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PURIFICATION OF GUANOSINE TRIPHOSPHATE CYCLOHYDROLASE I FROM *ESCHERICHIA COLI*

THE USE OF COMPETITIVE INHIBITORS *VERSUS* SUBSTRATE AS LIGANDS IN AFFINITY CHROMATOGRAPHY

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SUMMARY

Different affinity chromatography ligands have been compared for the purification of guanosine triphosphate (GTP) cyclohydrolase I, an enzyme that catalyses the transformation of GTP into formate and dihydroneopterin triphosphate, the first metabolite in the biosynthetic pathway of the pterins. When this enzyme is purified by affinity chromatography on GTP-Sepharose a major fraction of the activity is lost and the yield of enzyme decreases as the amount of enzyme applied to the column decreases. The use of nucleotide competitive inhibitors (UTP and ATP) as ligands in the affiity column has shown that the extent of inactivation of the enzyme is related to the affinity of the enzyme for the ligand. Further, the extent of inactivation was reduced by reducing the length of the columns when using the same volume of GTP-Sepharose. Dihydrofolate-Sepharose gave consistently higher yields of GTP cyclohydrolase I regardless of the amount of enzyme applied, but several other proteins were also obtained. For a high purification of GTP cyclohydrolase I the best yield may be obtained with UTP as the affinity ligand and with the shortest length possible of the affinity column, and the purity of enzyme is comparable with that obtained with GTP-Sepharose.

INTRODUCTION

Guanosine triphosphate (GTP) cyclohydrolase I (E.C. 3.5.4.16) catalyses the transformation of GTP into two products, formate and D-*erythro*-7,8-dihydroneo-

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pterin 3'-triphosphate[1]. This is the first enzyme in the biosynthesis of riboflavin, tetra-hydrofolate and tetrahydrobiopterin, the natural cofactor for aromatic amino acid hydroxylases[2]. The lack of the latter pterin leads to a type of phenylketonuria in humans with decreased levels in the neurotransmitters norepinephrine, serotonin and dopamine. At least two cases of GTP cyclohydrolases I deficiency have been reported in humans[3,4]. Because this enzyme catalyses the first step in the biosynthetic pathway of pteridines it may play a key role in the regulation of the biosynthesis of pteridines in most organisms.

        GTP cyclohydrolase I has been purified from many sources, including bacteria[5], *Drosophila melanogaster*[6], chicken[7], rat[8] and man[9]. However, the use of affinity chromatography to purify this enzyme has been restricted to GTP cyclohydrolases from *Escherichia coli*[5], *Lactobacillus plantarum*[10] and *Serratia indica*[11] as the enzyme from other sources was either destroyed or not bound by conventional affinity chromatography columns[6,7,9]. Only recently has GTP cyclohydrolase from human liver been purified by affinity chromatography using a GTP analogue as ligand[9].

        For several years we have purified GTP cyclohydrolase I of *E. coli* by affinity chromatography[12-14] and regularly found this system to behave in a non-ideal manner but not always for the same reason. At times the enzyme was not retained and at times it was retained but not released, much like the enzyme from other sources[6,7]. This paper evaluates different ligands for the purification of GTP cyclohydrolase I of *E. coli* and offers suggestions for obtaining improved yields.

EXPERIMENTAL

        Sephadex G-25 (fine) and Sepharose 4B were obtained from Pharmacia. Agarose–hexane–guanosine 5'-triphosphate (GTP-agarose), agarose–hexane–adenosine 5'-triphosphate (ATP-agarose) and agarose–hexane–uridine 5'-triphosphate (UTP-agarose) (each nucleotide was periodate oxidized) were purchased as Type 4 from P-L Biochemicals, Bio-Gel A-0.5m from Bio-Rad Labs., GTP, ovalbumin, myoglobin, chymotrypsinogen and ammonium sulfate (grade III) from Sigma, ε-aminocaproic acid methyl ester from Vega Chemical, cyanogen bromide from Aldrich, bovine albumin (crystallized twice) from Nutritional Biochemical Corp. and [8-^{14}C]guanosine 5'-triphosphate (58 Ci/mol) from Amersham. Adipic acid dihydrazide was synthesized according to Lamed *et al.*[15]. Folic acid (crystalline) was obtained from Sigma and 1,6-diaminohexane from Aldrich.

