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Binding of 3-Methylcholanthrene to Anionic Gluthathione-S-Transferase

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INTRODUCTION

Since the first finding of cytosolic binding protein for 3-methylcholanthrene (3-MC) in rat liver by Bresnick, *et al.* (3), considerable interest has been shown in this protein with regard to its possible role in carcinogenesis.

Recent evidence by Tierney, *et al.* (10), in fact, demonstrated the transloca- tion of 3-MC bound to protein which to the nucleous in the same manner as steroid receptors, suggesting an intracelluar carrier function of this protein for carcinogenesis. Although this cytosolic protein has not yet been isolated in a pure form, certain physicochemical characteristics of it, hitherto clarified, are quite analogous to those of an anionic glutathione-S-transferase (GST) which has also recently been found in rat liver cytosol. Both proteins have a molecular weight of 45000, and binding capacity for polycyclic aromatic hydrocarbons (7, 10).

In the present paper, therefore, attempts were made to purify anionic GST and to examine its binding properties of 3-MC.

MATERIALS

Animals: Sprague-Dawley male rats, weighing 80 g to 100 g, were sacrificed to obtain livers.

Chemicals: Most chemicals used in this study, including reduced glutathione, BrCN, 1-chloro-2,4-dinitrobenzene, 3-methylcholanthrane, bilirubin, and deoxy-ribonucleic acid from calf thymus, were purchased from Sigma Chemical Co.

Materials for Chromatography: Sephadex G 100, Sepharose 6B-CL, and BrCN-activated Sepharose 4B were obtained from Pharmacia Chemical Co. DEAE cellulose (DE52) was obtained from Whatman Chemical Co. ^{3}H-3-methy-

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cholanthrene was purchased from Amersham Seales. Co.

METHODS

Enzyme assay: GST acitivities were determined spectrophotometrically at 343 nm according to the method of Bhargava, *et al.* (2). Reactions were initiated at 25° C by the addition of 10–50μl of sample in a reaction mixture containing 0.1 M sodium phosphate buffer, pH 6.5, 1.3mM 1-chloro-2,4-dinitrobenzene (CDNB) and 2.5 mM reduced glutathione. All assays were based on the linear functions of protein concentration and reaction rate was linear with time for at least 3 min. Non enzymic increases of absorbance due to nonenzymic reactions were subtracted from the reaction rates. The unit of enzyme activity was defined as 1μmol of substrate utilized per min per mg of protein at 25°C.

Purification of Anionic and Cationic GST: Rat livers was homogenized in 4 volumes of 0.25 M sucrose, 10 mM sodium phosphate buffer, pH 7.4, with a Teflon glass homogenizer (Arthur H. Thomas, Co.). The homogenate was then centrifuged at 10000 g for 30 min and the resulting supernatant was further centrifuged at 105000 g for 2 hrs in a Beckmann Spinco Model L ultracentrifuge to remove residual cell debris. The liver cytosol thus obtained was then dialysed against 10 mM Tris-HCl buffer, pH 8.0 (equilibrating buffer) for 2 nights, after which an aliquot (100 ml) was applied to a column of DEAE cellulose (DE 52, Whatman, Co., 3×20 cm) which has been preequilibrated with the same buffer. After allowing the sample to flow through under gravity, the column was washed with the equilibrating buffer until the eluate was free of transferase activity.

The eluate and wash, which contained cationic GST, were pooled and subjected to further purification of the enzyme according to published procedures (4). The other enzyme fraction (anionic GST) absorbed to the column was eluted with a linear gradient consisting of equal parts of the equlibrating buffer (500 ml) and the same buffer containing 80 mM NaCl (500ml). The activity of anionic GST was recovered as a single broad peak between 25 mM and 42 mM of NaCl. The eluate containing crude anionic GST fraction was then concentrated on Amicon membrane PM 10 and subsequently subjected to affinity chromatography of glutathione.

The preparation of the glutathione affinity column was done according to the method of Inoue, *et al.* (4). First, a 30 ml sample containing 400 to 500 units of anionic GST was added to the affinity column (3×10 cm). Then the column was washed throughly with 10 mM sodium phosphate buffer, after which the washing was discarded. Next, GST bound to immobilized glutathione was eluted from the column with 10 mM reduced glutathione in 10 mM Tris-HCI buffer, pH 8.0.

The final step of purification was carried out by gel filtration on a Sephadex G

100 column (2.5×100 cm) with 10 mM Tris-HCI buffer, pH 8.0. The peak of GST activity was eluted in fractions with an estimated molecular weight of 45000. The specific activity of the thus purified anionic GST was 19.5 μmol/min/mg.

