Human glutathione S-transferases
Characterization of the anionic forms from lung and placenta

Dat D. DAO, Catherine A. PARTRIDGE, Alexander KUROSKY and Yogesh C. AWASTHI*
Department of Human Biological Chemistry and Genetics, C-316 Child Health Center, University of Texas Medical Branch, Galveston, TX 77550, U.S.A.

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Anionic glutathione S-transferases were purified from human lung and placenta. Chemical and immunochemical characterization, including polyacrylamide-gel electrophoresis, gave strong evidence that the anionic lung and placental enzymes are chemically similar, if not identical, proteins. The electrophoretic mobilities of both proteins were identical in conventional alkaline gels as well as in gels containing sodium dodecyl sulphate. Gel filtration of the intact active enzyme established an $M_r$ value of 45000; however, with sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under dissociating conditions a subunit $M_r$ of 22500 was obtained. Amino acid sequence analysis of the N-terminal region of the placental enzyme revealed a single polypeptide sequence identical with that of lung. Results obtained from immunoelectrophoresis, immunotitration, double immunodiffusion and rocket immunoelectrophoresis also indicated the anionic lung and placental enzymes to be closely similar. The chemical similarity of these two proteins was further supported by protein compositional analysis and fragment analysis after chemical hydrolysis. Immunochemical comparison of the anionic lung and placental enzymes with human liver glutathione S-transferases revealed cross-reactivity with the anionic $\omega$ enzyme, but no cross-reactivity was detectable with the cationic enzymes. Comparison of the N-terminal region of the human anionic enzyme with reported sequences of rat liver glutathione S-transferases gave strong evidence of chemical similarity, indicating that these enzymes are evolutionarily related. However, computer analysis of the 30-residue N-terminal sequence did not show any significant chemical similarity to any other reported protein sequence, pointing to the fact that the glutathione S-transferases represent a unique class of proteins.

GSH S-transferases (EC 2.5.1.18) are a family of enzymes that play an important role in the detoxification of a number of electrophilic compounds by conjugating them to GSH (Booth et al., 1961; Boyland & Chasseaud, 1969; Chasseaud, 1979). These enzymes are present in relatively high concentrations not only in liver but also in tissues such as placenta, lung, lens and erythrocytes. Kamisaka et al. (1975) have reported five cationic forms of GSH S-transferases in human liver with pI values in the range 7.8–8.8. In addition to the five cationic GSH S-transferases, several anionic GSH S-transferases having pI values in the range 4.5–5.4 were isolated from human liver (Awasthi et al., 1980), erythrocytes (Marcus et al., 1978), placenta (Guthenberg et al., 1979; Polidoro et al., 1980; Awasthi & Dao, 1981; Vander Jagt et al., 1981) and lung (Koskelo et al., 1981).

Previous evidence indicated that the anionic GSH S-transferases of human erythrocytes, placenta and lung are homodimers of subunits of $M_r$ about 22000–24000 (Marcus et al., 1978; Awasthi & Dao, 1981; Partridge et al., 1984). On the other hand, the anionic GSH S-transferase $\omega$ of human liver was suggested to be a heterodimer of subunits with $M_r$ values of approx. 22500 and 24500 (Dao et al., 1982). Reported amino acid compositions and subunit $M_r$ values of the anionic forms of GSH S-transferase isolated from human tissues showed significant differences (Marcus et al., 1978;...
Awasthi et al., 1980; Awasthi & Dao, 1981). The underlying direct chemical differences that give rise to the multiple forms of these enzymes have not heretofore been elucidated, and the structural interrelationship among various GSH S-transferases of human tissues is not clearly understood.

In the present paper we have examined the structural interrelationship of these enzymes by comparing partial amino acid sequences of anionic GSH S-transferases isolated from human placenta and lung. Further structural comparison was achieved by comparative fragment analysis of the placental and lung GSH S-transferases after hydrolysis with CNBr and N-chlorosuccinimide. In addition, we have compared the immunological interrelationship among various anionic GSH S-transferases of human tissues by using antibodies raised against either purified GSH S-transferase of human placenta or the cationic GSH S-transferases of human liver.