Growth of bacteria
        E. coli B cells were grown in 200-l batches on a glucose carbon source in a mineral salts media, harvested at late log, and stored at −80°C.

Composition of buffers
        Buffer A is composed of 50 mM potassium phosphate–5 mM EDTA (pH 7.0), buffer B of 10 mM potassium phosphate–2.5 mM EDTA (pH 7.0), buffer D of 20 mM potassium phosphate–2.5 mM EDTA (pH 7.0), buffer E of 200 mM Tris–HCl (pH 8.0)–20 mM 2-mercaptoethanol–1 M potassium chloride, and buffer F of 10 mM sodium phosphate–2.5 mM EDTA–20 mM 2-mercaptoethanol (pH 7.0).

Preparation of enzyme for affinity chromatography

Frozen cells (500 g) were allowed to thaw at 4°C for 16 h and suspended in 1 l of buffer A. The cells were disrupted at 11 000 psi in a Gaulin press previously cooled with cold distilled water. The suspension was passed twice through the press to ensure complete breakage of the cells and the long fibers of DNA. Alternatively, the enzyme was extracted from the cells by incubating 300 g of *E. coli* at 30°C with 300 mg of lysozyme dissolved in 600 ml of 0.2 *M* Tris–HCl (pH 8.0) for 15–45 min. Then 30 mg of DNase I were added and incubation was continued for 15 min. The reaction was stopped by adding 300 g of ice. In either procedure for lysis, after chilling the suspension was centrifuged at 6000 *g* for 90 min at 4°C to remove the cell debris. Unless indicated otherwise, all further manipulations of the enzyme were performed at 4°C.

With continuous stirring, the supernatant was adjusted to 35% saturated ammonium sulfate by dropwise addition of saturated ammonium sulfate solution. The mixture was stirred for 20 min and then centrifuged at 6000 *g* for 45 min. The supernatant was treated again to bring it to 50% saturated ammonium sulfate, and stirred for 20 min. It was centrifuged as before (supernatant volume = 1600 ml). The pellet was suspended in buffer A and dialysed for 3 h *vs.* 6 l of buffer A and then for 12 h after replacing the buffer. After dialysis most of the protein was dissolved and the volume of the solution inside the bags was 100–200 ml. Any undissolved protein was removed by centrifugation.

The dialysed fraction was applied to a column (97 × 5 cm I.D.) of Bio-Gel A-0.5m (100–200 mesh) equilibrated with buffer A. The enzyme was eluted with the same buffer. The fractions that contained GTP cyclohydrolase I activity were pooled and either used immediately or stored in 50-ml batches at −20°C or −80°C. This pool was used for subsequent experiments on affinity columns.

Preparation of nucleotide-Sepharose

AMP-, ATP-, UTP- and GTP-Sepharose were synthesized following the procedure of Jackson *et al.*[10] with slight modifications. Sepharose 4B (45 g) was washed on a sintered-glass funnel with 1 l of cold water and suspended in 120 ml of water. The temperature was maintained at <5°C in an ice-bath, the pH was adjusted to 11 with 5 *M* potassium hydroxide solution and 12 g of crushed cyanogen bromide were added. The pH was maintained at *ca.* 11 (± 0.3) with potassium hydroxide solution until the rate of consumption of the latter had decreased significantly (75 min). The cyanogen bromide-activated Sepharose was then washed with 1 l of cold water and 1 l of cold 0.1 *M* sodium hydrogen carbonate buffer (pH 9.5) and suspended in 120 ml of the same buffer. Adipic acid dihydrazide (6.7 g) was added to the suspension and allowed to mix on a rotator for 22 h in the dark at 4°C (unless indicated otherwise, the columns used for this work were synthesized with adipic acid as spacer). Alternatively, ε-aminocaproic acid methyl ester (4 g) was added in place of the adipic acid dihydrazide, together with 70 ml of hydrazine hydrate (98–100%); the mixture was heated at 70°C for 15 min[10]. The gel with the extender arm was washed with 500 ml of cold water and 1 l of 0.1 *M* sodium acetate solution (pH 6.0) and then mixed with periodate-oxidized nucleotide and rotated for 2 h in the dark at 4°C. The oxidized nucleotide-gel was filtered, rinsed with water and stored at −20°C in 50% aqueous glycerol. The oxidation of the nucleotides was carried out as follows: 45 mg