Double immunodiffusion: Ouchterloney double immunodiffusion was carried out in 1% agarose gel in a 0.15 M NaCl solution, pH 7.4. Samples containing 0.5 mg/m*l* of protein were applied to each well and diffusion was allowed to proceed for 24 hours at 20°C in an airtight moist atmosphere. Rabbit anti-rat anionic GST antiserum was prepared according to procedures described earlier. (8).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE): SDS-PAGE was performed in 8-22% polyacrylamide gradient slab gel containing 0.1% sodium dodecyl sulfate. Protein samples (0.5 mg/m*l*) were heated at 100°C for 3 min in 10 mM Tris-HCI buffer, pH 6.8, containing 1%-β-mercaptoethanol, 1% SDS, 5% glycerol before application into the gel. The gel was then stained with Coomassie brilliant blue and destained by the stepwise procedure described by Drysdale and his coworkers (1). The molecular weights of the components were estimated from relative electrophoretic mobility using myoglobin, cytochromec, ovalubumin, and bovine serum albumin as standards.

Circular Dichroism (*CD*): A Cary model 60 spectropolarimeter with a 6001 CD attachment was employed for CD studies. Cells of 1.0 and 0.1 cm path length were used and the dynode voltage of the instrument was less than 600 volts for all measurements. Protein samples, bilirubin and 3-MC were dissolved in 10 mM sodium phosphate buffer, pH 7.4, 10 mM NaOH and 100% dioxane, respectively.

Preparation of DNA Affinity Column: Two mg of calf thymus DNA (Sigma Co.) was dissolved in 0.1 M NaHCO3, pH 7.5 and was coupled to 6 m*l* of packed BrCN -Sepharose 4B gel as described by Schabort (9).

RESULTS

SDS-PAGE Pattern of Anionic and Cationic GST

The purity and subunit composition of anionic GST as compared with those of cationic GST. were analysed by SDS-PAGE

As shown in Fig. 1, only one component with electrophoretic mobility corresponding to a molecular weight of 23000 was observed in the preparation of anionic GST, suggesting that this preparation was highly pure in electrophoretic entities. It was also suggested that the native molecules of anionic GST consisted of two subunits of similar size, since the molecular weight of the native enzyme estimated by gel filtration was 45000 (cf. purification prodedure).

On the other hand, cationic GST was composed of two types of subunits with molecular weights of 25000 and 22000, respectively.

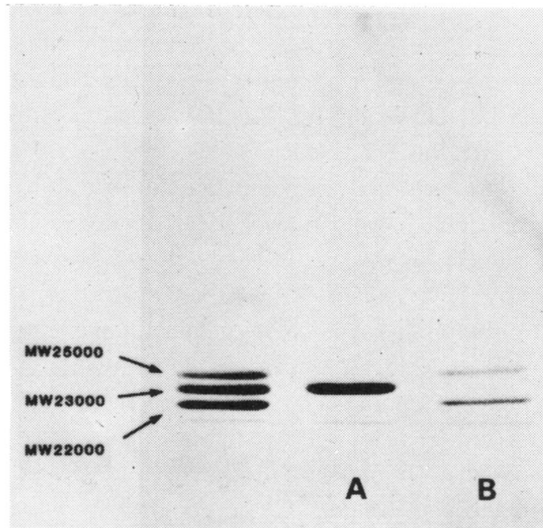
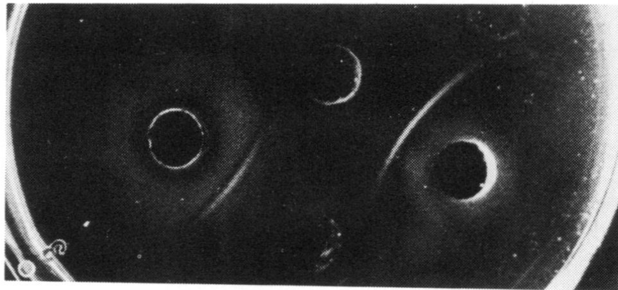


Fig. 1 SDS Polyacrylamide Gel Electrophoresis. Anionic glutathione-S-transferase (GST) (A) has only one component with a molecular weight of 23000. Cationic GST (B), however, has two subunits with molecular weights of 25000 and 22000.



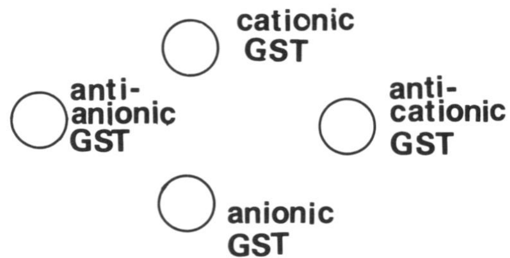


Fig. 2 Double Immunodiffusion: Note that there is no cross reaction between anti-anionic GST antiserum and cationinc GST or between anti-cationic GST antiserum and anionic GST.

