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SUBUNIT COMPOSITION OF RAT LIVER GLUTATHIONE S-TRANSFERASES

Chen-Pei D. Tu*, Mitchell J. Weiss and C. Channa Reddy†

Department of Biochemistry, Microbiology, Molecular and Cell Biology
and †Center for Air Environment Studies
The Pennsylvania State University, University Park, PA 16802

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SUMMARY: The plasmid pGTR112 contains partial coding sequences for one of the rat liver glutathione S-transferase subunits. We have used immobilized pGTR112 DNA to select for complementary and homologous liver poly(A)-RNAs under conditions of increasing stringency for hybridization. Each fraction of selected poly(A)-RNAs was assayed by in vitro translation followed by immunoprecipitation. A total of four distinct polypeptides precipitated by antiserum against rat liver glutathione S-transferases were resolved by $NaDodSO_4$ polyacrylamide gel electrophoresis. They are separated into two pairs according to the sequence homology of their poly(A)-RNAs with the pGTR112 DNA. Purified rat liver glutathione S-transferases can be resolved on gradient $NaDodSO_4$ polyacrylamide gels into four polypeptides. There should be ten isozymes of different binary combinations from four distinct subunits for the rat liver glutathione S-transferases.

INTRODUCTION

The rat liver glutathione S-transferases (E.C. 2.5.1.18) are a group of dimeric, multifunctional proteins which can be induced to a higher level by many xenobiotics such as phenobarbital (1-3). The subunit molecular weights of liver glutathione S-transferases range from 22,000 to 27,500. Approximately 90% of the rat liver glutathione S-transferases can be adsorbed onto CM-cellulose cationic ion exchanger and at least six peaks of activities are resolved by elution with a salt gradient. They have been referred to as transferases E, D, C, B, A and AA based on their order of elution off a CM-cellulose column. All forms of transferase isozymes have been recovered from a single rat liver (4). Several groups have suggested that the glutathione S-transferases consist of various pairs of three subunits designated as Ya, Yb, and Yc with molecular weights of 22,000, 23,500, and 25,000 respectively (5-7). Two groups have provided evidence that transferases C, B, A and AA are composed of YbYb, YaYc, YbYb and YcYc respectively (7,8). Also it has been suggested that a selective proteolytic cleavage may contribute to the multiplicity of glutathione S-transferases (6,9). However, evidence presented by Pickett et al. on the differential induction of Ya

*To whom correspondence should be addressed.

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and Yc subunits suggests that their mRNAs are under independent regulation (10). The glutathione S-transferase activities in the "flow through" fraction of CM-cellulose column chromatography are presumably anionic isozymes but very little is known about them (11-13). The three-subunit hypothesis is not sufficient to account for all the known liver glutathione S-transferases. Proteolytic mechanisms might generate further multiplicity, but it is difficult to explain why only transferase A and transferase C have immunological cross reactivity (14). In this communication, we report the detection of poly(A)-RNA species for various glutathione S-transferase subunits by a cDNA plasmid pGTR112 containing partial coding sequences for one of the rat liver glutathione S-transferase subunits (molecular weight 26,000). Our results clearly indicate that there is sequence homology among the various liver poly(A)-RNA species for the glutathione S-transferases and that the minimum number of subunits for liver isozymes is four.

MATERIALS AND METHODS

Purification of glutathione S-transferases from rat livers, preparation of rabbit antiserum against mixtures of total isozymes, characterization and sequence analysis of pGTR112 DNA have been reported earlier (15). The rabbit reticulocyte lysate system for *in vitro* translation was obtained from BRL, Inc. (Gaithersburg, MD). The ^{35}S-methionine (specific activity > 800 Ci/mmole) was from New England Nuclear (Boston, MA). *In vitro* translation, poly(A)-RNAs purification, immunoprecipitation, polyacrylamide gel electrophoresis and fluorography were carried out as previously described (15). Procedures for hybrid-selected translation are described in the figure legend.