of nucleoside triphosphate or 31 mg of AMP were dissolved in 15 ml of 0.1 M acetate buffer (pH 6.0); 1.5 ml of 0.1 M sodium metaperiodate solution were added and the solution was incubated in the dark on ice for 60 min. Ethylene glycol (45 μl) was added and allowed to react for 30 min to destroy the excess of oxidant. The formaldehyde formed was removed by bubbling with an inert gas [nitrogen or helium–butane (98.7:1.3)] for 5 min.

Measurement of enzyme activity

The procedure of Burg and Brown[1] was slightly modified. The reaction mixture of 200 μl contained 0.1 M Tris–HCl (pH 8.5)–0.1 M sodium chloride–0.01 M EDTA (pH 7.5), 0.1 mM [8-^{14}C]GTP (376 000 cpm/μmole) and enzyme and was incubated at 42°C for 30 min. The reaction was stopped by adding 0.25 ml of 0.5 M formic acid. Activated charcoal (15 mg) suspended in water (Darco G-60, acid and alkali washed) was added and the mixture was transferred into a Pasteur pipet with a cotton plug where the charcoal was retained forming a column about 0.5–1 cm high. The column was washed twice with water (0.5 ml each). The radioactive GTP was retained in the charcoal, and the radioactive formate that had been released in the enzymatic reaction was eluted from the column. The original solution and two washes that passed through the column were combined and mixed with ten volumes of 0.27% 2,5-bis[2-(5-*tert.*-butylbenzoxalyl)]thiophene (BBOT) in toluene–Triton X-100 (2:1, v/v), and analysed for radioactivity in a scintillation counter. The amount of formate released was linear with the enzyme concentration. One unit of enzyme released 1 nmole of formate per minute[5]. Specific activity is defined as units per milligram of protein.

Dihydrofolate-Sepharose affinity chromatography

Folic acid was bound to Sepharose 4B through 1,6-diaminohexane and subsequently reduced to the dihydrofolate state as described by Then[16]. The adsorption of GTP cyclohydrolase and subsequent washing of the column followed the published procedure and the enzyme was eluted with 3.3 mM dihydrofolic acid–1 M potassium chloride–0.2 M Tris–HCl–20 mM 2-mercaptoethanol (pH 8.0). A method to reduce folic acid to the dihydro form was described by Then[16]. A 3-ml aliquot of the eluted enzyme was passed over a Sephadex G-25 column (18 × 2.4 cm I.D.) equilibrated with buffer D to remove dihydrofolate, an inhibitor of *E. coli* GTP cyclohydrolase.

Determination of protein content and purity

Protein was determined by the method of Miller[17] with slight modifications. The samples were dialysed against water to remove EDTA, which interferes in the reaction. Electrophoresis was performed using 5% polyacrylamide in sodium dodecylsulfate-containing buffer as described by Weber and Osborn[18].