Materials and methods

Materials

Human placenta were collected soon after parturition and processed within 24 h. Unless otherwise stated the sources of chemicals used in this study were the same as in the previous studies (Awasthi et al., 1980; Partridge et al., 1984). Acetonitrile, tetrahydrofuran and water used in high-pressure liquid chromatography were HPLC grade purchased from Burdick and Jackson Laboratories. Reagents used in automated sequencing were products of Beckman Instruments, Pierce Chemical Co. and Burdick and Jackson Laboratories. Iodo[1-14C]acetic acid was obtained from Amersham-Searle (specific radioactivity 54 mCi/mol).

Purification of GSH S-transferases

All purification steps were carried out at 4°C. The enzyme activity was monitored with 1-chloro-2,4-dinitrobenzene as substrate. For purification of placental GSH S-transferase, placenta were cleaned of amniotic membrane and connective tissues and washed thoroughly with cold deionized distilled water. From 600 g of tissue, placental GSH S-transferase was purified by using (NH₄)₂SO₄ fractionation, chromatography over DEAE-cellulose (DE-52), affinity chromatography over GSH bound to epoxo-activated Sepharose 6B and Sephadex G-100 gel filtration. Details of the purification steps (Table 1) were similar to those reported previously (Awasthi et al., 1980) for the liver enzymes. Anionic lung GSH S-transferase was purified by using essentially the same protocol as that used for the placental enzyme. Cationic GSH S-transferases and anionic GSH S-transferase ω of human liver were purified as described previously (Awasthi et al., 1980).

Enzyme assays

GSH S-transferase activity was determined with 1-chloro-2,4-dinitrobenzene and other substrates according to the procedures described by Habig et al. (1974), Keen et al. (1976) and Keen & Jakoby (1978). One unit of GSH S-transferase utilized 1 μmol of substrate/min at 25°C. Protein content was measured by the method of Bradford (1976), with bovine serum albumin as a reference standard.

CNBr hydrolysis

Proteins were hydrolysed in 70% (v/v) formic acid with a 50-fold molar excess of CNBr over methionine residues essentially as described by Gross (1967). Resulting peptides were analysed by using urea/SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970).

Reaction with N-chlorosuccinimide

GSH S-transferases were treated with N-chlorosuccinimide as described by Lischwe & Ochs (1982). Samples were dissolved in a urea/acetic acid mixture [1 g of urea/2 ml of 50% (v/v) acetic acid] to a protein concentration of 3 mg/ml. A small volume of N-chlorosuccinimide in urea/acetic acid was added to the samples to achieve a 10-fold molar excess of N-chlorosuccinimide over tryptophan residues. The reaction was allowed to

<table>
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<tr>
<th>Activity</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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<td>346</td>
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</table>

Table 1. Purification of GSH S-transferase from human placenta

The purification steps were performed similarly to those used previously (Awasthi et al., 1980) for the liver enzymes (see the text).
Human glutathione S-transferases

proceed for 30 min at 25°C. An excess of methio-
nine was added to stop the reaction, and the
samples were then dialysed against water over-
night. After being freeze-dried, the samples were
subjected to urea/SDS/polyacrylamide-gel slab
electrophoresis.

Electrophoresis

Polyacrylamide-gel disc electrophoresis was per-
formed by the method of Davis (1964). The gels
were stained for protein by the procedure of
Reisner et al. (1975), and destained by a rapid-
destaining procedure (Saneto et al., 1980). Urea/
SDS/polyacrylamide-gel electrophoresis was
performed similarly to that described by Laemmli
(1970). The gels were stained with Coomassie
Blue R-250 by the method of Matsudaira & Burgess
(1978).

Carboxymethylation

Purified samples of GSH S-transferases were
reduced for 4 h with dithioerythritol in 0.1M-N-
ethylmorpholine buffer, pH 8.1, containing 5.2M-
guanidinium chloride, essentially as described by
Hirs (1967). The reduced enzyme preparations
were subsequently alkylated with iodo[1-14C]-
acetic acid, dialysed and freeze-dried.

