Purification and characterization of the major glutathione transferase from adult toad (*Bufo bufo*) liver

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Five forms of glutathione transferase (GST) were resolved from the cytosol of adult common toad (*Bufo bufo*) liver by GSH-affinity chromatography followed by isoelectric focusing. The major enzyme (GST-7.64; 55% of total activity bound to the column) has a pI value of 7.64, is composed of two subunits each with a molecular mass of 23 kDa, and has the N-terminal amino acid residue blocked. GST-7.64 has also been characterized with respect to amino acid composition, substrate specificity, inhibition characteristics, c.d. spectra and immunological reactivity. The N-terminal sequence of some peptides obtained after tryptic digestion has also been determined. All together the results obtained suggest that the major toad liver GST is distinct from any known GST, including microbial, plant and mammalian GSTs.

INTRODUCTION

The cytosolic glutathione transferases (GSTs; EC 2.5.1.18) comprise a family of multifunctional proteins which catalyse the conjugation of glutathione to a large variety of endogenous and exogenous hydrophobic electrophiles (Jakoby and Habig, 1980; Mannervik, 1985; Ketterer et al., 1988). In mammalian species the multitude of cytosolic GSTs so far characterized are dimeric and can be divided into four principal classes named Alpha, Mu, Pi and Theta according to their structures and catalytic properties (Mannervik et al., 1985; Meyer et al., 1991). Theta-class GSTs, unlike the GSTs of other classes, are not retained by the GSH-affinity matrices (Hiratsuka et al., 1990; Meyer et al., 1991). Cytosolic GSTs have also been investigated in other species, including bacteria (Di Ilio et al., 1988a, 1991), plant (Mozer et al., 1983), insect (Toung et al., 1990) and fish (Dominey et al., 1991), and their structural relationships to mammalian GSTs have been established.

There is little information on amphibian GST. In a previous study we have purified and characterized a GST isoenzyme from common toad (*Bufo bufo*) embryos at an early developmental stage (Di Ilio et al., 1992). The results indicated that the embryonic GST could be included in the Pi class (Di Ilio et al., 1992). Further studies investigating the expression of GST subunits during *Bufo bufo* embryo development have found that the Pi-type embryonic GST, which is continuously expressed at high levels up to the end of development, declines to very low levels in adult toad liver (Del Boccio et al., 1987a; A. Aceto, B. Dragani, P. Sacchetta, T. Bucciarelli, S. Angelucci, M. Miranda, A. Poma, F. Amicarelli, G. Federici, and C. Di Ilio, unpublished work). In this latter tissue, however, an elevated amount of GST activity is present (Del Boccio et al., 1987a; A. Aceto, B. Dragani, P. Sacchetta, T. Bucciarelli, S. Angelucci, M. Miranda, A. Poma, F. Amicarelli, G. Federici, and C. Di Ilio, unpublished work).

The aim of the present study was to characterize the adult toad liver GST subunits and establish what relationship they have to the *Bufo bufo* embryo GST, as well as with the GSTs of other species. Evidence is presented that the major toad liver GST is structurally, immunologically and kinetically distinct from the embryonic GST, as well as from any other GST isoenzymes so far characterized from other species.

MATERIALS AND METHODS

Purification

Adult toads were collected near L'Aquila, Italy. Liver tissue was homogenized at 25% (w/v) in 10 mM potassium phosphate buffer, pH 7.0, supplemented with 1 mM dithiothreitol (buffer A) and homogenized in a Potter homogenizer by hand with ten pestle strokes. The extract was centrifuged at 105000 *g* for 1 h at 4 °C, and the resulting supernatant was applied to a GSH-affinity column (Simons and Vander Jagt, 1977) that had been previously equilibrated with buffer A. After the column was washed with buffer A supplemented with 200 mM KCl, GST was eluted with 50 mM Tris/HCl, pH 9.6, containing 10 mM GSH. The active fractions were pooled, concentrated by ultrafiltration, dialysed against buffer A and subjected to isoelectric focusing on a column (110 ml; LKB Produkter, Stockholm, Sweden) containing 1% Ampholine, pH 3.5–10, plus 1% ampholine, pH 9–11, in a 0–40% (w/v) sucrose-density gradient. After focusing for 72 h at a final voltage of 700 V (4 °C), the contents of the column were eluted and collected in 1.0 ml fractions. The peaks of activity thus separated were concentrated by ultrafiltration, dialysed against buffer A and used for further characterization. All operations in the purification procedure were performed at 4 °C.