RESULTS

Affinity chromatography on GTP-Sepharose and dihydrofolate-Sepharose

When various amounts of enzyme activity were applied to several GTP-Sepharose columns, the recovery decreased as the amount of enzyme decreased, as shown in Table I. The specific activity of each enzyme preparation also showed a

TABLE I

RECOVERY OF GTP CYCLOHYDROLASE I FROM GTP-SEPHAROSE AND DIHYDROFO-LATE-SEPHAROSE

Column	Enzyme activity (units)		Recovery (%)	Specific activity
	Applied	Recovered		
GTP-Sepharose*	190	35	18	465.0
	133	12	11	8.0
	56	3.5	6	3.5
	19	0	0	0
Dihydrofolate-Sepharose**	768	462	60	14.0
	563	442	79	24.7
	152	117	77	11.9
	75	64	85	17.0

* The enzyme was obtained after gel filtration in buffer A. The GTP-Sepharose column (5 ml in the barrel of a 10-ml syringe) was washed with 80 column volumes of buffer B. The enzyme pool from gel filtration passed through the GTP-Sepharose slowly (20 ml/h); then the column was washed with buffer B + 0.3 M KCl (500 ml) and then with buffer B (15 ml). The enzyme was eluted with buffer B plus GTP (0.3 mg/ml) and it emerged in a sharp peak (8–10 ml) with the leading edge of the GTP. To determine the recovery, the GTP was removed by passing 3 ml of the enzyme solution over a Sephadex G-25 column (24 × 1.8 cm I.D.). The recovery from the Sephadex G-25 column was greater than 90%. When a 300-ml sample (700 units of enzyme) was applied to GTP-Sepharose, the recovery was 50–60% of the activity applied.

** Conditions for chromatography followed those of Then[16]. The folate column (14 × 0.6 cm I.D.) was converted into the dihydrofolate form by passing sodium dithionite (40 mg/ml)–sodium ascorbate (200 mg/ml) solution through at 25°C. The column was then operated at 4°C. The reducing solution was removed by washing with buffer F. The enzyme was applied and the column then washed with buffer F with 0.1, 0.5 and finally 1.0 M NaCl added. The enzyme was eluted with 3 mM dihydrofolate in buffer E. Recovery was measured after an enzyme sample was passed over Sephadex G-25 to remove the dihydrofolate. Flow-rates were maintained at 0.5 ml/min.

marked decrease as a function of the amount of enzyme loaded. However, when dihydrofolate-Sepharose columns were used, neither the recovery nor the specific activity of the enzyme decreased with decreasing amounts of enzyme applied to the column (Table I). The cause of the diminishing recovery as smaller amounts of enzyme are used with the GTP-Sepharose columns could be irreversible adsorption or inactivation of the enzyme by the column.

Affinity chromatography on GTP-, ATP- and UTP-Sepharose

Four Sepharose columns were compared: ATP-, UTP- and GTP-Sepharose from P-L Biochemicals and GTP-Sepharose synthesized in this laboratory. The columns were equilibrated in buffer B and eluted at the same flow-rate in each instance. Using a linear GTP gradient with buffer B kept constant, the enzyme eluted from each of the columns in the first 16 fractions, with the maximum around fraction No. 7 (where the concentration of GTP was 70 mg/l). Table II shows the results obtained in the elution of the different columns. The percentage recovery of the initial amount of enzyme was higher in the UTP-Sepharose column than in the ATP-Sepharose or GTP-Sepharose columns, which were comparable to each other. The specific activity

TABLE II

GTP CYCLOHYDROLASE I PURIFIED BY DIFFERENT AFFINITY CHROMATOGRAPHY COLUMNS

The column (3.8 × 1.0 cm I.D.) contained 3 ml of oxidized nucleotide-Sepharose equilibrated in buffer B. The enzyme purified from the Bio-Gel A-0.5m column was applied (50 ml) to the affinity column at a flow-rate of 1 ml/min. The column was washed with 100 ml of 0.3 M KCl in buffer B, and the enzyme eluted with a linear gradient of GTP using 100 ml of 0.3 M KCl in buffer B and 200 mg of GTP in 100 ml of the same solution at a flow-rate of 1.5 ml/min. Fractions (4 ml each) that contained enzyme activity were pooled; to remove the GTP and KCl, 5 ml of the pooled fractions were loaded on to a Sephadex G-25 column (29 × 1.7 cm I.D.) equilibrated with buffer B and eluted with the same buffer. The final volume of fractions that contained enzyme activity after the Sephadex column was 8.2 ml.

