Characterization of Glutathione S-Transferases from Day-Old Chick Livers†

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ABSTRACT: Glutathione S-transferases (GSTs, EC 2.5.1.18) were isolated from the liver cytosolic fraction of 1 day old Leghorn chicks by S-hexylglutathione and glutathione affinity columns arranged in tandem. After sample loading, the affinity columns were detached from each other and developed separately. Four groups of GSTs (CL 1, 2, 3, and 4) were eluted from the hexylglutathione column, and an additional group of GSTs (CL 2 and 5) was eluted from the glutathione affinity column. CL 2, CL 3, and CL 5 were further purified to homogeneity by chromatofocusing, and the substrate specificities of each group were determined. Fractions from the chromatofocusing column were analyzed by native IEF electrophoresis. Protein bands were electroblotted onto PVDF membrane for N-terminal sequence analysis or extracted from IEF gel and rerun on SDS-PAGE to determine the subunit composition of each GST dimer. CL 2, CL 3, and CL 5 can form homodimers, whereas CL 1 and CL 4 exist only as CL 1-2 and CL 3-4 heterodimers. CL 2 and CL 5 have N-terminal amino acid sequences homologous to rat liver Yb and Ya GSTs, respectively. CL 1 has a unique N-terminal sequence that is not homologous to any known GSTs.

Glutathione S-transferases (GSTs,[1] EC 2.5.1.18) comprise a group of functionally similar dimeric proteins that catalyze the conjugation of hydrophobic, electrophilic substrates to glutathione (Habig et al., 1974; Mannervik, 1985). This reaction is considered to be the initial step in the formation of mercapturic acids, a pathway through which hydrophobic xenobiotics are inactivated and eliminated from the body (Jakoby & Habig, 1980; Chasseaud, 1979).

The rat and human cytosolic glutathione S-transferases have been the subject of numerous studies. GSTs exist as isoenzymes that can be distinguished on the basis of their physical, chemical, immunological, enzymatic, and structural properties (Warholm et al., 1983; Tahir et al., 1985; Alin et al., 1985a). In rat liver, there are at least eight homo- or heterodimeric GSTs composed of subunits of 24-30 kDa that are products of a gene superfamily (Abramovitz & Listowsky, 1987; Lai & Tu, 1986; Ding et al., 1986; Satoh et al., 1985; Telakowski-Hopkins et al., 1985). The amino acid sequence of several GSTs from human or rat tissues has been deduced from cDNA clones or from direct sequencing (Frey et al., 1983; Pickett et al., 1984; Lai et al., 1984; Taylor et al., 1984; Dao et al., 1984).

Avian glutathione S-transferases have received less attention. Yeung and Gidari (1980) purified to homogeneity a dimeric protein of p*I* 8.9 from chicken liver by a combination of ion-exchange chromatography and preparative isoelectric focusing. Several peaks of transferase activity were eluted from the column, implying the presence of isozymes as in mammalian systems.

In the present paper, we report the purification of different isozymes of glutathione S-transferases from day-old chick liver by S-hexylglutathione and glutathione affinity chromatography, followed by chromatofocusing. These enzymes were characterized according to their isoelectric point, subunit composition, substrate specificity, immunological cross-reactivity with antisera against rat liver GSTs, and partial N-terminal amino acid sequencing.

Experimental Procedures

Materials. Male white Leghorn chicks were obtained from Taiwan University Experimental Station and sacrified 1 day after hatching. Ampholytes pH 5-7 and pH 3-10 (Bio-Lyte) were electrophoretic-grade chemicals from Bio-Rad Laboratories. Ampholytes pH 7-9 and pH 9-11 were from LKB. Pharmalyte pH 8-10.5, epoxy-activated Sepharose 6B, Polybuffers, and the prepacked Mono P column were from Pharmacia. ^{125}I-Protein A was purchased from Amersham. Chemicals used in sequencing were obtained from Applied Biosystem Inc. (ABI). Poly(vinylidene difluoride) (PVDF) membranes were from Millipore Co. All other chemicals were obtained from Merck and Sigma and were reagent grade or better.

Buffers. Buffer A contained 10 mM Tris-HCl, pH 8.0, and 6 mM 2-mercaptoethanol. Buffer B contained 22 mM potassium phosphate, pH 7.0, and 6 mM 2-mercaptoethanol. Buffer C contained 10 mM potassium phosphate, pH 7.0, and 1 mM DTT. Buffer D contained 10 mM sodium phosphate, pH 7.0, 140 mM NaCl, and 2.7 mM KCl.

Purification of GSTs from Chick Liver. Approximately 90 g of fresh livers from 1 day old chicks was used in a typical enzyme preparation. The procedures were those used to purify GSTs from sheep liver up to the affinity column step (Reddy et al., 1983). S-Hexyl-GSH-linked Sepharose 6B and GSH-linked Sepharose 6B were prepared according to Mannervik and Guthenberg (1981) and Simons and Vander Jagt (1981), respectively. Crude liver extracts from 100 chicks in buffer A (390 mL) were first passed through an S-hexyl-GSH-linked Sepharose 6B column (2.8 × 14 cm) preequilibrated with the

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[1] Abbreviations: GSTs, glutathione S-transferases; GSH, glutathione; IEF, isoelectrofocusing; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; TEA, triethylamine; CDNB, 1-chloro-2,4-dinitrobenzene.

