Glutathione Transferase in Bacteria: Subunit Composition and Antigenic Characterization

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The presence of glutathione transferase (GST; EC 2.5.1.18) in *Escherichia coli* ATCC 25922, *E. coli* ATCC 25422, *Proteus vulgaris* ATCC 8427, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella oxytoca* CIP 666, *K. oxytoca* AF 101, *Enterobacter cloacae* CIP 6085, *Serratia marcescens* CIP 6755, and *Proteus mirabilis* AF 2924 was investigated. Using 1-chloro-2,4-dinitrobenzene as substrate, GST activity was found in the glutathione- (GSH-) affinity-purified fraction of all strains tested. SDS-PAGE analysis of GSH-affinity-purified enzyme indicated that the GSTs of all these bacteria are dimers of two identical subunits of *M*_r about 22500. Rabbit antiserum directed against the major isoenzyme present in *Proteus mirabilis* AF 2924, Pm-GST-6-0, was used to investigate the antigenic properties of bacterial GSTs. Western blot analysis indicated that a GST antigenically identical to Pm-GST-6-0 is present in *Enterobacter cloacae* CIP 6085, *Escherichia coli* ATCC 25422 and *Proteus vulgaris* ATCC 8427, but absent in *Escherichia coli* ATCC 25922, *Klebsiella oxytoca* CIP 666, *K. oxytoca* AF 101 and *Serratia marcescens* CIP 6755. The presence of Pm-GST-6-0, but not mammalian GST, increased the MIC values of amikacin, ampicillin, cefotaxime, cephalothin and nalidixic acid for *E. coli* ATCC 25922. It is suggested that bacterial GST may represent a defence against the effects of antibiotics.

INTRODUCTION

Glutathione transferases (GSTs; EC 2.5.1.18) are a family of multifunctional proteins that catalyse the conjugation of glutathione (GSH) to the electrophilic centre of a large number of active molecules (Awasthi & Singh, 1985; Chasseaud, 1979; Jacoby & Habig, 1980; Ketterer, 1986; Mannervik, 1985; Mannervik & Danielson, 1988). GSTs can probably also function as intracellular transport and binding proteins by virtue of their capacity to bind covalently or non-covalently a wide number of hydrophobic compounds such as bilirubin, haem, drugs, steroid hormones, bile acids and other xenobiotics including carcinogens (Jacoby & Habig, 1980; Kamisaka *et al.*, 1975*a, b*; Mannervik & Danielson, 1988; Maruyama *et al.*, 1983; Smith & Litwack, 1980; Takikawa *et al.*, 1986). In general they are thought to play a key role in detoxification processes. GSTs have been most extensively studied in man, rat and mouse, in which multiple isoforms all composed of two subunits are present (Awasthi & Singh, 1985; Jacoby & Habig, 1980; Ketterer, 1986; Mannervik, 1985; Mannervik & Danielson, 1988). By

Abbreviation: GST, glutathione transferase.

the use of various substrates, inhibitors, SDS-PAGE and antisera, and by determining the N-terminal amino acid sequences, it was proposed that the cytosolic GSTs of rat, mouse and man should be grouped into at least three distinct families: alpha, mu and pi (Mannervik *et al.*, 1985). GSTs have also been purified from fish (Ramage *et al.*, 1986) and insects (Clark *et al.*, 1986; Cochrane *et al.*, 1987), and have been studied in a number of micro-organisms (Casalone *et al.*, 1988; Di Ilio *et al.*, 1988a; Kumagai *et al.*, 1988; Lau *et al.*, 1980). A GST composed of two subunits of M_r 22000 has been isolated from the fungus *Mucor javanicus* (Ando *et al.*, 1988). Two structurally and antigenically distinct GSTs have been recently purified and characterized from the yeast *Issatchenkia orientalis* (Tamaki *et al.*, 1989). However, very little information is available on the presence, the subunit composition, the antigenic properties and the role of bacterial GSTs.

We have recently purified and characterized an isoenzyme of GST from *Proteus mirabilis* AF 2924 (Pm-GST-6-0) (Di Ilio *et al.*, 1988a) having structural and antigenic properties which distinguished it from the GSTs characterized from mammalian sources. In the present study, by using a GSH-affinity-purification technique, the amount of GSTs in several bacterial strains was estimated and, by using antiserum raised against Pm-GST-6-0, the presence of an antigenically similar GST in several other bacterial strains was investigated. To better understand the role of bacterial GST, we studied the binding capacity of Pm-GST-6-0 to several antibiotics, and determined the effect of Pm-GST-6-0 on their MIC values.