SDS/PAGE and immunoblotting analysis

Subunit molecular masses of GST were determined by SDS/PAGE as described by Laemmli (1970). The concentration of SDS was 0.1%, and those of the spacer gel and the separating gel were 3 and 12.5% acrylamide respectively. Phosphorylase *b* (94 kDa), BSA (67 kDa), ovoalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as standards for characterization of subunit molecular mass. The Western blot was done essentially as described by Towbin et al. (1979) and Di Ilio et al. (1991).

Abbreviations used: GST, glutathione transferase; PTH, phenylthiohydantoin.

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Antisera

Antiserum against GST-7.64 was raised in rabbits via two injections of 100 μg of protein in Freund's complete adjuvant. Antisera against members of mammalian Alpha-, Mu- and Pi-class GST, antisera against *Bufo bufo* embryonic GST and antisera against bacterial GST were available in our laboratory and were the same as those used in previous studies (Del Boccio et al., 1987b; Di Ilio et al., 1988a,b, 1992; Aceto et al., 1989). These antisera recognize GST isoenzymes belonging to the same classes but do not recognize members of the other classes.

Enzyme assay

GST activity with 1-chloro-2,4-dinitrobenzene, ethacrynic acid, Δ^5-androstene-3,17-dione and *trans*-4-phenyl-3-buten-2-one was measured as described by Habig and Jakoby (1981). GST activity with 4-nitroquinoline 1-oxide was determined as described by Stanley and Benson (1988). GST activity with *trans*-non-2-enal as substrates was measured as described by Brophy et al. (1989). GST activity with cumene hydroperoxide as substrate was measured as previously reported (Di Ilio et al., 1986). Protein concentration was determined by the method of Bradford (1976). Inhibitory studies with characteristic inhibitors were made as described by Tahir et al. (1986).

Amino acid analysis

Amino acid analysis was determined on protein samples precipitated with trichloroacetic acid (Marcus et al., 1978) and carboxymethylated with iodoacetate (Barra et al., 1984). Protein hydrolysis was performed in 6 M HCl at 110 °C for 24, 48 and 72 h in evacuated sealed tubes. HCl was removed under vacuum and the sample, dissolved in citrate buffer, was applied to an LKB 4151 Alpha Plus amino acid analyser. Amino acid composition was calculated using a molecular mass of 46 kDa.

Analysis of subunits by reverse-phase h.p.l.c.

H.p.l.c. analysis of toad liver GST subunits was performed by using the method described by Ostlund-Farrants et al. (1987). Samples of GST were injected on to a Waters μBondapak C_{18} (0.39 cm × 30.0 cm) attached to a Kontron h.p.l.c. apparatus that had previously been equilibrated with 35% acetonitrile in 0.1% (v/v) trifluoroacetic acid. The column was developed at 1 ml/min by a 45 min gradient from 35 to 55% acetonitrile in 0.1% (v/v) trifluoroacetic acid; this was followed by a 55–70%-acetonitrile gradient in 0.1% trifluoroacetic acid formed over 2 min. The eluate was monitored at 220 nm.

C.d. spectra

The near-u.v. c.d. spectrum at a protein concentration of 1 mg/ml was recorded on a Jasco J-600 instrument with cuvettes of 1 cm path length. All measurements were repeated five times.

Fluorescence spectra

The intrinsic fluorescence of the protein was measured with a Kontron SFM 25 fluorimeter equipped with a thermostatically controlled sample holder. The spectra of GST-7.64, and *Bufo bufo* embryonic GST at the same concentration (3 μM), were recorded at 25 °C in buffer A. For each sample the spectrum was corrected by subtraction of the spectrum for buffer A alone. The sample were excited at 280 ± 3 nm.