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same buffer and then loaded directly onto a GSH-linked Sepharose 6B column (2.8 × 4 cm) that had been preequilibrated with buffer B. After sample loading, the affinity columns were detached from each other and developed separately. The *S*-hexyl-GSH-linked affinity column was washed with 0.2 M NaCl in buffer A, and the enzymes were eluted with 5 mM *S*-hexylglutathione, 2.5 mM glutathione, and 0.2 M NaCl in buffer A. The GSH-linked Sepharose 6B column was first washed with buffer B and then eluted with 5 mM glutathione and 6 mM 2-mercaptoethanol in 50 mM Tris-HCl, pH 9.6. Fractions with UV absorption at 280 nm were pooled and thoroughly dialyzed against buffer C before storage.

Chromatofocusing. Samples from the *S*-hexylglutathione affinity column were subjected to chromatofocusing by a modification of the procedure of Alin et al. (1985b). Sample in buffer C (15 mg, approximately 10 mL) was adjusted with 25 mM TEA to pH 10 and applied immediately onto a Mono P column preequilibrated with 25 mM TEA, pH 10.6. Elution was first performed with 50 mL of 1:100 diluted Pharmalyte (8–10.5), pH 9, followed by 50 mL of a mixture of 1 mL of Pharmalyte (8–10.5) and 5 mL of Polybuffer 96 diluted to 100 mL with water and adjusted to pH 7.0 with HCl. Proteins eluted from the column were detected by UV absorption at 280 nm and were dialyzed against buffer C before storage.

Enzymatic Assay. GST activity was detected directly on IEF gels according to the method of Ricci et al. (1984) or assayed with CDNB and other substrates according to the methods of Habig and Jakoby (1981), Habig et al. (1974), and Lawrence and Burk (1976). A unit of activity is defined as that amount of enzyme that catalyzes the formation of 1 μmol of product/min at 25 °C. Protein concentration was determined by the dye-binding method of Bradford (1976). Specific activity is expressed as units of activity/mg of protein. Rat liver GSTs used in the assay were prepared according to the method of Jensson et al. (1985).

Electrophoresis. Samples for IEF were incubated with 6 mM 2-mercaptoethanol for 30 min at room temperature before being dried down in a Speed-Vac concentrator. Samples were then resuspended in sample buffer containing 2% ampholytes, 20% glycerol, and 6 mM 2-mercaptoethanol. Isoelectric focusing in the acidic range was performed according to Hsieh et al. (1988) on a 7.5% (w/v) acrylamide vertical slab gel with 2% pH 3–10 ampholytes. The gel solution for basic isoelectric focusing analysis was similar to that of acidic IEF except that 1.33% pH 7–9 ampholytes and 0.67% pH 9–11 ampholytes were used. The anionic and cationic buffers were substituted with 0.1 N H_2SO_4 and 0.1 N KOH, respectively (Hjelmeland et al., 1979). Samples were loaded on the anionic side and ran at 200 V for 16 h without prefocusing. The pH gradient formed in the gel was measured according to Hjelmeland et al. (1979) and confirmed with p*I* markers. For direct protein visualization, gels were fixed with 3.5% (w/v) TCA for 30 min before staining with Coomassie blue R-250.

Proteins focused on IEF gels were visualized with Coomassie blue dye staining, and protein bands were excised from the gel with a razor blade. Proteins were extracted from the gel with 60 μL of SDS buffer (62.5 mM Tris-HCl, pH 6.8, 2.0% SDS, 10% glycerol, and 5% 2-mercaptoethanol) overnight and boiled at 90 °C for 5 min. Samples (20 μL) were then loaded on a 12.5% polyacrylamide–SDS gel according to Laemmli (1970).

Electroblotting. Transfer of proteins from IEF gel onto PVDF membranes was carried out in an apparatus described by Gibson (1981) with graphite slabs as electrodes. Electroblotting was carried out in 1.5% (v/v) acetic acid and 20% (v/v) methanol. Experimental details will be published elsewhere (Tam et al., submitted for publication). Transfer of proteins from SDS gels onto PVDF membranes was carried out similarly, except that 150 mM sodium borate, pH 9.6, 20% methanol, and 0.5% SDS were used as a blotting buffer. Membranes were used directly for immunoblotting or stained with 0.5% Coomassie blue R-250 in 30% 2-propanol and 10% acetic acid for 2 min for protein visualization. Destaining was carried out in the same solution without the dye. The protein bands were then excised from the membrane for N-terminal sequence analysis.

Protein Sequencing. Automated cycles of Edman degradation were performed with an ABI gas/liquid-phase Model 470A/900A sequencer with an on-line Model 120A PTH-amino acid analyzer according to Hewick et al. (1981). Sequencing of proteins from PVDF membranes was performed according to Hsieh (1988).

Antisera. Antisera raised in rabbits against rat glutathione *S*-transferases separated on a CM-cellulose column were available in the laboratory (Tu & Reddy, 1985). The procedure of Ramanathan et al. (1979) was used in preparing the antisera against GSTs from the chick liver.