Double Immunodiffusion of Anionic and Cationic GST

Double immunodiffusion was used to investigate the immunological difference between anionic and cationic GST. Anti-anionic GST antiserum and anti-cationic GST antiserum reacted to form precipitation lines with anionic GST and cationic GST, respectively. No cross reaction, however, was observed between antianionic GST antiserum and cationic GST or between anti-cationic GST antiserum and anionic GST. It was, therefore, evident that anionic GST and cationic GST were immunologically different from each other (Fig. 2).

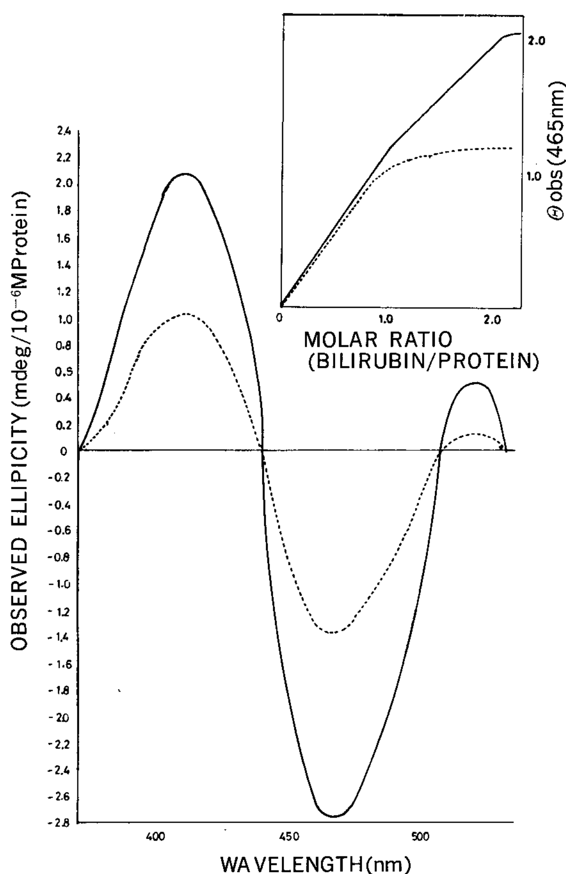


Fig. 3 Circular Dichroism Spectrum of Bilirubin-GST Complex : The solid line (———) indicates the bilirubin-anionic GST complex and the dotted line (·········) indicates the bilirubin-cationic GST complex. The ellipticity extrema at wave lengths of 405 nm, 465 nm and 515nm, are noted. The insert figure shows the titration of GSTs with bilirubin as reflected by ellipticity bands.

CD Studies of the 3-MC-Anionic GST Complex

Binding of 3-MC to anionic GST was investigated by CD titration. Because the binding of 3-MC to anionic GST itself did not produce any ellipticity, competitive binding studies between bilirubin and 3-MC were performed. The CD spectra of the bilirubin anionic GST complex featured three ellipticity extrema at wave lengths of 405 nm, 465 nm, and 515 nm (Fig. 3). At the band of 465 nm, the magnitude of ellipticities of bilirubin-anionic GST and that of bilirubin-cationic GST increased up to a molar ratio of 2 : 1 and 1 : 1, respectively. (Fig. 3. insert).

3-MC was added to the solutions of these complexes. As the concentration of 3-MC increased, ellipticity at 465 nm of bilirubin-anionic GST complexs decreased linearly, indicative of the displacement of bilirubin by 3-MC. With the bilirubin -cationic GST complex, on the other hand no change in ellipticity was observed following by the addition of 3-MC (Fig. 4).

Binding of ^3H 3-MC-Anionic GST Complex to DNA Affinity Column

Anionic GST (0.7 mg) was incubated with 50μM ^{3}H 3-MC in 10 mM sodium

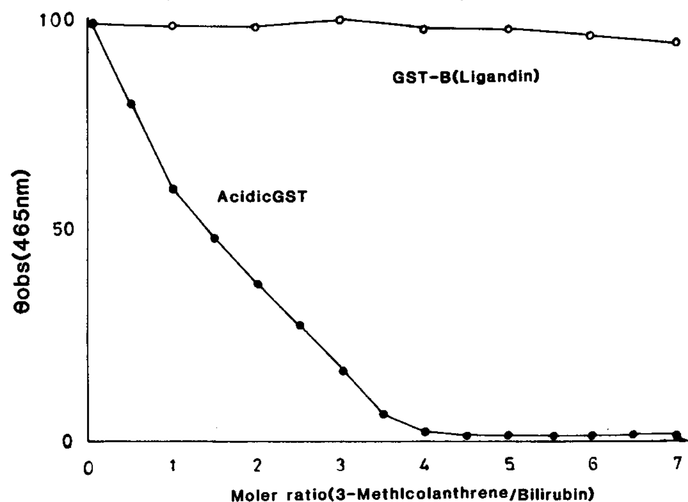


Fig. 4 The effect of 3-MC on CD Spectrum of the bilirubin-Cationic GST and the bilirubin-anionic GST.