Peptide preparation and amino acid sequencing

About 0.5 mg of GST-7.64 was alkylated with iodoacetate as previously described (Barra et al., 1984). The S-carboxymethylated protein was first resuspended in 0.2 ml of 0.1 M ammonium bicarbonate buffer, pH 7.8, and then incubated at 37 °C for 2 h after addition of 20 μg of trypsin. The reaction was stopped by the addition of trifluoroacetic acid (0.2% final concn.). The resulting peptides were injected on to a macroporous reversed-phase column (Aquapore RP-300; 4.6 mm × 250 mm; 10 μm pore size; Brownlee Labs) attached to a Kontron h.p.l.c. apparatus and eluted with a linear gradient run from 5% to 70% acetonitrile in 0.2% (v/v) trifluoroacetic acid at flow rate of 1.0 ml/min. The eluate was monitored at 220 nm. Amino acid sequencing was performed on an Applied Biosystems model-475A gas-phase protein sequencer equipped with an Applied Biosystems model-120A phenylthiohydantoin (PTH) analyser for the on-line detection of PTH-amino acids.

RESULTS

Purification

Table 1 summarizes the results of a typical purification of GST present in adult toad liver cytosol monitored with 1-chloro-2,4-dinitrobenzene as substrate. GST was purified about 28-fold with a total recovery of about 65% after the affinity-chromatography step. Isoelectric focusing in pH range 3.5–10 resolved five different activity fractions centred respectively at pH 4.76 ± 0.09 (peak I), pH 5.45 ± 0.05 (peak II), pH 5.98 ± 0.06 (peak III), pH 6.73 ± 0.07 (peak IV) and pH 7.6 ± 0.05 (peak V) (Figure 1). GST-7.64 was the major fraction, accounting for about 55% of total activity. Essentially similar results were obtained in four different enzyme preparations. To allow for a more accurate estimation of GST subunits present in adult toad liver the affinity-purified material was also subjected to reverse-phase h.p.l.c. (Figure 2a). By using a 35–55%-(v/v)-acetonitrile gradient to develop the μ-Bondapak column, the GST were resolved into six peaks, which were eluted at 26.27 ± 0.18, 33.23 ± 0.18, 36.15 ± 0.21, 42.72 ± 0.19, 45.61 ± 0.21 and 52.0 ± 0.23 min respectively. The major peak, which was eluted at 42.12 ± 0.19 min, represents about 85% of total protein. When the GST in peak V (GST-7.64) was examined by reverse-phase h.p.l.c. the results reported in Figure 2(b) were obtained. The GST-7.64 form gave a single peak at 42.72 ± 0.19 min, indicating that it consists of two identical polypeptides. H.p.l.c. analysis of the other GST forms resolved by isoelectric focusing indicated that GST in peak IV (GST-6.73) contained the subunits eluted

Table 1 Purification of GST from adult toad liver

Step		Specific activity (μmol/min per mg)	Total activity (μmol/min)	Total protein (mg)	Yield (%)
Cytosol		1.56	450	288	100
Affinity chromatography		44	300	6.8	6.7
Isoelectric focusing					
Peak I	(pI 4.76)	26.8	9.10	0.34	
Peak II	(pI 5.45)	23.6	5.30	0.25	
Peak III	(pI 5.98)	42.3	19.9	0.47	27
Peak IV	(pI 6.73)	48.1	8.17	0.17	
Peak V	(pI 7.64)	54.7	87.6	1.60	

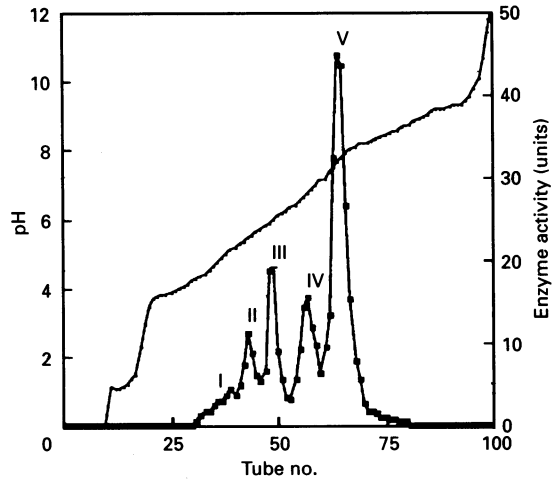

Figure 1 Separation of cytosolic GST from adult toad liver by isoelectric focusing in a 110 ml column