Immunoblotting. Proteins from IEF or SDS gels were electroblotted onto PVDF membranes as described above. The membrane was first blocked with 10% (w/v) nonfat dry milk in buffer D for 1 h and then incubated with antiserum (1:200 dilution) and 10% nonfat dry milk in buffer D for 1 h, followed by washing with buffer D containing 0.05% Tween 20. The blot was then incubated with ^{125}I-protein A (10^6 cpm) and 10% nonfat dry milk in buffer D for 1 h, washed with buffer D containing 0.05% Tween 20, and prepared for autoradiography.

Results and Discussion

Isolation of Glutathione S-Transferases from Chick Liver. The first step in the purification of GSTs from the 100000*g* supernatant fraction is chromatography on an *S*-hexylglutathione affinity column. Approximately 70% of the enzymatic activity bound to the column. The amount of enzyme retained on the column did not increase with column size, indicating the binding capacity of the column had not been exceeded. The 0.2 M NaCl salt wash removed about 15% of total enzymatic activity from the column whereas elution with *S*-hexylglutathione desorbed in a single peak about 30% of the units contained in the 100000*g* supernatant. The enzyme recovered had a specific activity of about 25 units/mg. The activity and recovery of a typical preparation are shown in Table I.

It has been previously shown that *S*-hexylglutathione affinity columns do not bind rat GST 5–5 and bind only weakly rat Ya GSTs (Mannervik, 1985). Similarly, the GSH conjugation activity in the flow-through fraction of the *S*-hexylglutathione affinity column may represent classes of chick liver GSTs that have low affinity for *S*-hexylglutathione. Therefore, a glutathione affinity column was connected to the *S*-hexylglutathione affinity column in tandem during sample loading. The glutathione affinity column retained about 10% of the total enzymatic activity. About 0.5% of the activity was recovered in the buffer B wash, whereas about 2% of the activity was eluted in a single peak from the column with 5 mM GSH in 50 mM Tris, pH 9.6 (about 80 units). The rest of the activities were coeluted with contaminants and were not collected. The enzymes thus obtained were purified more than 40-fold and have a specific activity of 45.7 units/mg.

Chick liver GST subunits were classified according to their electrophoretic mobility in SDS–polyacrylamide gel. Proteins eluted from the *S*-hexyl-GSH affinity column can be resolved