Amino acid analysis

Amino acid analysis was performed on a
Beckman model 121M automatic acid analyser
with AA20 resin as described by Kurosky et al.
(1977). Tryptophan analysis was carried out with
the use of mercaptoethanesulphonic acid as de-
scribed by Benke et al. (1974).

Automated sequence analysis

Automated amino acid sequence analysis was
performed with an updated Beckman 890B Se-
quenator with the 1.0M-Quadrol program (Beck-
man no. 060275). Sequenator methodology and the
methods for the identification of amino acid
phenylthiohydantoin derivatives by using high-
pressure liquid chromatography and amino acid
analysis after back-hydrolysis with HI have been
described previously (Fader et al., 1982).

Immunological studies

Antibodies against purified human placental
GSH S-transferase were raised in goat as described
previously (Partridge et al., 1984). Double-
immunodiffusion studies were performed on agar
plates (Ouchterlony, 1958). Immunelectro-
phoresis and rocket immunoelectrophoresis were
performed by using the methods of Grabar &
Williams (1953) and Laurell (1966) respectively.
Production of rabbit anti-(human liver cationic
GSH S-transferases) serum was achieved as
described previously (Awasthi et al., 1980).

Results

Purification and properties of GSH S-transferase

The elution of placental GSH S-transferase from
the Sephadex G-100 column in the final step of
purification is shown in Fig. 1. Protein absorbance
at 280 nm was coincident with enzymic activity,
and the enzyme was eluted with an apparent $M_r$ of
45,000 when compared with protein standards.
The yield of the enzyme was about 40% (Table 1). The
enzyme gave a single stained band of $M_r$ 22,500
when subjected to urea/SDS/polyacrylamide-gel
electrophoresis under reducing conditions (Fig. 2).
Further evidence of protein homogeneity was
provided by automated protein sequence analysis,
which established the occurrence of only a single
amino acid sequence (Table 2 and Fig. 3). Al-
though some of the SDS/polyacrylamide-gel
electrophoretograms indicated possible trace con-
tamination by higher-$M_r$ proteins, quantitative
sequence analysis did not demonstrate any detect-
able amounts of another sequence, providing
evidence that the enzyme preparations were
reasonably homogeneous for chemical
characterization.

The purification of human lung GSH S-transfer-
ase was also achieved by using the same protocol
described above for the placental enzyme. The
purified lung enzyme was obtained in an overall
yield of about 30%, and its homogeneity was
established by the same criteria described for the
placental enzyme (Fig. 2). When a mixture of
anionic lung and placental GSH S-transferases
was subjected to either conventional polyacryl-

![Diagram](https://via.placeholder.com/150)
Fig. 2. Urea/SDS/2-mercaptoethanol/polyacrylamide-gel electrophoresis of GSH S-transferases of human lung and placenta

Experimental details are indicated in the text. Lanes 1 and 5, standard protein markers; lane 2, GSH S-transferase of lung; lane 3, anionic GSH S-transferase of lung; lane 4, mixture of GSH S-transferases of lung and placenta.

Protein compositional and sequence analyses

The amino acid composition of the placental enzyme obtained by time-course compositional analysis is given in Table 3. This is quite similar to the amino acid composition of the anionic lung enzyme previously reported from this laboratory (Partridge et al., 1984). The difference index calculated in accordance with the method of Metzger et al. (1968) for the composition of the placental enzyme reported here and that of the anionic lung enzyme was found to be 4.7.

Automated sequence analysis of the N-terminal region of human placental GSH S-transferase is given in Table 2. Only a single polypeptide amino acid sequence was obtained. Prediction of protein conformation by using the method of Chou & Fasman (1978) indicated considerable secondary structure in this region, especially between residues 3 and 24. No prediction was put forth for the region 25–30, since position 27 was not determined. Sequence analysis of human lung GSH S-transferase gave results identical with those obtained for human placenta. Comparison of the human enzyme partial sequence with sequences reported for rat liver GSH S-transferases (Frey et al., 1983) is given in Fig. 4. With the exception of the rat liver enzymes, computer analysis of the 30-residue segment, utilizing the program SEARCH (Dayhoff, 1978) of the National Biomedical Re-
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Table 2. Automated sequence analysis of human placental GSH S-transferase with prediction of conformation