RESULTS

The cDNA plasmid pGTR112 contains partial coding sequences for one of the rat liver glutathione S-transferase subunits, most probably the larger subunit of ligandin (15). In addition to their common requirement for GSH, various isozymes of glutathione S-transferases have different but overlapping substrate specificities. Therefore, we assumed that there are some sequence homologies among the subunits of rat liver glutathione S-transferases. DNA sequencing results and hybrid-selected translation experiments presented by us, (15) and by Kalinyak and Taylor (16), clearly indicate that two of these subunits have extensive sequence homologies. We therefore used pGTR112 DNA immobilized on diazophenylthioether (DPT) paper to select for complementary and partially homologous liver poly(A)-RNAs. The poly(A)-RNAs bound to the DPT-paper were recovered under increasing stringency by stepwise elution at various temperatures from 60° to 100°. Each fraction of poly(A)-RNAs was assayed for its ability to synthesize glutathione S-transferase subunits *in vitro* in the rabbit reticulocyte lysate system (17) followed by immunoprecipitation with antiserum against total rat liver glutathione S-transferases. As shown in Figure 1 (Panel a), the poly(A)-RNAs selected by pGTR112, especially those eluted at 80° or higher, programmed the synthesis of

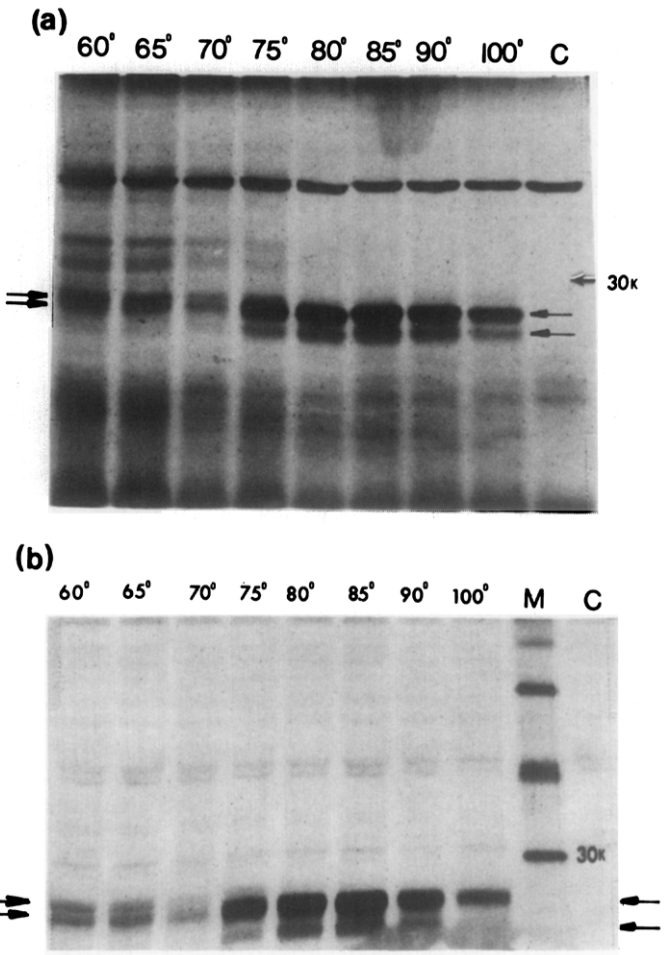

Figure 1. In vitro translation of rat liver poly(A)-RNA selected at different temperatures by pGTR112 DNA-DPT paper. Approximately 40 µg of liver poly(A)-RNAs from pentobarbital treated rats was incubated with pGTR112 DNA (10 µg) covalently bound to DPT-paper at 37° for 16 hours in 0.6 ml volume of 50% formamide, 0.6 M NaCl, 75 mM sodium citrate, 100 µM sodium phosphate pH 7.0, 0.1% $NaDodSO_4$, 200 µg/ml poly(adenylate) and 200 µg/ml tRNA. After hybridization, the DPT-paper was washed at 37° in 5 ml of 50% formamide, 0.2 M NaCl, 8 mM sodium citrate, 0.2% $NaDodSO_4$ for 10 minutes three times. Poly(A)-RNA hybridized to DPT-paper was successively eluted at 60°, 65°, 70°, 75°, 80°, 85°, 90°, and 100° by incubation with 0.4 ml of 10 mM EDTA (pH 7.8) and 0.1% $NaDodSO_4$ for four minutes. At the end of each incubation, 0.4 ml of liquid was removed very quickly, adjusted to 0.3 M NaCl and 50 µg/ml yeast tRNA and precipitated with ethanol. The pellet was washed once in 70% ethanol, redissolved in 0.3 ml of 0.3 M potassium acetate (pH 7.2) and precipitated with ethanol. The pellet was washed once in 70% ethanol, dried under vacuum and resuspended in 10 µl of sterile H_2O for in vitro translation in a final volume of 30 µl. Aliquots (5 µl) were analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis and visualized by fluorography as previously described (Panel a). The majority of the total in vitro translation mixture (23 µl) for each fraction of eluted poly(A)-RNA was immunoprecipitated by antiserum against total rat liver glutathione S-transferases according to Gilman et al. as previously described. Aliquot (60%) of each fraction of immunoprecipitated polypeptides was analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis and fluorography (Panel b). C lanes are control experiments without poly(A)-RNA. M lane is the ^{14}C molecular weight markers (Amersham Corp.). Positions of 30K molecular weight are labeled. Glutathione S-transferase polypeptides are labeled by horizontal arrows.