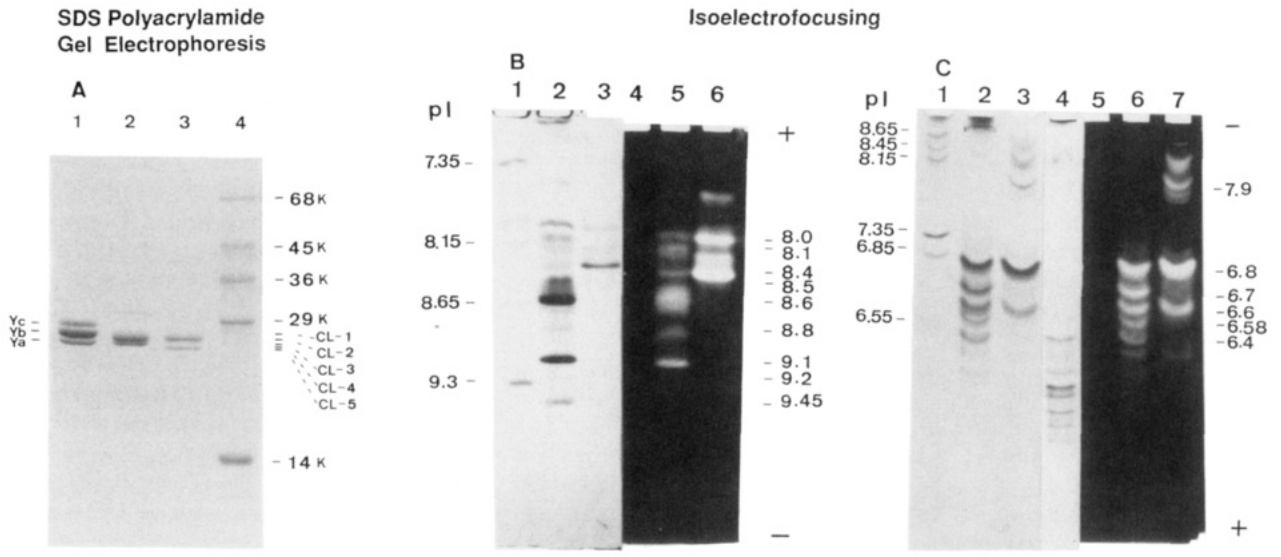

FIGURE 1: Polyacrylamide gel electrophoresis of chick liver GSTs. (A) Samples were analyzed on a 12.5% SDS–polyacrylamide gel, and proteins were visualized with Coomassie blue staining: lane 1, rat liver GSTs; lane 2, chick liver GSTs eluted from an *S*-hexylglutathione affinity column; lane 3, chick liver GSTs eluted from a glutathione affinity column; lane 4, molecular mass markers. (B) Isoelectrofocusing patterns of chick liver GSTs eluted from an *S*-hexylglutathione affinity column (lanes 2 and 5) and a glutathione affinity column (lanes 3 and 6) on a basic IEF polyacrylamide gel (pH 7–11). Proteins were visualized with Coomassie dye (lanes 1–3) or activity (lanes 4–6) staining. Lanes 1 and 4 were p*I* markers. (C) Chick liver GSTs were analyzed on an acidic IEF polyacrylamide gel (pH 3–10). Lanes 2 and 6 were samples eluted from an *S*-hexylglutathione column. Lanes 3 and 7 were samples eluted from a glutathione affinity column. Lane 4 was GSTs eluted from an *S*-hexylglutathione column and stored at −70 °C for 1 month without reducing agents. Sample was loaded without treatment with 2-mercaptoethanol. Lanes 1 and 5 were p*I* markers. Proteins were visualized with Coomassie dye (lanes 1–4) or activity (lanes 5–7) staining.

Table I: Purification of Chick Liver Glutathione *S*-Transferases

fraction	vol (mL)	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)
liver supernatant	390	4212	4867	1.15	100
S-hexylglutathione-Sepharose 6B	42	57.5	1397	24.3	29
glutathione-Sepharose 6B	7	1.75	80	45.7	1.6
Mono P					
peak 1	16	4.1	96	23.9	2
peak 2	4	0.44	48	10.8	1
peak 3	4	0.44			
peak 4	1.2	0.15	1.44	10.1	0.02
peak 5	8	9.2	135	13	2.8
peak 6	3.2	2.56	32	12.5	0.67
peak 7	16	9.6	121	12.7	2.5
peak 8	10	2.2	34	11.8	0.67
peak 9	16	1.9	22	11.6	0.45

into two major bands (CL 2 and CL 3) and two minor bands (CL 1 and CL 4) by electrophoresis on a 12.5% polyacrylamide–SDS gel (Figure 1A, lane 2). The molecular masses of CL 1 to CL 4 are estimated to be 27, 26, 25, and 24.5 kDa, respectively, and are within the range of the molecular mass reported for mammalian cytosolic GSTs (24–30 kDa) (Mannervik & Danielson, 1988). Several high molecular mass (>30 kDa) contaminants always existed in the preparation. The minor species CL 1 and CL 4 were identified more clearly in samples eluted from the chromatofocusing column (Figure 3A, lanes 5 and 3, respectively) or samples excised from the IEF polyacrylamide gel (data not shown). Two groups of proteins, CL 2 and CL 5, were eluted from GSH-linked column (Figure 1A, lane 3). CL 5 has an estimated molecular mass of 24 kDa by SDS–PAGE.

On the basis of SDS–PAGE and chromatographic (Mono P) analysis, an age-related change in isoenzyme pattern was observed. The same five groups of GST subunits were expressed in 1 day old and 4 week old chick. CL 2 subunits were the major GSTs expressed in 1 day old chick, while CL 3 subunits were the abundant species in livers of 4 week old chick (Chang et al., unpublished results).

Proteins eluted from affinity columns were analyzed by IEF PAGE. Even though the sample was separated into five bands by SDS–PAGE, a greater number of species were resolved by native IEF PAGE (Figure 1B,C). The protein bands were visualized by Coomassie blue dye staining or activity staining (Ricci et al., 1984). The p*I* markers were used as negative controls for activity staining. The fact that the same protein patterns were observed on the IEF polyacrylamide gel with either staining method indicates that the proteins isolated were mainly glutathione *S*-transferases.

Regular acidic IEF PAGE (pH 3–10) cannot adequately resolve proteins with p*I* above 8. Therefore, samples from affinity columns were analyzed by both acidic IEF PAGE (pH 3–10) and basic IEF PAGE (pH 7–11). Enzymes eluted from the *S*-hexylglutathione affinity column were resolved into six major bands by basic IEF PAGE and five major bands by acidic IEF PAGE (Figure 1B, lane 2, and Figure 1C, lane 2, respectively). The p*I*'s of these proteins eluted from the *S*-hexylglutathione column are estimated to be 9.45, 9.2, 9.1, 8.8, 8.6, 8.5, 8.1, 8.0, 6.8, 6.75, 6.6, 6.58, and 6.4. Proteins eluted from the glutathione affinity column were resolved into one major (p*I* 8.4) and two minor bands by basic IEF PAGE (Figure 1B, lane 3). In an acidic IEF polyacrylamide gel, the sample focused into several bands with p*I* values below 7 and an extra band at p*I* 7.9 (Figure 1C, lane 3). The two bands above p*I* 7.9 have p*I*'s identical with those of the minor bands in the basic IEF polyacrylamide gel.