The results were obtained with an 890B Sequenator. The protein was sequenced three times, the initial amount applied to the Sequenator being 30 nmol, as determined by amino acid analysis and $M_r$, 22,500. The phenylthiohydantoin derivatives of amino acids were analysed by high-pressure liquid chromatography and by amino acid analysis after back-hydrolysis with HI. The quantitative amounts shown are those obtained by high-pressure liquid chromatography except for that for threonine, which was calculated as $\alpha$-aminobutyric acid by amino acid analysis. The value for cysteine was also confirmed by measurement of radioactivity resulting from alkylation with iodo[1-$^{14}$C]acetic acid (Fig. 3). Parameters for prediction of protein conformation are taken from Chou & Fasman (1978).

Abbreviation: N.D., not determined.

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Fig. 4. Comparison of human placental GSH S-transferase N-terminal sequence with sequences reported for rat liver GSH S-transferases by Frey et al. (1983)

Details for prediction of the conformation are given in Table 2. Residue identities are boxed in. Abbreviation: N.D., not determined.

search Foundation, did not reveal any significant sequence similarity to any other reported protein sequence to date.

Comparison of chemical hydrolytic fragments of lung and placental GSH S-transferases

CNBr hydrolysates of both lung and placental
GSH S-transferases were subjected to polyacrylamide-gel electrophoresis comparatively as shown in Fig. 5. The CNBr hydrolytic patterns, as evidenced by the gels, were identical for the lung and placental enzymes and demonstrated three major fragments of M, values of about 21800, 12600 and 13600 as well as the unhydrolysed protein. Incomplete hydrolysis may have been due to the oxidation of methionine residue(s). Minor bands seen in lanes 3 and 4 of the gel (Fig. 5) were probably contaminants of a result of heavily overloading the sample to ensure adequate staining of the smaller-M, fragments. Moreover, some minor fragmentation could also have occurred as a result of hydrolysis of tryptophyl bonds (Kurosky et al., 1976). Importantly, even these minor cleavage products were consistent for both the lung and plaecental enzymes and provided even further evidence of chemical similarity. The occurrence of three major CNBr-cleavage fragments gave evidence of two methionine residues per subunit rather than one as indicated by compositional data (Table 3). A low methionine value in the compositional results could have occurred as a result of oxidation. Thus the possibility of two methionine residues per subunit cannot be ruled out. Compositional analysis of the anionic lung enzyme did indicate two methionine residues (Partridge et al., 1984). Comparative hydrolysis of the lung and placental enzymes with N-chlorosuccinimide was also evaluated by polyacrylamide-gel electrophoresis, as shown in Fig. 6. Both the lung and placental proteins gave virtually identical gel-electrophoretogram patterns after N-chlorosuc-
cinimide hydrolysis. In addition to the unhydrolysed protein five peptide fragments having $M_r$ values of 19900, 17000, 14000, 12100 and 10000 were obtained for each protein.

**Immunological studies**

Results obtained from immunotitration with antibodies raised in goat against purified human placental GSH S-transferase completely precipitated anionic GSH S-transferase $\omega$ of human liver (results not shown). These antibodies, however, did not cross-react with the cationic GSH S-transferases of human liver. Cross-reactivity of these enzymes was also confirmed by immunoelectrophoresis (Fig. 7). Further immunotitration studies revealed that antiserum raised against the cationic GSH S-transferases of human liver did not cross-react with the placental or the lung enzyme. This antiserum, however, cross-reacted with the anionic GSH S-transferase $\omega$ of human liver. Double-immunodiffusion results obtained with anionic GSH S-transferases of placenta and lung with antiserum raised against placental GSH S-transferase demonstrated a reaction of identity; however, when the antibodies raised against the placental enzyme were fully absorbed with the anionic lung enzyme, no precipitation reaction with placental GSH S-transferase was observed. The results of rocket immunoelectrophoresis with the antiserum raised against the placental enzyme also indicated immunological identity between the anionic GSH S-transferases of lung and placenta. Lung and placental enzymes were, however, immunochemically distinct from the liver cationic GSH S-transferases.