METHODS

Bacterial strains. These were as follows: *Escherichia coli* ATCC (American Type Culture Collection) 25922, *E. coli* ATCC 25422, *Proteus vulgaris* ATCC 8427, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella oxytoca* CIP (Collection of the Institut Pasteur) 666, *Enterobacter cloacae* CIP 6085, *Serratia marcescens* CIP 6755, and *Klebsiella oxytoca* AF 101 and *Proteus mirabilis* AF 2924 of the Type Culture Collection of the Institute of Experimental Medicine, Chieti, Italy. All strains were stored at -70°C , and fresh subcultures were regrown in sterile Mueller-Hinton broth (MH broth; BBL).

Antimicrobial agents. The antibiotics were obtained as follows: amikacin, Bristol Italiana (Latina, Italy); cefotaxime, Hoechst Italia (L'Aquila, Italy); ampicillin, Beecham, cephalothin and nalidixic acid, Sigma. Nalidixic acid was initially dissolved in 0.1 M-NaOH and subsequently diluted in MH broth. Amikacin, ampicillin and cephalosporins were prepared in distilled water and sterilized by membrane filtration. Fresh dilutions of each compound were prepared daily and according to the manufacturers' instructions.

Preparation of cell-free extracts and GSH-affinity purification. Washed cells were grown aerobically for 18 h at 37°C in Trypticase Soy Broth (BBL), suspended in 10 mM-potassium phosphate buffer, pH 7.0 (buffer A), and disrupted by sonication (five bursts of 3 min each, at 300 W) with a Labsonic 1510 (Braun) sonicator. The particulate material was removed by centrifugation at $105000g$ for 1 h and the supernatant applied to a GSH-Sepharose affinity column (1 cm $\times$ 10 cm) (Simmons & Van der Jagt, 1981) pre-equilibrated with buffer A. The column was exhaustively washed with buffer A supplemented with 50 mM-KCl. The enzyme was eluted with 50 mM-Tris/HCl buffer, pH 9.6, containing 5 mM-GSH. The fractions showing GST activity were pooled, concentrated by ultrafiltration, dialysed against buffer A supplemented with 1 mM-dithiothreitol and used for further studies.

SDS-PAGE. SDS-PAGE in discontinuous slab gels was done by the method of Laemmli (1970). The SDS concentration was 0.1% (w/v), and the spacer and the separating gels contained 3% and 12.5% (w/v) acrylamide respectively. Bovine serum albumin (M_r 66000), ovalbumin (M_r 45000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 29000), trypsinogen (M_r 24000), soybean trypsin inhibitor (M_r 20100) and α -lactalbumin (M_r 14200) were used as standards for determination of subunit molecular size.

Antiserum. Antiserum against Pm-GST-6-0 (an isoenzyme of GST from *Proteus mirabilis* AF 2924) was raised in rabbits via two injections of 100 μg protein in Freund's complete adjuvant and was the same as that used by us previously (Di Ilio *et al.*, 1988a).

Assay of GST activity. This was done as described by Habig & Jakoby (1981) using 1 mM-1-chloro-2,4-dinitrobenzene and 2 mM-GSH. Protein concentrations were determined by the method of Bradford (1976) with γ -globulin (Bio-Rad) as standard.

Immunoblot analysis. Proteins were electrophoretically transferred from polyacrylamide gels onto nitrocellulose membranes (Bio-Rad Transblot System) according to the method of Towbin *et al.* (1979). Electroblothing was done for 16 h at 30 V in 25 mM-Tris base/192 mM-glycine, pH 8.3, containing 20% (v/v) methanol. All incubations were for 1 h at 25°C with intermediate rinses in 50 mM-Tris base buffer, pH 7.5, 400 mM-NaCl (buffer B) containing 0.05% Tween 20 (buffer C). Non-specific binding was blocked by placing membranes in buffer B supplemented

with 3% (w/v) bovine serum albumin. Membranes were incubated with primary antiserum (antiserum raised against Pm-GST-6-0 at optimum dilution, 1:250), in buffer B containing 3% bovine serum albumin. The membranes were washed with buffer C and then incubated for 1 h at room temperature, with gentle shaking, in the same buffer containing 1% (w/v) gelatin and a horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) diluted 1:3000. After treatment with peroxidase-conjugated antibody, the membranes were washed three times in buffer C (5 min each) and twice in buffer B, then immersed in development solution (100 ml buffer B containing 60 mg 4-chloro-1-naphthol (Bio-Rad) and 60 μ l 30% H_2O_2). The blot was then washed once with distilled water, air-dried and photographed.