Upon sample storage, the protein bands were shifted toward the acidic end in the IEF polyacrylamide gel (Figure 1C, lane 4). This shift was accompanied by a decrease in specific activity. The observation that treatment with 6 mM 2-mercaptoethanol can reverse the p*I* shift suggests that the change of pattern is caused by oxidation of a free cysteine residue(s) (Gan & Wells, 1987). However, the specific activity of the oxidized enzyme could not be raised to the level prior to storage. A similar phenomenum was described by Fjellstedt

Table II: Characterization of Chick Liver GSTs

		CL 2–2					CL 3–3							CL 5–5	
	CL 1–2	a	b	c	d	e	a	b	c	d	e	f	CL 3–4	a	b
subunit MW	27K	26K	26K	26K	26K	26K	25K	25K	25K	25K	25K	25K	25K	24K	24K
	26K	26K	26K	26K	26K	26K	25K	25K	25K	25K	25K	25K	24.5K	24K	24K
p*I*	7.7	6.8	6.75	6.6	6.58	6.4	9.45	9.1	8.6	8.5	8.1	8.0	8.8	8.7	7.9
immunochemical reactivity antibody															
YaYa	–	–	–	–	–	–	+	++	++	++	++	++	+	+	+
YbYb	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–
YcYc	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CL 1–CL 4	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
CL 5, CL 2	+	+	+	+	+	+	–	–	–	–	–	–	–	+	+

Table III: Substrate Specificities of Glutathione *S*-Transferases

chromatofocusing fraction:	specific activity (μmol min^{-1} mg^{-1})											
	1	2	3	4	5	6	7	8	9			
isozymes:	CL 3–3	CL 3–4		CL 1–2	CL 2–2	CL 2–2	CL 2–2	CL 2–2	CL 2–2	CL 5–5	YaYa[a]	Yb_1Yb_1[a]	YaYc[d]
1-chloro-2,4-dinitrobenzene	23.9	10.8		10.1	13	12.5	12.7	11.8	11.6	21	31.5	15.7	15.7
1,2-dichloro-4-nitrobenzene					0.36	0.36	0.24		0.24			1.68	
1,2-epoxy-3-(*p*-nitrophenoxy)propane	0.8			0.8	4.4	7	6.6	5.2	5.4	1.6		1.2	
ethacrynic acid	1.5	1.8		0.48	0.44	0.6	0.6	0.6	0.62	0.6	0.06		0.5
Δ^5-androstene-3,17-dione	0.1	0.17		0.006	0.018	0.018	0.015	0.014	0.012	0.29	0.104	0.012	0.067
cumene hydroperoxide	2	3		0.6	0.24	0.25	0.33	0.3	0.48	0.72	1.48	0.48	3.28

[a] Prepared according to Jensson et al. (1985).

et al. (1973) for glutathione *S*-transferase A. These authors suggested that treatment of the sample with 30% glycerol, 5 mM glutathione, and 5 mM EDTA at room temperature for 6 h could increase the enzymatic activity toward 1,2-epoxy-3-(*p*-nitrophenoxy)propane by as much as 3 times after prolonged storage at −90 °C. The enzymatic activity of chick liver glutathione *S*-transferases increased by 150% after the same treatment.

The subunit composition of chick liver GSTs, purified by affinity columns and further separated in IEF polyacrylamide gels, was determined by SDS–PAGE. The molecular mass of subunits present in each species is summarized in Table II. CL 2, CL 3, and CL 5 subunits can form CL 2–2, CL 3–3, and CL 5–5 homodimers whereas CL 1 and CL 4 can be isolated only as CL 1–2 and CL 3–4 heterodimers. The result for CL 1–2 was obtained from samples enriched by chromatofocusing as described below.

CL 1–1 or CL 4–4 homodimers were not detected. CL 2–2 homodimers focused into five major and several minor protein bands with a p*I* value below 7. These CL 2–2 homodimers were electroblotted from IEF polyacrylamide gel onto PVDF membranes for direct N-terminal sequencing. Identical sequences for the first 30 residues were obtained for each subspecies. Although these results suggest that the CL 2–2 homodimers differ in oxidative states, we cannot exclude the possibility that the differences of this family of isozymes may reside beyond residue 30. Most protein bands that appeared on basic IEF polyacrylamide gels were composed of CL 3–3 homodimers except for the two minor protein bands at p*I* 8.9 and 9.25, which were CL 3–4 heterodimers. CL 5–5a and CL 5–5b homodimers were focused electrophoretically at pH 8.4 (basic IEF) and pH 7.9 (acidic IEF), respectively. Proteins from the glutathione affinity column that focused below pH 7 in acidic IEF polyacrylamide gels were CL 2–2 homodimers (on the basis of molecular mass and N-terminal sequence analysis).

Immunoreactivity. Proteins were separated on IEF gels, electroblotted onto PVDF membranes, and reacted with antisera against rat and chick liver GSTs. The results are summarized in Table II. CL 2–2 homodimers and CL 1–2 heterodimers cross-reacted with Yb antisera [antisera raised against protein from peak I (Yb_2Yb_2 or isozyme 4–4) (Tu & Reddy, 1985)] while CL 3–3, CL 3–4, and CL 5–5 cross-reacted with Ya antisera [antisera raised against protein from peak IV (Tu & Reddy, 1985)]. We could not detect cross-reactivity of Yc antisera [antisera raised against protein from peak X (Tu & Reddy, 1985)] with chick GSTs, even though antisera raised against a mixture of CL 1 to CL 4 subunits cross-reacted with Yc. This observation probably reflects the low reactivity of the Yc antisera since reaction with rat liver GSTs was also weak. Sample used to raise antisera against CL 5 was contaminated with CL 2 subunits. Consequently, CL 1–2 and CL 2–2 also reacted with this batch of antisera on an immunoblot.