phosphate buffer, pH 7.4, at either 0℃ or 27℃ for 20 min. Separation of the bound form from the free form was performed by the charcoal-dextran method (1% Norit A and 0.1% Dextran T 70).

^{3}H 3-MC-anionic GST complexes were then applied to a DNA affinity column at 0°C or 27°C (Fig. 5) At 0°C, most complexes passed through the column after washing with 10 mM sodium phosphate buffer, pH 7.4. At 27°C, in contrast, approximately 80% of complexes bound to the column and were eluted by application of a linear gradient of NaCl (0-200 mM) in the buffer mentioned above.

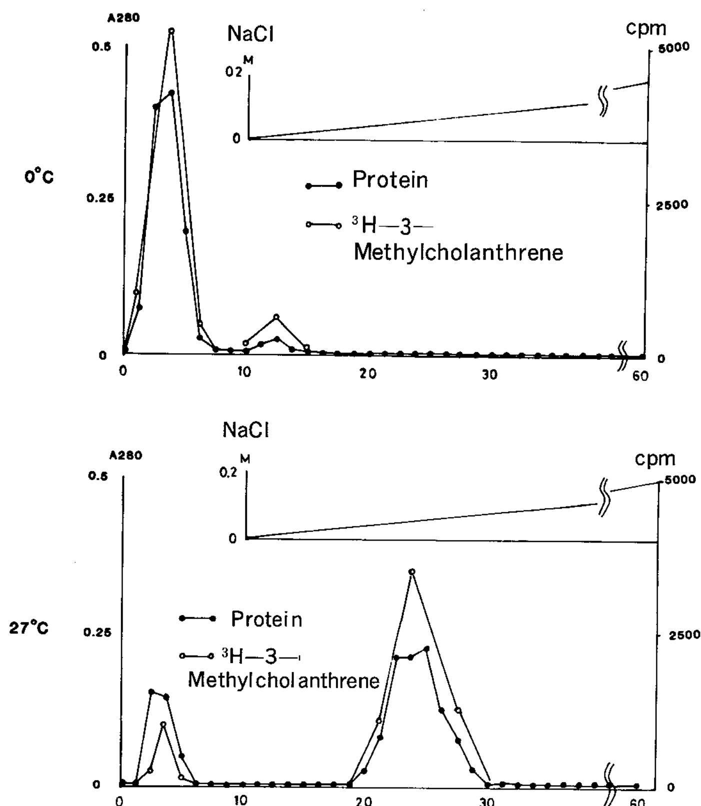


Fig. 5 DNA Affinity Chromatography of ^{3}H 3-MC-Anionic GST Complex : The solid circles represent protein absorbance at 280 nm and open circles represent radioactivity of the complex with ^{3}H 3-MC. The insert is of a gradient of NaCl concentration.

DEAE-Cellulose Chromatography of ^{3}H 3-MC-Anionic GST Complex

^{3}H 3-MC-anionic GST complexes were subjected to a DEAE cellulose column at 0°C or 20°C. Chromatographic procedures were essentially the same as that used for purification of anionic GST (cf. experimental methods). At 0°C, the main peak of complexes absorbed on the column was eluted by increasing the concentration of NaCl to 200 mM in 10 mM sodium phosphate buffer, pH 7.4. On the other hand, at 20°C, most complexes were not absorbed on the column, but were recovered in fractions passed through the column (Fig. 6).