Fractions (1.0 ml each) were collected, and the enzymic activity with 1-chloro-2,4-dinitrobenzene (■) as well as the pH (——) were measured.

respectively at 26.27±0.18, 42.72±0.19 and 52.0±0.23 min; the GST in peak III (GST-5.98) contained the subunits eluting respectively at 42.72±0.19 and 45.61±0.21 min; the GST in peak II (GST-5.45) contained the subunits eluting respectively at 42.72±0.19, 45.61±0.21 and 52.0±0.21 min; the GST in peak I (GST-4.76) contained the subunits eluting respectively at 36.15±0.21, 42.72±0.19, 45.61±0.21 and 52.0±0.23 min. Only the major isoenzyme (GST-7.64) was further characterized in the present study.

Structural properties

Size-exclusion chromatography of GST-7.64 gave a molecular mass of about 50 kDa (results not shown). The electrophoretic mobility on SDS/PAGE of adult GST-7.64 is reported in Figure 3. For comparison, the electrophoretic mobility of the *Bufo bufo* embryo GST is also reported. A single polypeptide band was obtained, indicating the absence of contaminating proteins. It can be noted from Figure 3 that both GST-7.64 and *Bufo bufo* embryonic GST subunits have the same electrophoretic mobility. The molecular mass of GST-7.64 subunit was estimated to be 23 kDa.

Substrate specificities

The substrate specificity of GST-7.64 was determined and the results compared with that of *Bufo bufo* embryo GST (Table 2). Remarkably different substrate specificities can be noted between the two enzymes. For example, the specific activity of GST-7.64 toward ethacrynic acid was about 50-fold lower when compared with embryonic GST. Unlike embryonic GST, GST-7.64 possesses a high capacity to reduce cumene hydroperoxide. The specific activity of GST-7.64 toward *trans*-non-2-enal was also much lower than that of *Bufo bufo* embryonic GST.

Inhibitor-sensitivities

The inhibitory effect of a number of non-substrate ligands on the enzymic activity of GST-7.64 are reported in Table 3. For comparison, the IC_{50} values of *Bufo bufo* embryonic GST are also given in Table 3. It was found that large differences exist in the sensitivities of the two enzymes. GST-7.64 was about 300-

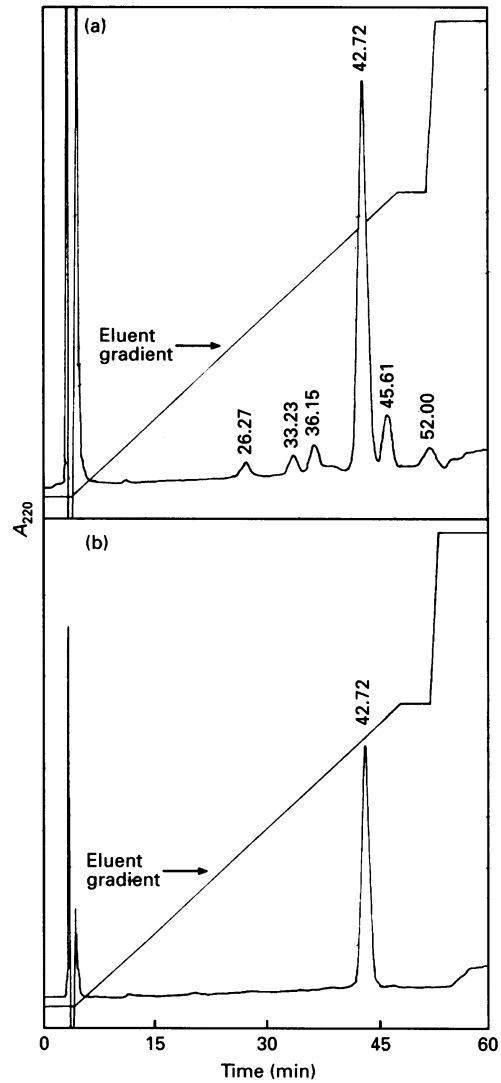

Figure 2 Separation of toad liver GST subunits by reverse-phase h.p.l.c.