Chromatofocusing. Proteins eluted from the *S*-hexylglutathione affinity column were further purified by chromatofocusing. About 15 mg of enzyme was loaded onto a Mono P column and resolved into nine fractions. The chromatogram is shown in Figure 2. A protein peak was eluted from the column immediately after the application of the first elution buffer (pH 9). Proteins from this fraction have identical electrophoretic patterns (SDS and IEF gels) and specific activity (with CDNB as substrate) as fraction 1 and were not further characterized. Two peaks eluted from the column at pH 10 and 9.9 and were designated as fractions 1 and 2, respectively. Seven peaks were eluted with pH 7.0 buffer. Peak 3 (pH 8.5) did not have glutathione *S*-transferase activity. A small peak (fraction 4) eluted from the column at pH 8.2, followed by three major peaks (pH 8.1, 7.9, and 7.8 corresponds to fractions 5–7) and two moderate peaks (pH 7.4 and 7.3, corresponding to fractions 8 and 9). The resolution of fractions 4 and 5 increased with the use of a 0.5× second elution buffer (pH 7). Proteins eluted from the column at a pH higher than their isoelectric point. About half of the protein and one-third of the enzymatic activity were recovered (Table I).

The subunit composition of each fraction was analyzed by SDS–PAGE (Figure 3A), and the results are presented in

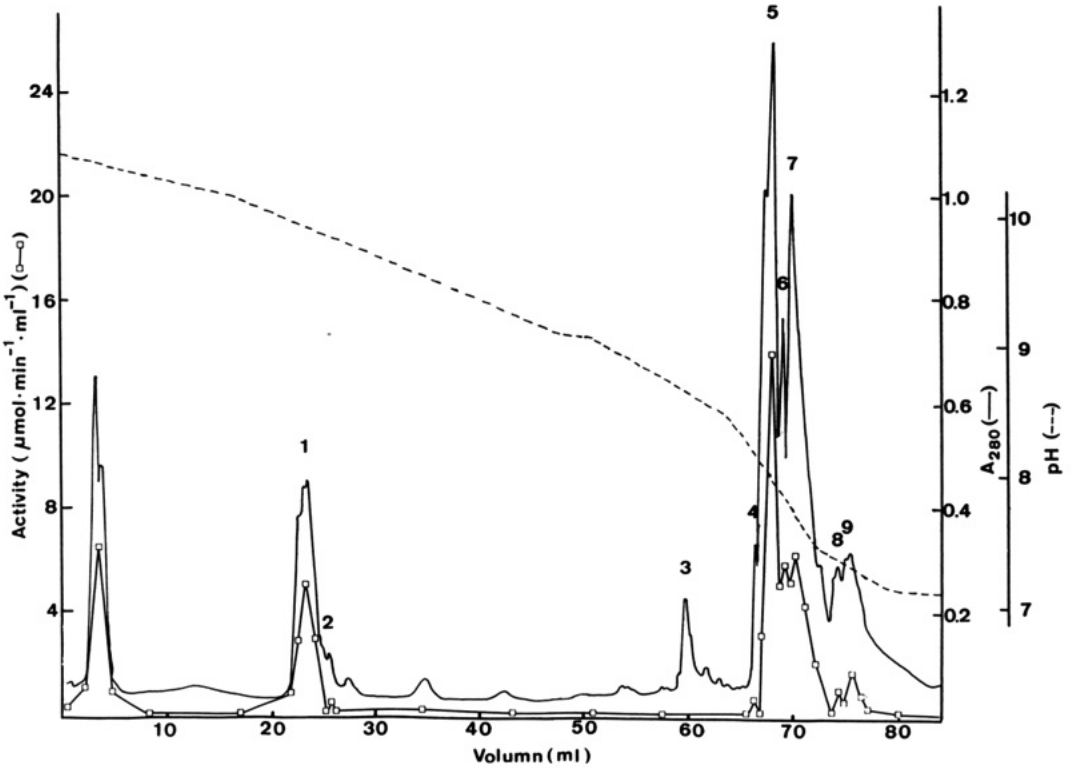

FIGURE 2: Chromatofocusing pattern of the chick liver glutathione *S*-transferase sample (15 mg) obtained from an *S*-hexylglutathione affinity column. Fractions collected for further analysis were marked 1–9. CDNB was used for activity determination of column fractions.