**Discussion**

We have provided considerable chemical evidence that human anionic lung GSH S-transferase is chemically similar to, if not identical with, that isolated from placenta. This evidence included (1) automated sequence analysis of the N-terminal region, (2) polyacrylamide-gel electrophoresis, (3) isoelectric focusing, (4) compositional analysis, (5) comparative chemical fragmentation with CNBr and N-chlorosuccinimide, and (6) immunochemical studies. The native enzyme gave an apparent $M_r$ of 45000 by gel filtration. Results from SDS/polyacrylamide-gel electrophoresis established an apparent $M_r$ of 22500 with or without reduction. Since quantitative sequence analysis established only a single polypeptide sequence that yielded residues at a molar level compatible with the molar amount of enzyme applied to the Sequenator (on the basis of an $M_r$ of 22500; Table 2), the subunit arrangement of the native enzyme must be a dimer composed of identical polypeptide subunits held together by non-covalent bonding.

Interestingly, N-terminal sequence comparison (Fig. 4) of the human placental transferase enzyme with GSH S-transferases isolated from rat liver as reported by Frey et al. (1983) revealed that the human placental and rat liver enzymes have chemical similarities and are therefore evolutionarily related, at least in part. In the N-terminal region the human lung and placental enzymes were 29–39% identical with the rat liver enzymes. The percentage identity of the human enzyme with rat liver GSH S-transferases B, A and X was in the order $B > A > X$. Although Frey et al. (1983) had reported that the subunit sequence of enzyme B bore no resemblance to the sequences of enzymes A and X, if an insertion is allowed in enzyme B

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**Fig. 7. Immunoelectrophoresis of GSH S-transferases of human liver, placenta and lung with antibodies raised against placental GSH S-transferase**

Experimental details are indicated in the text. 1. Cationic GSH S-transferases of human liver; 2. anionic GSH S-transferase $\omega$ of human liver; 3. placental GSH S-transferase; 4. anionic lung GSH S-transferase.
corresponding to positions 6 and 7 the comparison of enzyme B with enzymes A and X becomes striking. Similarly, an insertion of valine at position 6 of the placental enzyme is required to optimize the comparison. Notably the sequence comparisons are more pronounced in the predicted β-turn region of residues 11–14 (Fig. 4 and Table 2). Computer analysis of the 30-residue region of the human placental enzyme did not reveal any significant chemical similarity to any other protein except for the rat liver transferases. Taken together, these results provide strong evidence that the GSH S-transferases represent a unique family of homologous enzymes.

Antibodies raised against either the lung or the placental enzyme quantitatively precipitated equal amounts of both enzymes, which confirmed the complete immunological identity of these proteins. Guthenberg & Mannervik (1981) have suggested that erythrocyte and placental GSH S-transferases are also similar. Thus it appears likely that the lung, placental and erythrocyte enzymes may be chemically similar or identical proteins. Antibodies raised against either the placental or the lung enzyme recognized the anionic GSH S-transferase ω of human liver but did not cross-react with the cationic GSH S-transferases of human liver. On the other hand, antibodies raised against cationic GSH S-transferases of human liver did cross-react with the anionic GSH S-transferase ω (Awasthi et al., 1980) but not with either the lung or the placental enzyme. We have previously shown that the anionic GSH S-transferase ω and the cationic enzymes of human liver are heterodimers that may share a common subunit of M, 24500 (Dao et al., 1982). In addition, the anionic GSH S-transferase ω and the cationic GSH S-transferases of human liver have two unique subunits with M, values of about 22500 and 26500 (Dao et al., 1982). Viewed together, these results can be explained if we consider that the 22500-M, subunit is common to the GSH S-transferases of placenta and lung and to GSH S-transferase ω of human liver.

Thus our results strongly suggest that the various GSH S-transferases of human tissues studied to date probably arise from at least three gene products: subunit A, M, 26500; subunit B, M, 24500; subunit C, M, 22500. In this context, the subunit composition of the placental and lung GSH S-transferases would be designated as CC, and the anionic GSH S-transferase ω and the cationic enzymes of human liver would be designated as BC and AB respectively.

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