Binding of antibiotics to Pm-GST-6-0. This was determined by following the quenching of the intrinsic tryptophan fluorescence upon addition of drugs. Measurements were made in 10 mM-potassium phosphate buffer, pH 7.0, using an Aminco Bowman spectrofluorometer. The decrease in the intrinsic fluorescence upon addition of antibiotic was measured at 350 nm while exciting at 280 nm, with a slit width of 3 nm. The absorbance of solutions with antibiotics was sufficiently low that correction for inner filter effects were of minor significance; correction was made for dilution. Dissociation constants (K_D) were calculated from a plot of the reciprocal of the change in fluorescence (ΔF) against the reciprocal of the total ligand concentration. Enzyme concentration was 1 μ M.

Determination of minimal inhibitory concentrations (MICs). These tests were done in 96-well microtitre trays, using a Titertek manual multidiluter (Flow Laboratories) for dispensing and inoculating 100 μ l volumes of MH broth containing doubling dilutions of each antibiotic (range 32–0.06 μ g ml^{-1} ; except cefotaxime, for which the range was 0.015–8 μ g ml^{-1}). For amikacin, the medium was supplemented with calcium and magnesium ions (National Committee for Clinical Laboratory Standards, 1983). The volume of the inoculum was 100 μ l, with a final bacterial concentration of 5×10^5 c.f.u. ml^{-1} . The wells were inoculated simultaneously with a Titertek multichannel pipette, 50–200 μ l (Flow Laboratories). The drug dilutions with enzyme (Pm-GST-6-0) were done as described above, mixing 50 μ l volumes of MH broth containing four times the final concentration of antibiotic (range 512–0.015 μ g ml^{-1}), 50 μ l enzyme, and 100 μ l bacterial inoculum. The 96-well microtitre trays were incubated at 37 °C for 18 h. The MIC is defined as the lowest concentration of antimicrobial agent that inhibited the development of visible growth in the well. -

RESULTS

Purification of GST

Table 1 shows the GST activity values toward 1-chloro-2,4-dinitro-benzene and the percentage of enzyme obtained after purification by GSH-affinity column in several bacterial strains; no measurable activity appeared in the column void volume, suggesting that all enzymes remained bound to the column. It has to be noted that the activity values reported for bacteria are much lower than those found in mammalian tissues (Di Ilio *et al.*, 1986, 1988*b*; Del Boccio *et al.*, 1987; Kamisaka *et al.*, 1975*a*; Ketterer, 1986; Mannervik *et al.*, 1985). Relatively high activity was found in *Klebsiella oxytoca* AF 101, *K. oxytoca* CIP 666 and *Enterobacter cloacae* CIP 6085. *Serratia marcescens* CIP 6755 showed the lowest activity. Intermediate values were found in *Proteus vulgaris* ATCC 8427, *Escherichia coli* ATCC 25422, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Proteus mirabilis* AF 2924.

Table 1. GST activity in several bacterial strains after affinity chromatography and percentage of protein retained on the affinity column and eluted by 5 mM-GSH

Strain	Activity [μ mol min^{-1} (mg protein) $^{-1}$]	Protein (%)
<i>Klebsiella oxytoca</i> AF 101	4.80	0.007
<i>Klebsiella oxytoca</i> CIP 666	4.18	0.01
<i>Enterobacter cloacae</i> CIP 6085	3.47	0.05
<i>Proteus vulgaris</i> ATCC 8427	1.47	0.04
<i>Escherichia coli</i> ATCC 25422	1.01	0.003
<i>Proteus mirabilis</i> AF 2924	0.79	0.06
<i>Escherichia coli</i> ATCC 25922	0.76	0.01
<i>Pseudomonas aeruginosa</i> ATCC 27853	0.74	0.002
<i>Serratia marcescens</i> CIP 6755	0.435	0.04
Rat liver	16.1	4.6
Human liver	18.1	2.7
Mouse liver	86.2	3.7

Fig. 1. (a) Slab SDS-PAGE of purified Pm-GST-6-0. Lane 1, standards and their M_r values, from top to bottom, are: bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), α -lactalbumin (14,200); lane 2, Pm-GST-6-0. (b) Slab SDS-PAGE of GSH-affinity-purified GST from several bacterial strains. Lanes 1, 7 and 13, standards; lanes 2 and 12, mouse GSTs; lane 3, rat GST; lane 4, *Klebsiella oxytoca* AF 101; lane 5, *K. oxytoca* CIP 666; lane 6, *Proteus vulgaris* ATCC 8427; lane 8, *Escherichia coli* ATCC 25422; lane 9, *E. coli* ATCC 25922; lane 10, *Serratia marcescens* CIP 6755; lane 11, *Enterobacter cloacae* CIP 6085.