A μBondapak C_{18} column (0.39 cm × 30 cm) were eluted at flow rate of 1 ml/min with a 35–55% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid formed over 45 min; this was followed by a 55–70% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid formed over 2 min. (**a**) GST fraction isolated from toad liver cytosol by GSH-affinity chromatography; (**b**) GST-7.64.

fold more sensitive to inhibition by Rose Bengal than *Bufo bufo* embryonic GST. GST-7.64 was consistently more sensitive to inhibition by tributyltin acetate (90-fold) and *S*-hexylglutathione (10-fold) than was embryonic GST. It is noteworthy that the IC_{50} value of GST-7.64 for Rose Bengal is significantly lower than the values obtained for the mammalian Alpha-, Mu- and Pi-class GST (Tahir et al., 1986).

Immunological properties

The antiserum raised against *Bufo bufo* embryo GST did not recognize GST-7.64 in immunoblotting experiments. In addition, the immunological properties of GST-7.64 were also studied by performing immunoblotting experiments with antisera raised against mammalian Alpha-, Mu- and Pi-class GSTs, as well as against bacterial GST. No cross-reaction was detected between these antisera and GST-7.64, indicating that it is an immunologically distinct protein. On the other hand, antiserum prepared

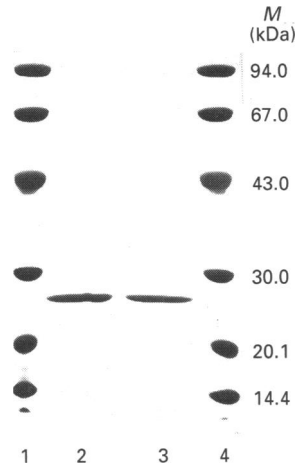

Figure 3 Comparison of electrophoretic mobility on SDS/PAGE between GST-7.64 and *Bufo bufo* embryonic GST

Lanes 1 and 4, standard proteins with molecular masses (*M*) (from top to bottom) of 94 kDa (phosphorylase *b*), 67 kDa (BSA), 43 kDa (ovoalbumin), 30 kDa (carbonic anhydrase), 20.1 kDa (soybean trypsin inhibitor) and 14.4 kDa (α-lactalbumin); lane 2, *Bufo bufo* embryonic GST; lane 3, GST-7.64.

Table 2 Comparison of specific activity between GST-7.64 and *Bufo bufo* embryonic GST toward different substrates

Substrate	Specific activity (μmol/min per mg)	
	Toad liver GST-7.64	*Bufo bufo* embryo GST*
1-Chloro-2,4-dinitrobenzene	55.0	46
Ethacrynic acid	0.02	1.1
4-Nitroquinoline 1-oxide	2.34	6.2
Cumene hydroperoxide	2.24	0.09
p-Nitrophenyl acetate	0.08	–
trans-Non-2-enal	0.006	0.28
Δ^2-Androstene-3,17-dione	N.D.†	N.D.
trans-4-Phenyl-3-buten-2-one	N.D.	0.05

* Data taken from Di Ilio et al. (1992).
† N.D., no detectable activity

Table 3 Comparison of inhibitor characteristics between GST-7.64 and *Bufo bufo* embryo GST

Inhibitor	IC_{50} (μM)	
	Toad liver GST-7.64	*Bufo bufo* embryo GST*
Cibacron Blue	0.25	0.1
Haematin	5	25
Bromosulphthalein	6	100
Tributyltin acetate	0.5	45
S-Hexylglutathione	15	150
Quercetin	150	> 100
Ellagic acid	37.5	37.5
Gossypol acetic acid	3	20
Alizarin	> 160	50
Rose Bengal	0.05	14

* Data taken from Di Ilio et al. (1992).

Table 4 Amino acid composition of GST-7.64

Amino acid analyses were carried out on S-carboxymethylated protein. The values for threonine and serine were obtained by extrapolation of hydrolysis data to zero time.

Amino acid	Composition (residues/molecule)
S-Carboxymethlcysteine	8.0
Aspartic acid	31.4
Threonine	29.4
Serine	60.8
Glutamic acid	48.4
Proline	16.6
Glycine	59.4
Alanine	15.0
Valine	17.0
Methionine	3.2
Isoleucine	15.0
Tyrosine	13.4
Phenylalanine	14.6
Histidine	5.2
Lysine	24.2
Arginine	18.0

against GST-7.64 did not recognize Alpha-, Mu- and Pi-class GSTs of mammalian origin or bacterial GST.