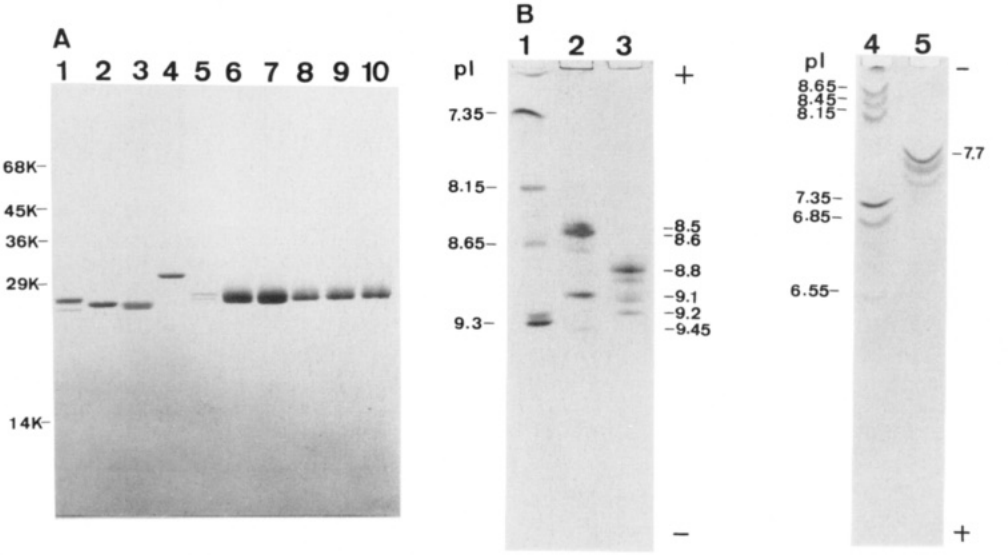

FIGURE 3: (A) SDS–PAGE of fractions eluted from the chromatofocusing column: lane 1, chick liver GSTs eluted from a glutathione affinity column; lanes 2–10, fractions 1–9 from the chromatofocusing column (Figure 2), respectively. (B) Fractions 1 and 2 (lanes 2 and 3) and fraction 4 (lane 5) from the chromatofocusing column (Figure 2) were analyzed by basic IEF PAGE and acidic IEF PAGE, respectively. Lanes 1 and 4 were p*I* markers.

Table III. Proteins in fraction 1 were composed of CL 3 subunits only (Figure 3A, lane 2) whereas CL 4 coeluted with CL 3 in fraction 2 (lane 3). Peak 2 was resolved into one major (p*I* 8.8) and three minor bands (p*I* 8.9, 9.1, and 9.2) upon basic IEF PAGE (Figure 3B, lane 3). When these bands were excised from the IEF polyacrylamide gel and analyzed by SDS–PAGE, they were all shown to contain bands with molecular masses corresponding to those of CL 3 and CL 4 (data not shown). Protein bands at p*I* 8.8 and 9.2 have been identified as CL 3–4 heterodimers in samples before chromatofocusing (Figure 1B, lane 2). Fraction 3 had no activity and corresponded to the 30-kDa contaminant that coeluted with GSTs from the *S*-hexylglutathione affinity column. Another heterodimer, CL 1–2, was eluted from the column in fraction 4 (Figure 3A, lane 5). This sample focused into a major and three minor bands with p*I*'s from 7.5 to 7.7 on an acidic IEF gel (Figure 3B, lane 5). Analysis of these bands, excised from an IEF polyacrylamide gel, by SDS–PAGE confirmed that they are all CL 1–2 heterodimers (data not shown). Proteins eluted below pH 8.1 were all CL 2–2 homodimers.

A GST sample from the glutathione affinity column, containing subunits CL 2 and CL 5, was also included in the same gel (Figure 3A, lane 1) for comparison. The protein pattern of this sample, together with fraction 2 (CL 3 and CL 4, Figure 3A, lane 3) and fraction 4 (CL 1 and CL 2, Figure

Table IV: N-Terminal Amino Acid Sequences of Glutathione *S*-Transferases[a]

Transferases	Amino acid sequences
CL 1	G L E L - Y L D L L S Q P S R A V Y I F A R S N
Yc (cDNA)[1]	P G K P - V L H Y F D G R G R M E P I R W L L A A A G V E F E E Q
Yp (cDNA)[2]	P P Y T V V Y F P V R G R C A A L R M L L A D Q G Q S W K E E
Maize III[3]	A P L K L - Y G M P L S P N V V R V A T V L N E K G L D
CL 2	V V T L G Y W D I R G L A H A I R L L L E Y T E T P Y Q E R R Y K A
Yb1 (cDNA)[4]	P M I L G Y W N V R G L T H P I R L L L E Y T D S S Y E E K R Y A M
Yb2[5]	P M T L G Y W D I R G L A H A I R L F L E Y T D T S Y E D K K Y S M
Hb[6]	P M I L G Y W D I R G L A H A I R L L L E Y T D S S Y E E K K Y T M
CL 5	P N - - Y K L T Y F N L R G R A E I S R Y L F A Y A G I K Y
Ya (cDNA)[7]	S G K P - V L H Y F N A R G R M E C I R W L L A A A G V E F D E K
Ha (cDNA)[8]	A E K P - K L H Y F N A R G R M E S T R W L L A A A G V E F E E K
Yc (cDNA)[1]	P G K P - V L H Y F D G R G R M E P I R W L L A A A G V E F E E Q

[a] References: (1) Telakowski-Hopkins et al. (1985); (2) Suguoka et al. (1985); (3) Grove et al. (1988); (4) Lai and Tu (1986); (5) Alin et al. (1986); (6) DeJong et al. (1988); (7) Lai et al. (1984); (8) Tu and Qian (1986).

