel was added and the suspension was stirred at room temperature for 2 hours. All reaction mixtures thus contained 2.37 μmoles of GTP per ml of hydrazide gel. The gel was filtered onto a Millipore filter (pore size 0.45μ) and washed with cold 0.05 M phosphate buffer, pH 6.8

TABLE I

Effect of periodate concentration on coupling yields

Sodium periodate/GTP µmoles/µmole	Oxidized GTP bound µmoles/ml gel	γ-^{32}P bound %	Recovery of radioactivity %
0.5	0.34	14.4	99.7
1.0	0.49	20.6	101
2.0	0.33	13.9	100
4.0	0.08	3.4	95

containing 5 x 10^{-3}M EDTA to remove unbound ligand. Aliquots of the filtrate and resuspended gel were counted in a Packard model 3375 Scintillation Spectrometer in 10 ml of Bray's (6) solution.

RESULTS AND DISCUSSION

The results of the initial experiments indicated that approximately 0.49 µmoles of oxidized GTP were bound per ml of settled gel. Subsequent experiments were performed to determine whether increased coupling yields could be attained. The conditions for examining the effect of periodate concentration of GTP oxidation were those described in Materials and Methods with the exception that the argon gassing procedure was omitted. One ml aliquots of the hydrazide gel were added to reaction mixtures containing the various ratio's of periodate to GTP listed in Table 1. As shown in the table, equimolar amounts of periodate and GTP proved most effective. A large excess of periodate resulted in depressed coupling yields presumably due to the competition of oxidized nucleotide with formaldehyde (produced by destroying the excess periodate with ethylene glycol) for the hydrazide sites on the gel.

TABLE II

Influence of pH on binding of oxidized GTP to hydrazide gel

pH	Oxidized GTP bound µmoles/ml gel	γ-^{32}P bound %	Recovery of radioactivity %
3.0	0.94	39.6	94.7
4.0	0.83	34.9	95.4
5.0	0.65	27.6	95.3
6.0	0.49	20.6	100.1
7.0	0.30	12.7	101.6

The optimum pH for coupling the oxidized GTP derivative to the Sepharose was also examined. GTP was oxidized in 0.01 M citrate-phosphate buffer pH 5.0 with an equimolar amount of periodate for 30 minutes. Aliquots were removed and pipetted into gel solutions containing 0.05 M citrate-phosphate buffer (final concentration) at the pH values shown in Table II. As shown in the table, optimum coupling of the periodate oxidized GTP to the gel occurred at pH 3. Under these conditions, 0.94 µmole of oxidized GTP (39.6%) was bound per ml of gel. We have noticed variations in coupling efficiency with different batches of hydrazide gel and this could account for the slightly higher coupling efficiency at pH 5.0 in this experiment as opposed to the previous experiment,(Table 1).

In order to determine whether extensive loss of GTP phosphate residues occurred during the oxidation and coupling reactions, the following experiment was performed. Equal amounts of the hydrazide gel in 0.1 M citrate-phosphate buffer pH 3.0 were placed in two separate tubes. To one tube 2.37 µmoles of oxidized [8-^{14}C] GTP (4.37×10^5 dpm) was added and to the other, an equal amount of oxidized [γ-^{32}P] GTP (3.83×10^5 dpm). The

TABLE III

Phosphate loss during oxidation and coupling

Radioactive GTP	Oxidized GTP bound µmoles/ml gel	Radioactivity bound %	Recovery of radioactivity %
[8-^{14}C]	1.28	48.7	98.9
[γ-^{32}P]	1.10	41.7	102.2

TABLE IV

Purification of dihydroneopterin synthetase

Enzyme preparation	Volume ml	Total Protein mg	Total units*	Units/ mg protein	Yield %
Crude extract	680	13,702	93,173	6.8	100
Rnase-treated extract	630	9,324	80,745	8.6	86.6
45 to 70% ammonium sulfate fraction	109	4,114	66,235	16.1	71.0
Sephades G-200 40-80% ammonium sulfate fraction	40	870	48,372	55.6	51.9
Affinity column concentrate	7.5	26.25	27,956	1,065	30.0
DEAE-cellulose concentrate	15.3	4.97	26,057	5,243	27.9

* 1 unit of enzyme is defined as the amount of enzyme capable of liberating 1 nanomole of formate per 15 min at 37° in a reaction mixture containing 0.4 µmoles [8-^{14}C] GTP (275,000 dpm per µmole), 100 µmoles phosphate buffer, pH 6.8 and enzyme in a total volume of 0.4 ml.

reaction mixtures were incubated and washed as previously described. The results which are given in Table III indicate that a minimum of 85% of the oxidized nucleotide bound to the gel is present as the triphosphate derivative.