M_r and subunit composition

The results of SDS-PAGE of GSH-affinity-purified GST from several bacterial strains are shown in Fig. 1. Duplicate experiments with two different enzyme preparations gave essentially the same results. A single polypeptide band was obtained in all the preparations, indicating the absence of contaminating proteins. For comparison, rat and mouse liver GSTs were included in the gel shown in Fig. 1(b). All the bacterial GSTs migrated slightly faster than the mammalian GSTs. On the basis of their relative electrophoretic mobilities the GSTs investigated can be divided in two groups. The GSTs of *Proteus mirabilis* AF 2924, *Proteus vulgaris* ATCC 8427, *Escherichia coli* ATCC 25422, *E. coli* ATCC 25922 and *Enterobacter cloacae* CIP 6085 migrated more slowly than the others, suggesting that the subunits of these enzymes have an M_r (22 500) slightly higher than that of *Klebsiella oxytoca* AF 101, *K. oxytoca* CIP 666 and *Serratia marcescens* CIP 6755 (22 000). As the approximate M_r of the bacterial GSTs, as determined by gel filtration on Sephadex G-100, was 50 000 (data not presented), the results of Fig. 1 indicate that the bacterial GSTs studied are all dimeric proteins composed of two subunits with the same M_r .

Antigenic studies

We have recently purified and characterized from *Proteus mirabilis* AF 2924 an isoenzyme of GST with acid properties (pI 6.0) (Di Ilio *et al.*, 1988a). This enzyme (Pm-GST-6.0) has several characteristics that distinguish it from all GSTs purified from mammalian sources. In particular, none of the antisera raised against numerous rat, mouse and human GSTs cross-reacted in immunodiffusion and immunoblotting experiments with this bacterial enzyme. On the other hand, identical results were obtained when anti-Pm-GST-6.0 serum was tested with human, rat and mouse isoenzymes (Di Ilio *et al.*, 1988a). To explore whether Pm-GST-6.0 is a

Fig. 2. Western blot analysis of GSH-affinity-purified GST of seven bacterial strains using antibodies raised against Pm-GST-6-0.

protein specific to *Proteus mirabilis* AF 2924 or is also present in other bacterial strains, the enzymes of *Klebsiella oxytoca* AF 101, *K. oxytoca* CIP 666, *Proteus vulgaris* ATCC 8427, *Escherichia coli* ATCC 25422, *E. coli* ATCC 25922, *Serratia marcescens* CIP 6755 and *Enterobacter cloacae* CIP 6085 recovered from GSH-affinity chromatography were subjected to SDS-PAGE and then transblotted on to nitrocellulose filter and tested against the anti-Pm-GST-6-0 serum (Fig. 2). Only the GSTs of *Proteus vulgaris* ATCC 8427, *Escherichia coli* ATCC 25422 and *Enterobacter cloacae* CIP 6085 cross-reacted with anti-Pm-GST-6-0 serum; no cross-reaction was seen with the GSTs of the other four strains tested, suggesting that Pm-GST-6-0 is not ubiquitously present in the strains examined.

Interaction with antibiotics

The effects of Pm-GST-6-0 on the antimicrobial activity of several antibiotics are reported in Table 2. Quadruplicate experiments with at least two different enzyme preparations gave essentially the same results. In the presence of Pm-GST-6-0 the MIC values for amikacin, ampicillin, cefotaxime, cephalothin and nalidixic acid were significantly increased (two to eight times the control values). This property was specific for bacterial GST, as no effects were seen in the presence of mammalian GSTs or other binding proteins such as human serum albumin.

Table 2. Effect of Pm-GST-6-0 on the antimicrobial activity (MIC) of several antibiotics against *Escherichia coli* ATCC 25922

The minimal inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial agent that inhibited the development of visible growth in the well. MIC values are from quadruplicate experiments with at least two different enzyme preparations. AK, Amikacin; AMP, Ampicillin; CTX, Cefotaxime; KF, Cephalothin; NAL, Nalidixic acid. The concentrations of proteins used are given in parentheses.