Amino acid composition

The amino acid composition of GST-7.64 is reported in Table 4. There appear to be significant amino acid compositional differences between the toad liver GST-7.64 and those of other species. In particular, the amino acid compositional similarities among toad liver GST-7.64 and the GSTs of Alpha, Mu- and Pi-classes were assessed by the difference index (Metzer et al., 1968); values higher than 20 were obtained.

C.d. spectrum

Figure 4 shows the c.d. spectrum, in the near-u.v. region of GST-7.64. It is characterized by relatively large rotatory strengths with vibronic fine structure in the aromatic region. The bands at the longer wavelengths (279–273 nm) probably reflect orientation of tryptophan and tyrosine residues respectively, whereas the

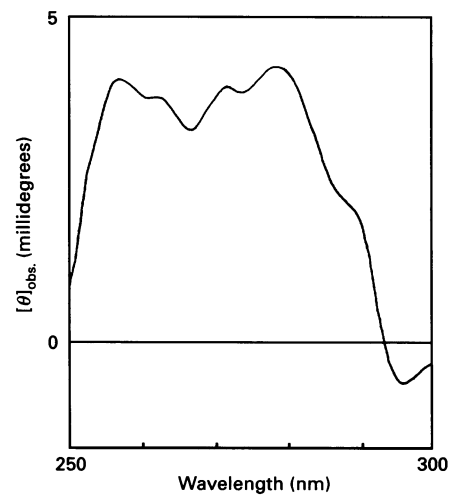

Figure 4 C.d. spectrum of toad liver GST-7.64 between 250 and 350 nm

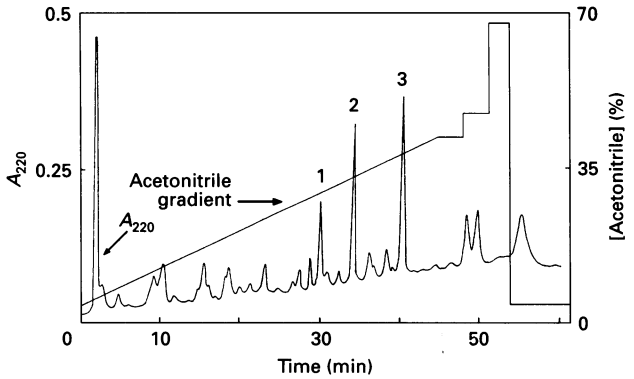

Figure 5 H.p.l.c. separation of tryptic-digested peptides of GST-7.64

Peptides were separated by using a linear gradient from 5 to 70% acetonitrile in 0.2% (v/v) trifluoroacetic acid at flow rate of 1 ml/min. The eluate was monitored at 220 nm. The number above the peaks refers to peptides for which the analytical data are given in Table 5.

Table 5 Partial *N*-terminal sequences of peptides derived from tryptic digestion of GST-7.64

Experimental details are given in the text.

Peptide	Sequence
1	F I F W D N E A N
2	N-terminal blocked
3	F L E E L A T

vibronic fine structure near 260 nm may be associated with phenylalanine residues. These data clearly indicate remarkable differences from the c.d. spectra of *Bufo bufo* embryonic GST (Di Ilio et al., 1992), bacterial GST (Di Ilio et al., 1988a) and those of Alpha-, Mu- and Pi-class GSTs (Warholm et al., 1983; Maruyama et al., 1984; Di Ilio et al., 1988b).

Fluorescence spectra

Analysis of the intrinsic fluorescence spectra of both GST-7.64 and *Bufo bufo* embryonic GST, indicated that the maximum emission wavelength of GST-7.64 was 9 nm red-shifted compared with that of *Bufo bufo* embryonic GST.