3A, lane 5) from the chromatofocusing column, clearly indicates that there are five different subunits of GSTs from chick liver.

CL 5–5 homodimers can be separated from CL 2–2 on a chromatofocusing column. CL 5–5a and CL 5–5b were eluted off the Mono P column in a broad peak at pH 8.8–8.5, while CL 2–2 eluted off the column at pH 7.8.

The elution profile from Mono P was altered by prolonged storage of the sample prior to chromatography. Addition of 6 mM 2-mercaptoethanol to the sample was not possible since GSTs did not bind to the chromatofocusing column in the presence of 2-mercaptoethanol. A reproducible pattern was, however, obtained by the addition of 6 mM 2-mercaptoethanol to buffers used prior to chromatography on Mono P. Fractions eluted from the affinity column were dialyzed against buffer containing 1 mM dithiothreitol, and samples were loaded immediately onto a Mono P column, or stored at −70 °C. Enzymes thus treated have the same chromatofocusing pattern even if stored for 1 week before loading onto a Mono P column. Upon prolonged storage, fractions 6 and 7 decreased in magnitude while fraction 9 increased. This phenomenom is in accordance with a decrease in p*I*'s of GSTs, as observed by IEF PAGE. The unresolved protein peak eluted from the column at the beginning of the gradient also decreased, while fraction 1 split into two peaks. A similar pattern was obtained with enzymes prepared in the absence of reducing agent and stored at −70 °C for over 1 week after elution from an *S*-hexylglutathione affinity column.

Substrate Specificities. The substrate specificities of fractions resolved by chromatofocusing are summarized in Table III. Data obtained with rat liver GSTs are also included for comparison. Rat liver GSTs were isolated by chromatofocusing according to Jensson et al. (1985). Peaks corresponding to YaYa homodimer, YaYc heterodimer, and Yb_1Yb_1 homodimer were used in substrate-specificity assays. The data for CL 5–5 were obtained from a preparation that had passed through a chromatofocusing column, which removed CL 2–2 from CL 5–5.

Six different substrates were used to classify GSTs from chick liver cytosol. The results are summarized in Table III and suggest the following conclusions: (1) Fraction 3 is not a glutathione *S*-transferase. (2) CL 2–2 homodimers have a high activity toward 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane, characteristic of a Yb family. (3) CL 3–3 and CL 3–4 have high activities toward cumene hydroperoxide and ethacrynic acid, characteristic of the Yc family. (4) CL 5–5 has high Δ^5-androstene-3,17-dione but low 1,2-dichloro-4-nitrobenzene activity, suggesting that it is related to the Ya family. (5) Except with cumene hydroperoxide, CL 1–2 heterodimer has a lower overall activity than CL 2–2 homodimers.

Partial N-Terminal Amino Acid Sequence Analysis. Fraction 4 from the Mono P column containing CL 1–2 heterodimers was resolved by SDS–PAGE and electroblotted onto a PVDF membrane for N-terminal sequence analysis. The results indicated two different polypeptides. The N-terminal sequence of each CL 2–2 band on acidic IEF polyacrylamide gel (p*I* 6.8, 6.75, 6.6, 6.58, and 6.4) was determined by similar methods. They all yielded identical sequences for the first 30 amino acids. CL 3 and CL 4 subunits have blocked N-termini. CL 5–5 focused at p*I* 8.4 yielded an N-terminal sequence that has high homology with rat liver Ya GSTs whereas the CL 5–5 band with p*I* 7.9 was N-terminal blocked. These two bands might represent two different members of the CL 5 family or two different products of posttranslational modification.

The partial N-terminal amino acid sequences of CL 1, CL 2, and CL 5 (p*I* 8.4) are listed in Table IV. The partial N-terminal amino acid sequences of rat GSTs (Lai & Tu, 1986; Telakowski-Hopkins, 1985; Lai et al., 1984; Fjellstedt et al., 1973; Suguoka et al., 1985), human GSTs (DeJong et al., 1988; Alin et al., 1986), and corn GST (Grove et al., 1988) are also listed for comparison. Beale et al. (1982) reported that Ya or Yc GSTs from rat liver do not contain free N-terminal amino acids. All the amino acid sequences obtained so far for Ya, Yc, or Ha proteins were deduced from cDNA sequencing. We reported here by direct protein sequencing that CL 5 is not N-terminal blocked. For the first 28 amino acids, this protein has more than 67% homology with Ya or Ha GSTs. The homology between CL 2 and Yb is even higher. Including conservative substitutions, CL 2 and Yb have more than 94% homology. CL 1 has a rather unique N-terminal sequence. It has less than 50% homology (including conservative substitutions) with either Yp, a placenta protein, or Yc GST. Interesting, it share 26% conserved sequence with maize III GST.

In summary, we have identified five classes of glutathione *S*-transferase subunits from chicken liver cytosol. CL 2, CL

3, and CL 5 form homodimers, while CL 1 and CL 4 exist only as CL 1–2 and CL 3–4 heterodimers. On the basis of immuno-cross-reactivity, substrate specificity, and N-terminal sequencing data, CL 2 is related to the Yb family, CL 3 and/or CL 4 is (are) related to the Yc family, and CL 5 is related to the Ya family.

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