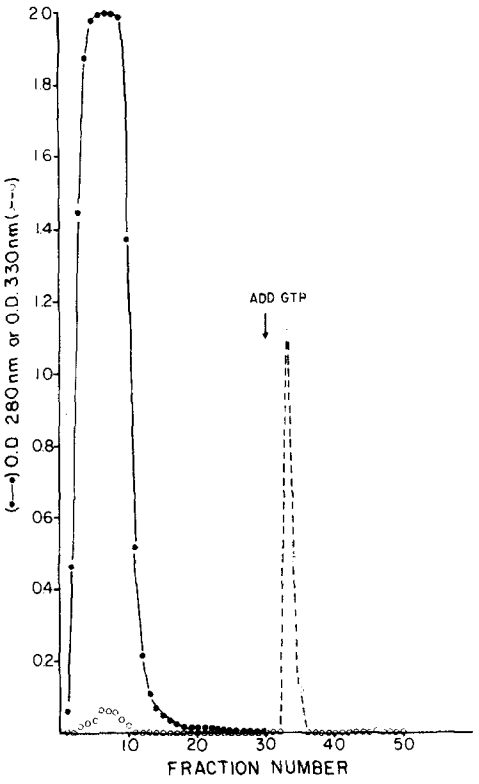

Figure I

Affinity chromatography of a partially purified enzyme preparation. A 40-80% ammonium sulfate fraction of a G-200 Sephadex purified enzyme preparation (13.2 ml) which was warmed to room temperature, was applied to the gel column (bed height 1 x 8.5 cm). The flow rate was approximately 0.5 ml/min. Two ml fractions were collected. The column was washed with 0.05M phosphate buffer pH 6.8 containing 5 x 10^{-3}M EDTA. Fractions 1-30 which were eluted with buffer were monitored at 280 nm for protein and assayed for enzyme activity in reaction mixtures consisting of 0.4 µmoles [8-^{14}C] GTP (275,000 dpm/µmole), 100 µmoles of phosphate buffer, pH 6.8 and 0.1 ml of each fraction in a total volume of 0.4 ml. After a 15 min. incubation at 37°, the reaction mixtures were extracted with ethyl acetate (7) and tested for the presence of radioactive formic acid. These fractions did not exhibit significant enzyme activity. Elution of the enzyme was accomplished by the addition of 4 ml of a 0.5mg/ml solution of GTP in buffer followed by additional washing with buffer. The enzyme was detected by monitoring the fractions at 330 nm since the enzyme product, D-erythrodihydroneopterin triphosphate, absorbs at this wavelength (5, 8). Since enzyme and substrate are present, the O.D. values increase with time. The presence of the minor 330 nm peak (fractions 3-10) is due to the high concentration of protein present in these fractions.

The modified GTP-substituted Sepharose was subsequently tested for its ability to bind the synthetase. Figure 1 depicts the elution pattern obtained when a partially purified preparation of the enzyme was applied to

the gel column. The major 330 nm peak (tubes 33-35) containing the enzyme and exhibiting bright blue fluorescence was pooled and dialyzed extensively to remove GTP and the pteridine product. The recovery of enzyme activity from the column ranges from 60 to 70%. In the experiment shown, a 20 fold increase in purification was achieved. Polyacrylamide gel electrophoresis of the concentrated eluate revealed 6 other proteins were also bound to the gel. The gel pattern of the enzyme preparation following a DEAE-cellulose chromatography exhibits one major and one minor protein band. Enzyme activity is associated with the major protein band. Incorporation of the affinity chromatographic step in our enzyme purification procedure (Table IV) has led to an overall yield of 28% and a purification of 765 fold as compared to an 8% yield and 100 fold purification as described earlier (5).

Partially purified enzyme preparations were used for the majority of the affinity chromatographic studies since the presence of phosphatases in less purified preparations might destroy the bound ligand. However, when small quantities (15 to 20 ml) of dialyzed crude extract were applied to 1 x 5 cm gel columns, purifications of 350 fold and yields of approximately 60% were obtained. Chromatography of larger quantities of crude extract is currently under investigation. The use of [8-^{14}C] or [γ-^{32}P] labeled gel columns indicated that only slight catabolism of the gel occurred during chromatography of partially purified enzyme. Gel columns have been used two or three times with little loss in efficiency of enzyme recovery. After each use, the gel was washed with approximately 1 liter of 0.05 M phosphate buffer pH 6.8 containing 5 x 10^{-3} M EDTA. Storage of the column at 4^{o} in the presence of 0.02% sodium azide for a period of four weeks results in little loss of enzyme recovery as compared to recently synthesized columns.

In addition to the periodate oxidized GTP Sepharose gel, we have prepared the oxidized guanosine and GDP derivatives with coupling efficiencies similar to that of the GTP column. These gels are not effective for the purification of the enzyme.

While this manuscript was in preparation, Berglund and Eckstein (9) prepared ATP and dATP substituted Sepharoses by coupling the p-aminophenyl-ester derivative to cyanogen bromide activated Sepharose. While less ligand is bound, their procedure extends the coupling of deoxynucleotides to Sepharose. The synthesis of such nucleoside and nucleotide columns may prove useful for the purification of other enzymes which have purine or pyrimidine nucleotides as substrates of effectors.

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