N-terminal amino acid sequencing and tryptic-digestion analysis

The N-terminus of GST-7.64 was found to be blocked. In this respect GST-7.64 is identical with the GSTs belonging to the Alpha class (Mannervik and Danielson, 1988). To obtain sequence information, GST-7.64 was digested with trypsin and the digestion mixture subjected to h.p.l.c. (Figure 5). Among the peptides eluted, three major fragments were analysed for their amino acid sequences (Table 5). Peptides 1 and 3 were sequenced up to the ninth and the seventh residues respectively (Table 5). As peptide 2 was found to be blocked, it could represent the N-terminal fragment of GST-7.64. In each case peptide 1 and peptide 3 gave a single sequence that could not be aligned with parts of known sequences of Alpha-class GSTs or with parts of the GSTs of other classes.

DISCUSSION

At least six different GST subunits can be resolved from the cytosolic fraction of adult toad liver. Altogether the different GST subunits constitute about 2.5% of total cytosolic proteins. This value is similar to the values (2–5%) found for mammalian livers GST (Mannervik, 1985; Jakoby and Habig, 1980).

The major toad liver GST, here called 'GST-7.64', was purified to apparent homogeneity by GSH-affinity chromatography and isoelectric focusing. GST-7.64 constitutes about 60% of total GST protein, has a pI value of 7.64 ± 0.05 and was found to be a homodimer of 23 kDa.

The results of the present investigation clearly indicate that the toad liver GST-7.64 significantly differs from the Alpha-, Mu- and Pi-class GSTs. Toad liver GST-7.64 has several characteristics not attributed to any of the GSTs belonging to the Alpha, Mu and Pi classes, the most noticeable being its antigenicity. In fact, none of the antisera raised against a large number of human, mouse and rat GSTs cross-reacted with the toad liver GST. This indicates that the antigenic domain of amphibian GST is different from those of mammalian origin. The analysis of their amino acid composition as well as the c.d. spectra confirm that toad liver GST and mammalian GST have major structural differences. As has been reported for most members of Alpha-class GSTs from different species, toad liver GST-7.64 has a blocked N-terminal amino acid residue. However, the analysis of its tryptic-digestion products, in addition to its c.d. spectrum and its immunochemical properties, clearly indicate that toad liver GST-7.64 does not correspond to any known Alpha-class GST. Substrate specificities and the effects of some inhibitors also strongly suggest that toad liver GST-7.64 is distinct from the GSTs of the Alpha class. For example, unlike Alpha-class GSTs, toad liver GST-7.64 exhibits a relatively high activity toward 4-nitroquinoline 1-oxide, an electrophilic compound found to be a preferred substrate of Mu- and Pi-class GSTs (Aceto et al., 1990). Thus the present study supports the idea that toad liver GST-7.64 is immunologically, kinetically and structurally distinct from Alpha-, Mu- and Pi-class GSTs.

We have previously purified and characterized GST from *Bufo bufo* embryo at an early developmental stage (Di Ilio et al., 1992). Later we found that this GST is continuously expressed at high levels up to the end of development (A. Aceto, B. Dragani, P. Sacchetta, T. Bucciarelli, S. Angelucci, M. Miranda, A. Poma, F. Amicarelli, G. Federici and C. Di Ilio, unpublished work). Despite the identical electrophoretic mobility of the subunits (Figure 3), *Bufo bufo* embryonic GST and toad liver GST are clearly distinct proteins, as indicated by their kinetic properties, amino acid compositions, immunological reactivities, c.d. spectra, intrinsic fluorescence and N-terminal amino acid sequences. On the other hand, the main conclusion of our previous investigation was that embryonic GST may be a member of the Pi class (Di Ilio et al., 1992).

It is at present unclear why adult toad liver expresses a GST form structurally and kinetically distinct from that expressed during embryonic life. Tentatively, it may be attributed to the changes in feeding and the transition to an amphibian life. A prominent kinetic feature which distinguishes the liver GST from the embryonic GST is its ability to eliminate organic hydroperoxides (Table 2). Thus it is plausible that these changes respond to the necessity of providing adult liver with a substantial protection against the toxic effect of compounds derived from oxygen metabolism. A change from an aquatic to an aerial respiratory environment may expose the animal to a higher oxygen partial pressure, which could promote increased formation of toxic metabolites. Thus it is possible that the changes could respond to the necessity of providing the liver with protection against the toxic products of oxygen metabolism.

In conclusion, the present work demonstrates that amphibian liver contains a near-neutral form of GST as a major constituent,

which is distinct from the GST forms so far characterized from other species.

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