Column*	Volume after elution (ml)	Protein content (µg/ml)	Enzymatic** activity after Sephadex column (units/ml)	Specific activity (units/mg)	Recovery (% of initial activity)
GTP-Sepharose (Lab)	45.0	22.5	$5.76 \cdot 10^{-3}$	$256 \cdot 10^{-3}$	6***
GTP-Sepharose (PL)	54.0	33.0	$8.64 \cdot 10^{-3}$	$262 \cdot 10^{-3}$	10
ATP-Sepharose (PL)	65.0	37.0	$10.8 \cdot 10^{-3}$	$292 \cdot 10^{-3}$	15
UTP-Sepharose (PL)	32.5	46.4	$52.1 \cdot 10^{-3}$	$1123 \cdot 10^{-3}$	36

    * The source of the oxidized nucleotide-Sepharose is as follows: Lab, prepared by us using adipic acid as the extender arm; PL, purchased from P-L Biochemicals.
    ** After elution from the affinity column the enzyme was passed through Sephadex G-25 to remove the GTP.
    *** Calculated as percentage of the enzyme applied to the column that was obtained by elution with GTP. Some of the enzyme was not retained and another portion lost due to inactivation.

of the purified enzyme was also higher in the UTP-Sepharose column than in the others. The experiment was repeated with each of the columns. No change was found between the first and second use.

When a large amount of enzyme was applied to a 1-ml nucleotide column synthesized in our laboratory, the column was overloaded and the excess of enzyme was measured in a pool of the five fractions of the flow-through. The recoveries in the flow-through from the Sepharose columns were as follows: from GTP 14%, from ATP 18% and from UTP 25% of the amount of enzyme applied to the column. These different recoveries may be due to different inactivation of the unadsorbed enzyme. An AMP-Sepharose column did not bind any enzyme and the enzyme concentration in the flow-through was the same as in the applied sample. When GTP-Sepharose was prepared using ε-aminocaproic acid instead of adipic acid as the extender arm, this affinity column performed similarly to the ones described.

This study of the loading of the affinity column is illustrated in a different way in Fig. 1. The column (5.5 ml) was washed in 50 ml of buffer B and the enzyme was added slowly. The capacity of GTP-Sepharose (prepared in our laboratory) was exceeded. The enzyme that appeared in the flow-through during sample application reached a concentration equivalent to 60% of the sample. When the flow-through fractions were pooled and reapplied to the same column, the same pattern as shown in Fig. 1 was again obtained; a significant amount of enzyme was not adsorbed and its concentration in the flow-through was only 50–60% of the concentration added.
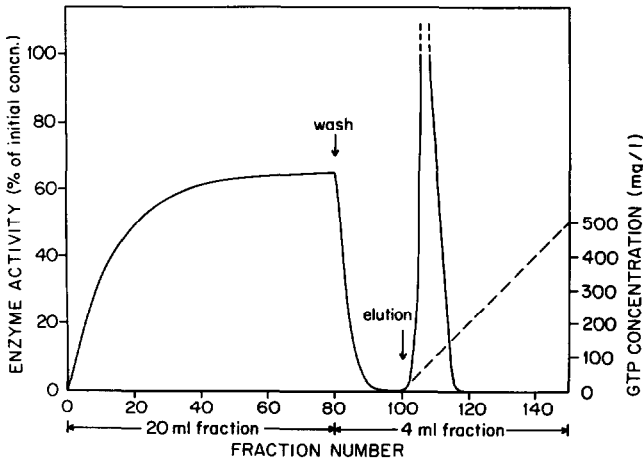
Fig. 1. Behavior of GTP cyclohydrolase I on GTP-Sepharose. The laboratory-prepared GTP-Sepharose (5.5 ml) column was equilibrated with buffer B. The enzyme was recovered in buffer A after the gel filtration column, diluted to 1800 ml in buffer B and added to the GTP-Sepharose at a rate of 2 ml/min. The column was washed with 25 ml of 0.3 M KCl in buffer B before the elution with a linear gradient of GTP in 0.3 M KCl in buffer B.