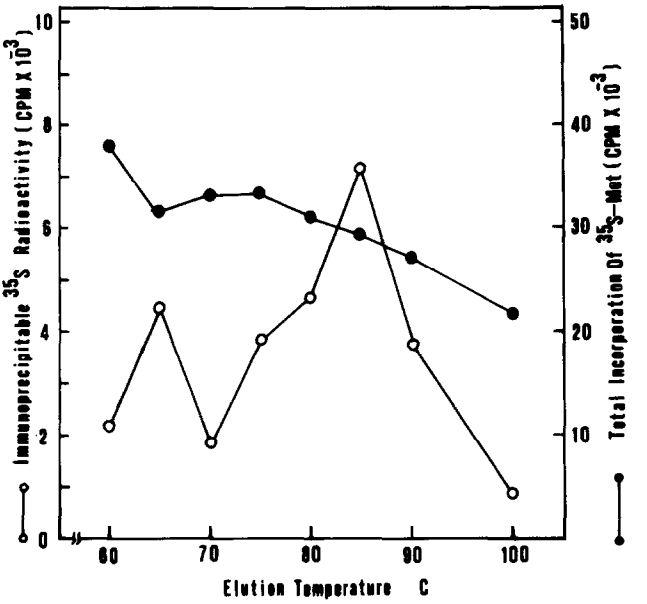

Figure 2. Acid precipitable ^{35}Sradioactivity of in vitro translation products programmed with poly(A)-RNAs eluted from pGTR112 DNA-DPT paper at various temperatures. These results were obtained from the same set of experiments described under Figure 1. Each point represented 5.4% of the total sample at the temperatures indicated. (●-●): total incorporation of ^{35}S-methionine; (○-○): immunoprecipitable ^{35}S radioactivity.

polypeptides less than 30,000 molecular weight. After immunoprecipitation, (Panel b), the predominant polypeptides show that the glutathione S-transferase subunits have molecular weights of 27,500, 26,000, and 24,000 relative to the ^{14}C molecular weight markers included in the same gel. It is obvious that the intensity of the 26,000 polypeptide bands decreases to a minimum in poly(A)-RNA fraction selected at 70° and increases to a maximum in poly(A)-RNAs selected at 85°. This difference was quantitated by measuring acid (trichloroacetic acid) precipitable ^{35}S radioactivity of the in vitro translation mixture before and after immunoprecipitation (Figure 2). While the total poly(A)-RNAs selected at each temperature were continuous with respect to total ^{35}S-methionine incorporation, acid precipitable radioactivity of the immunoprecipitated polypeptides was detected in two separate peaks. Therefore, two 26,000 molecular weight polypeptides differing from each other in their respective poly(A)-RNA sequence homology toward pGTR112 DNA exist in the liver glutathione S-transferase subunits. The total number of liver glutathione S-transferase subunits determined by in vitro translation of liver poly(A)-RNAs is four instead of three reported by others (5-9).

To substantiate this finding with purified rat liver glutathione S-transferases, we subjected a mixture of homogeneous isozymes purified from fresh rat livers to the same denaturing gel electrophoresis conditions (18) as the ones used in resolving the in vitro translation products. Our results