Protein in assay	MIC ($\mu\text{g ml}^{-1}$)				
	AK	AMP	CTX	KF	NAL
Control	1	2	0.03	4	2
Human serum albumin (2.5–100 $\mu\text{g ml}^{-1}$)	1	2	0.03	4	2
Mouse GST MI (2.5–100 $\mu\text{g ml}^{-1}$)	1	2	0.03	4	2
Mouse GST MII (2.5–100 $\mu\text{g ml}^{-1}$)	1	2	0.03	4	2
Pm-GST-6-0 (5 $\mu\text{g ml}^{-1}$)	1	2	0.03	4	2
(10 $\mu\text{g ml}^{-1}$)	1	2	0.03	4	32
(20 $\mu\text{g ml}^{-1}$)	1	4	4	8	64
(30 $\mu\text{g ml}^{-1}$)	8	8	4	8	256
(40 $\mu\text{g ml}^{-1}$)	128	8	4	8	512
(50 $\mu\text{g ml}^{-1}$)	128	8	4	8	512
(100 $\mu\text{g ml}^{-1}$)	256	8	4	8	512

The binding capacity of four antibiotics to Pm-GST-6-0 was determined by the quenching of the intrinsic tryptophan fluorescence. The dissociation constants obtained were as follows: cephalothin, 207 nM; ampicillin, 309 nM; amikacin, 434 nM; cefotaxime, 2400 nM. These results indicate that all the drugs tested have a high affinity for the bacterial GST. It is interesting that the dissociation constants for the binding of antibiotics to bacterial GST are quite comparable with the values for haematin and bilirubin binding to mammalian GSTs (Kamisaka *et al.*, 1975*a, b*; Ketley *et al.*, 1975; Maruyama *et al.*, 1983; Takikawa *et al.*, 1986).

DISCUSSION

The data presented here prove that GSTs are present in several bacterial strains. These results are of particular interest as until recently the presence of GSTs in bacteria was uncertain (Lau *et al.*, 1980). This was probably due to the limited number of strains investigated or to difficulties in testing the GSH conjugating activity in their cytosol fractions. In the bacterial strains investigated here, GST ranged from 0.002% of total cytosolic proteins for *Pseudomonas aeruginosa* ATCC 27853 to 0.06% for *Proteus mirabilis* AF 2924. These values are much lower than those (2–5%) found for GSTs of mammalian tissues (Jakoby & Habig, 1980; Ketterer, 1986; Mannervik, 1985). However, as with all other mammalian GSTs that have been examined, the bacterial GSTs we investigated are dimeric proteins in the native state. The mammalian enzymes are composed either of two identical subunits (homodimers) or combinations of two non-identical subunits (heterodimers) (Awasthi & Singh, 1985; Jakoby & Habig, 1980; Ketterer, 1986; Mannervik, 1985; Mannervik & Danielson, 1988). Homodimeric proteins have also been purified from the fungus *Mucor javanicus* (Ando *et al.*, 1988) and the yeast *Issatchenkia orientalis* (Tamaki *et al.*, 1989). The GST of *Tetrahymena thermophyla*, on the other hand, has been reported to be a monomer (Overbaugh *et al.*, 1988).

This paper also shows that bacterial GSTs have characteristics which distinguish them from mammalian GSTs. For example, SDS-PAGE indicates that the subunits of bacterial GSTs are smaller than those of mammalian GSTs. Immunoblotting analysis suggests that the presence of the enzyme we identified as Pm-GST-6-0 (Di Ilio *et al.*, 1988*a*) is not exclusive to *Proteus mirabilis* AF 2924, being also present in *Proteus vulgaris* ATCC 8427, *Enterobacter cloacae* CIP 6085 and *Escherichia coli* ATCC 25422. On the other hand, the lack of cross-reaction of GSH-affinity materials from *Klebsiella oxytoca* CIP 666, *K. oxytoca* AF 101, *Serratia marcescens* CIP 6755 and *Escherichia coli* ATCC 25922 with anti-Pm-GST-6-0 antiserum also suggests the possibility that in bacteria, as in mammals, a wide number of antigenically and structurally different isoenzymes of GST exist. We are currently verifying this hypothesis. The finding that bacterial GST, but not mammalian GSTs, reduces, *in vitro*, the antimicrobial activity of several classes of antibiotics is of particular interest. It is possible that by binding drugs (the dissociation constants obtained indicate that Pm-GST-6-0 avidly binds antibiotics), bacterial GSTs may represent a defence against the toxic effects of antibiotics.

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