This pattern has been found with enzyme prepared from different batches of *E. coli* and with different nucleotide-Sepharose columns (either prepared in our laboratory or commercial). The presence of 20% glycerol in the enzyme solution during the
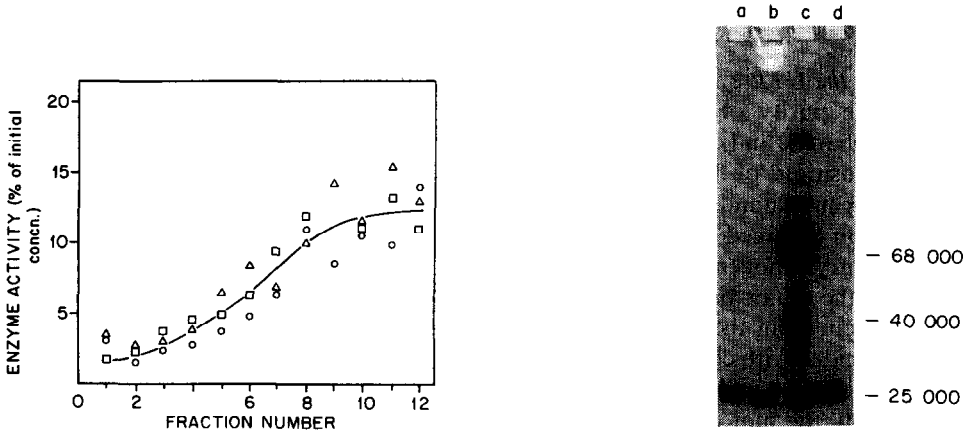

Fig. 2. GTP cyclohydrolase in flow-through from three GTP-Sepharose columns with different lengths. The bed volume was 3.0 ml but the diameter varied (see text). The sample volume was 50 ml and the fractions were 4 ml. (O) Short, (△) medium and (□) long columns. The flow-rate was 1 ml/min.

Fig. 3. Electrophoretic analysis of GTP cyclohydrolase I. The enzyme was purified from *E. coli* B on the following affinity columns: (a) GTP-Sepharose; (b) UTP-Sepharose; (c) dihydrofolate-Sepharose; (d) GTP-Sepharose. The enzymes in (a) and (d) were purified from separate batches of cells. The molecular weight markers (not shown) were bovine serum albumin (68 000), ovalbumin (40 000) and chymotrypsinogen (25 000).

TABLE III

COMPARISON OF THE EFFICIENCIES OF THE DIFFERENT SHAPED GTP-SEPHAROSE COL-
UMNS IN THE RECOVERY OF THE ENZYME

The values are the percentage of the amount of GTP cyclohydrolase applied to the column as measured
by enzyme activity. The values were obtained from chromatography experiments from Fig. 2. The flow-
rate was 1 ml/min for sample addition, washing and elution.

Shape of GTP-Sepharose column	Enzyme activity (%)	
	Flow-through	Eluted with GTP
Short	8	20
Medium	8	10
Long	8	3

loading step completely eliminated activity in the flow-through but produced no in-
crease in the amount recovered by elution with GTP. Presumably this means that
glycerol facilitated the inactivation of the unadsorbed enzyme when passing through
the GTP-Sepharose.