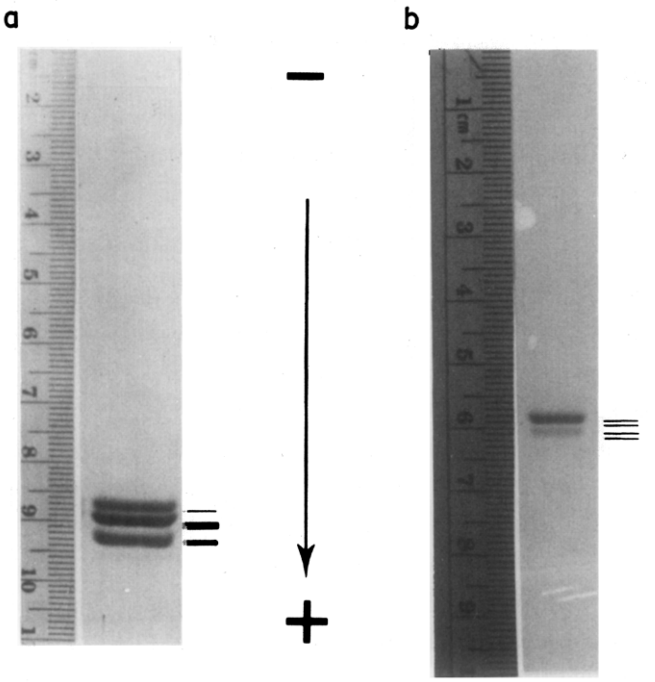

Figure 3. Subunit composition of purified rat liver glutathione S-transferases revealed by NaDodSO$_4$-polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue R. The glutathione S-transferases have been purified through the S-hexylglutathione-linked sepharose 6B column (15). The direction of electrophoresis is indicated by the arrow. The buffer system of Laemmli was used as previously described. Gradient polyacrylamide gels were made with an LKB gradient gel former (2001-500) and run with the LKB 2001-001 vertical electrophoresis unit. a. 11% polyacrylamide gel. b. 9% to 13% gradient polyacrylamide gel.

shown in Figure 3(a) gave the expected pattern: three bands can be resolved, with the middle band much more intense than the two neighboring bands. When a gradient polyacrylamide gel (19) was used under denaturing conditions, four distinct bands can be resolved as shown in Figure 3(b). Therefore, the total number of liver glutathione S-transferase subunits is four as determined from purified enzymes and *in vitro* translation of liver poly(A)-RNAs. This number is mathematically sufficient to generate ten different dimeric isozymes without involvement of any proteolytic mechanisms.

From the results in Figures 1 and 2, the four subunits can be grouped into two pairs. The poly(A)-RNAs of one of the pairs (i.e., for the 27.5K and 26K subunits) were selected by elution from pGTR112 DNA between 60° to 70° indicating lesser sequence homology. However, judging from the intensity of each subunit in the pair, those poly(A)-RNAs seem to have similar extent of relative sequence homology to pGTR112 DNA. The poly(A)-RNAs of the other subunits (i.e., for the 26K and 24K subunits) were removed mainly between 80° and 100° indicating extensive sequence homology with pGTR112 DNA. The

extent of sequence homology between this latter pair of poly(A)-RNAs has been demonstrated in an earlier report (15) based on DNA sequencing results of the pGTR112 DNA (for one of the 26K subunits) and pGST94 DNA (for the 24K subunit). Among a stretch of 352 base pairs, there are only nine nucleotide differences between the two cDNA clones which probably correspond to the large and small subunits of ligandin.

DISCUSSION

There are four different subunits for the rat liver glutathione S-transferases as determined by in vitro translation of poly(A)-RNAs selected by the pGTR112 DNA followed by immunoprecipitation with antiserum against total liver glutathione S-transferases. When purified rat liver glutathione S-transferases were resolved on gradient polyacrylamide gels under denaturing conditions, four polypeptides can be resolved. However, two of them migrate together when a uniform $NaDodSO_4$ gel was used. Since the glutathione S-transferases are known to be dimeric proteins, there should be ten isozymes of different binary combinations with these four different subunits. So far at least six cationic rat liver glutathione S-transferases have been purified and characterized (1,13). With inclusion of previously unresolved cationic glutathione S-transferases (20), and the reported anionic glutathione S-transferases (11,12), the three-subunit hypothesis cannot account for these experimental findings. Although proteolysis of specific subunits can serve as a means of generating additional subunits and isozymes, the experimental evidence for such specific proteases acting upon glutathione S-transferase subunits in vivo is lacking. By taking a molecular approach, with a cloned glutathione S-transferase coding sequence as hybridization probe, we have successfully demonstrated that there are enough poly(A)-RNA species to code for four different yet partially homologous glutathione S-transferase subunits. Although we do not have experimental evidence to rule out any specific protease activity in post-translational processing of glutathione S-transferases, our finding of four distinct subunits is sufficient to account for the number of known liver glutathione S-transferase isozymes resolvable by ion exchange chromatography (20). From the extent of DNA sequence homology between the known sequences of two subunits (15), it is interesting to speculate the genetic origins of these coding sequences. Whether they are generated at the level of DNA rearrangements or RNA processing, or simply from a multigene family, remains to be investigated.

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