Discussion

3-methycholanthrane (3-MC) is a chemical carcinogen which belongs to a

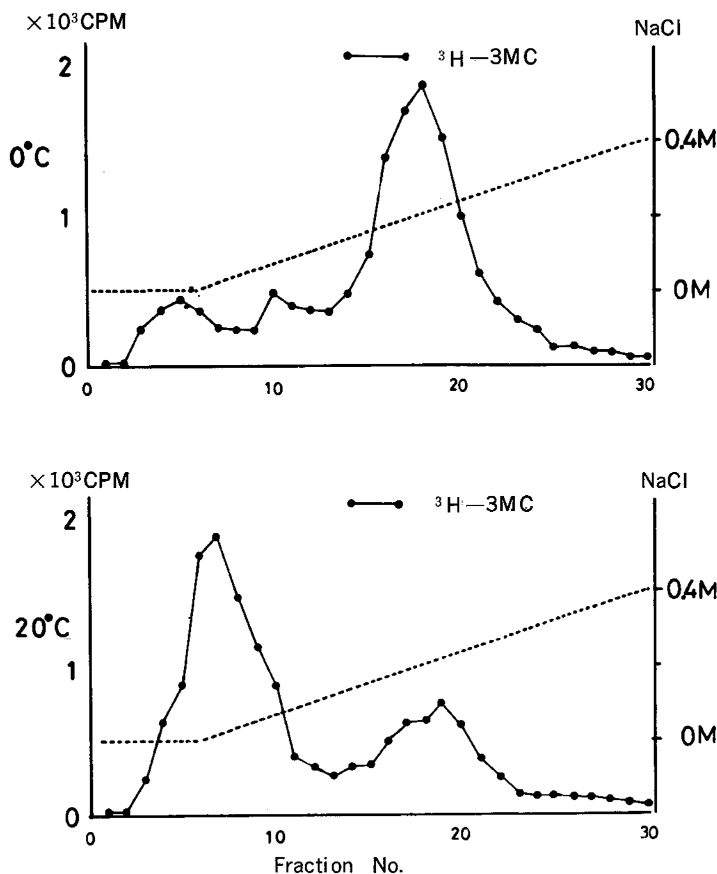


Fig. 6 DEAE Cellulose Chromatography of ^{3}H 3-MC-Anionic GST Complex : The solid line presents radioactivity of the complex, while the dotted line is a gradient of NaCl concentration.

family of carcinogenic polycyclic aromatic hydrocarbons. A carrier protein for 3-MC has been recently identified in rat liver cytosol (10). This cytosolic protein has been able to translocate the carcinogen into the nucleus when incubated with rat liver nuclei (10).

The physicochemical properties of the protein, have not been fully elucidated, since it has not yet been isolated in a pure form. In the present study, anionic glutathione-S-transferase (GST) was purified from rat liver and the interaction of 3-MC with this enzyme was investigated.

GSTs represent a family of multifunctional enzymes which share a number of common properties (5). Thus, all forms exhibit GST activity, catalyze conjugation reactions between glutathione (GSH) and a variety of organic molecules

bearing electropholic centers, and have broad specificity for binding of lipophilic substances (9).

Until quite recently all well characterized species of GST found in rat liver were cationic forms with pI values in the range of 8.1–9.6. More recent work, however, has revealed the presence of anionic form of GST with pI 6.7 in rat liver cytosol (7). One of the prominent properties of this anionic GST is its higher affinity for steroids as compared to the cationic forms (7). The complex of this enzyme with steroid was activated to interact with DNA by incubation at 27°C in a similar manner to the specific binder for 3-MC mentioned above (heat sentitive activation) (7).

This apparent similarity between the anionic form of GST and 3-MC binder convinced us to study the binding of 3-MC to anionic GST. The anionic GST prepared from rat liver was electrophoretically pure and had a similar molecular weight (45000) to that of 3-MC binding protein (10). The subunit structure of anionic GST was clearly different from that of the cationic form of GST (Glutathine-S-transferase B or Ligandin (7)). The former consisted of two homogeous subunits with molecular weight of 23000 whereas the latter was a heteropolymer molecule of two different subunits with molecular weights of 25000 and 22000. The immunological differences between these enzymes were also distinct as revealed by double immunodiffusion test (Fig. 2).

These structural and immunological difference were naturally reflected in their binding capacity to 3-MC. Two molecules of bilirubin attached to anionic GST were totally replaced by the addition of a 4 fold molar excess of 3-MC, suggesting that 3-MC probably bound to the enzyme by competing for the binding site with bilirubin. On the other hand, no replacement of bilirubin by 3-MC was observed for cationic GST (GST-B).

In addition, the temperature dependent activation of the complex of 3-MC -anionic GST was demonstrated using DNA affinity chromatography (Fig. 5). The apparent conformational change of the complex caused by the heat sensitive activation was also confirmed by the finding that, while at 20°C the complexes passed through the column of DEAE cellulose, at 4°C they stayed in the column even after extensive washings (Fig. 6). These results strongly suggest that anionic GST is carrier protein for 3-MC in rat liver cytosol.

The biological function of this 3-MC binding enzyme *in vivo* is currently under investigation in our laboratory.

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