Influence of the shape of the column on the recovery of enzyme activity

The GTP-Sepharose prepared in this laboratory was used to prepare three
columns, each of 3-ml bed volume but with heights and diameters as follows: short
(1.7 × 1.5 cm I.D.), medium (3.8 × 1.0 cm I.D.) and long (7.8 × 0.7 cm I.D.). The
amount of GTP cyclohydrolase that failed to bind to each of the three columns was
the same for each column (Fig. 2). However, the percentage of enzyme recovered
from the shortest column was 6.7 times that from the longest, as shown in Table III.

Purity of the GTP cyclohydrolase I from different affinity columns

The purity of the GTP cyclohydrolase I obtained from GTP-Sepharose,
UTP-Sepharose and dihydrofolate-Sepharose was assesed by gel electrophoresis (Fig.
3). The positions of the Coomassie Blue-stained bands were compared with those of
bovine serum albumin, ovalbumin, chymotrypsinogen and myoglobin. Over 95% of
the protein obtained from GTP- and UTP-Sepharose migrated at the same position
as chymotrypsinogen (molecular weight, M_r = 25 000), in agreement with earlier
results[5]; the minor bands corresponded to molecular weights of 57 000 and 73 000.
The protein from the dihydrofolate-Sepharose consisted of four strong bands, one
that coincided with chymotrypsinogen, the major band corresponding to a molecular
weight of 70 000 and the other two to 40 000 and 160 000; no band was observed at
M_r 18 000, the molecular weight of dihydrofolate reductase. We conclude that the
M_r 25 000 band represents the GTP cyclohydrolase and that this enzyme is obtained
in a high state of purity from affinity chromatography on GTP- and UTP-Sepharose.

DISCUSSION

Affinity chromatography provides the major purification step for GTP cyclo-
hydrolase I from *E. coli*[5] and from *Serratia indica*[11]. In these cases periodate-oxidized

GTP was linked to Sepharose through a six-carbon chain. From the published accounts it is not clear how reproducibly the GTP cyclohydrolase I is bound and released. In one of our laboratories (K.B.J.) we often experienced unpredictable performance with GTP-Sepharose; on the other hand, our other laboratory (J.J.Y.) seldom experienced such problems. Consequently, we examined this step in the purification and we also examined the behavior of GTP cyclohydrolase I on various affinity columns using competitive inhibitors as ligands.

The competitive inhibition of GTP cyclohydrolase I by dihydrofolate has been described by Then, and he subsequently demonstrated the use of this inhibitor in the purification of this enzyme[16]. ATP and UTP have also been reported to be competitive inhibitors for GTP cyclohydrolase I from *E. coli* with K_i values of 0.25 and 2.9 μM, respectively, compared with the K_m value of 0.2 μM for GTP as substrate, and AMP is not an inhibitor[5].

All the data related to the nucleotide-Sepharose columns suggest that part of the enzyme is inactivated while passing through the column. In the experiment shown in Fig. 1, the capacity of the column to remove enzyme from solution was exceeded but the enzyme that appeared in the effluent contained only 60% of the initial activity. As shown in Table III and Fig. 2, the amount of enzyme activity in the flow-through did not vary with the length of the bed but the overall recovery was still low. The amount of enzyme eluted by GTP varied more than six-fold as the column length varied from 1.7 to 7.8 cm. We found that a major fraction of the enzyme is unaccounted for on combining the activities found in the flow-through and the GTP eluate.

These observations can be explained if one assumes that the inactivation is a function of the number of times a molecule collides with the Sepharose-ligand complex. In a steady-state situation like the one found when the sample is passing through a column whose capacity is being exceeded, the extent of the inactivation would depend on the volume of the column (total number of sites where the enzyme can be bound) (Figs. 1 and 2 and Table III). Once the column has been loaded and the enzyme is being eluted, the extent of the inactivation depends on the length of the column, *i.e.*, the number of sites the enzyme is going to bind before it elutes from the column (Table III). This hypothesis was tested in another way with the use of the ATP-Sepharose and UTP-Sepharose columns. As the enzyme has less affinity for ATP and UTP than for GTP, it would be expected that affinity columns made with ATP and UTP as ligands should produce less inactivation because the enzyme would bind fewer times to the matrix while passing through the column. This is what indeed was found (Table II); the UTP-Sepharose gave the greatest recovery and highest specific activity (UTP is the inhibitor with the lowest affinity for the enzyme). A control with AMP-Sepharose gave no inactivation. This seems to exclude the possibility that either the cyanogen bromide-activated sites on Sepharose or the extender molecules, that were unoccupied with the affinity ligand, could be responsible for the inactivation.

Different mechanisms for inactivation could be proposed but only those which imply that the nucleotide ligand behaves in a catalytic way should be considered, as we found that a GTP-Sepharose column never ceased to inactivate the enzyme no matter how much sample was loaded. We suggest that the nucleotide affinity ligand induces a conformational change in the enzyme that results in irreversible inactivation as observed for other proteins[19,20].

Almost no inactivation was found when dihydrofolate-Sepharose was used, although a more poorly purified enzyme was obtained. It should be noted that the dihydrofolate-Sepharose chromatograms were obtained with different salt and buffer conditions than those for the nucleotide-Sepharose. The yield from this affinity column remained high with all amounts of enzyme tested and the specific activities varied no more than two-fold. When a highly pure enzyme preparation is required, the UTP-Sepharose, arranged in as short a column configuration as possible, would give better results. Both GTP-Sepharose and UTP-Sepharose can produce a homogeneous enzyme provided that the column is washed thoroughly with 0.3 M potassium chloride in buffer B after the enzyme is bound. It is also advisable to wash the column with buffer thoroughly before use (80 bed volumes at least), as the glycerol that is used to stabilize the nucleotide-Sepharose during storage or shipping enhances the inactivation of GTP cyclohydrolase I.

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REFERENCES

1 A. W. Burg and G. M. Brown, *J. Biol. Chem.*, 243 (1968) 2349.
2 G. M. Brown and J. M. Williamson, *Adv. Enzymol.*, 53 (1982) 345.
3 A. Niederwieser, N. Blau, M. Wang, P. Joller, M. Atares and J. Cardesa-Garcia, *Eur. J. Pediatr.*, 141 (1984) 208.
4 R. Matalon, *J. Pediatr.*, 104 (1984) 579.
5 J. J. Yim and G. M. Brown, *J. Biol. Chem.*, 251 (1976) 5087.
6 E. P. Weisberg and J. M. O'Donnell, *J. Biol. Chem.*, 261 (1986) 1453.
7 K. Fukushima, W. E. Richter, Jr. and T. Shiota, *J. Biol. Chem.*, 252 (1977) 5750.
8 Z. Bellahsene, J. L. Dhondt and J. P. Farriaux, *Biochem. J.*, 217 (1984) 59.
9 N. Blau and A. Niederwieser, *J. Clin. Chem. Clin. Biochem.*, 23 (1985) 169.
10 R. J. Jackson, R. M. Wolcott and T. Shiota, *Biochem. Biophys. Res. Commun.*, 51 (1973) 428.
11 M. Kohashi, T. Itadani and K. Iwai, *Agric. Biol. Chem.*, 44 (1980) 271.
12 D. Dorsett, J. J. Yim and K. B. Jacobson, *Biochemistry*, 18 (1979) 2596.
13 D. Dorsett, J. M. Flanagan and K. B. Jacobson, *Biochemistry*, 21 (1982) 3892.
14 D. Dorsett and K. B. Jacobson, *Biochemistry*, 21 (1982) 1238.
15 R. Lamed, Y. Levin and M. Wilchek, *Biochim. Biophys. Acta*, 304 (1973) 231.
16 R. L. Then, *Anal. Biochem.*, 100 (1979) 122.
17 G. L. Miller, *Anal. Chem.*, 31 (1959) 964.
18 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
19 D. F. Ollis and R. Data, *Methods Enzymol.*, 44 (1976) 444.
20 G. Kümel, H. Daus and H. Mauch, *J. Chromatogr.*, 172 (1979) 221.