Cysteine-86 is not needed for the enzymic activity of glutathione S-transferase 3–3

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Recombinant glutathione S-transferase 3–3 expressed in *Spodoptera frugiperda* (SF9) cells with the use of a baculovirus expression system was modified with 1 mM-iodoacetamide. Amino acid analysis indicated that 0.79 ± 0.15 cysteine residue was modified per enzyme subunit. The S-carbaminomethylated protein retains the GSH-conjugating activity. Glutathione S-transferase 3–3 modified with iodo[14 C]acetamide was digested with *Achromobacter* proteinase I and the resulting peptides were separated by h.p.l.c. The modified peptides were pooled and further digested with *Staphylococcus aureus* V8 proteinase. Isotope-labelled peptides were isolated and collected for N-terminal sequence analysis. By this procedure, cysteine-86 was identified as the major S-carbaminomethylated residue. Verification of this finding came from the use of site-directed mutagenesis in which this cysteine was replaced by serine (C86S mutant). The C86S mutant is enzymically active. Therefore cysteine-86 is not needed for the conjugation of GSH with electrophilic compounds on glutathione S-transferase 3–3.

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a group of enzymes that catalyse the conjugation of GSH with a wide variety of electrophilic alkylating agents. They are also involved in the reduction of organic hydroperoxides, the isomerization of prostaglandins and the binding of non-substrate hydrophobic ligands such as bile acids, bilirubin, a number of drugs and thyroid hormones (for reviews see refs. [1] and [2]).

Rat cytosolic GSTs have been well characterized and documented. At least 11 distinct subunits have been identified in rat tissues, and combinations of these subunits result in 14 homo- or hetero-dimeric GSTs [1,3]. These isoenzymes have been classified into three non-homologous multigene families, namely Alpha, Mu and Pi [4].

For each GST subunit, there is a binding site for GSH (G-site) and a separate binding site for the electrophilic substrate (H-site) [5]. Even though the occurrence of different isoenzymes and the role of GST in biotransformation have been the subject of many investigations, the chemical nature of the amino acid side chains located in the active sites is not known. Using chemical modification methods, Awasthi *et al.* [6] suggested that a histidine residue might be present at or near the G-site of a Pi-family GST from human liver. With a similar approach, van Ommen *et al.* [7] modified a rat liver Mu-class GST with tetrachloro-2,4-benzoquinone and claimed that a cysteine residue is at or in the vicinity of the active site of the enzyme. Several other groups also reported that modification of thiol group(s) decreased the catalytic activity of GSTs [8–10]. In these studies the modified amino acid(s) has not been identified.

Recently we expressed a full-length GST 3 cDNA clone in *Spodoptera frugiperda* (SF9) cells with the use of a baculovirus expression system [11]. This expression system has allowed isolation of large quantities of functionally active homogeneous GST 3–3 of undisputed purity without possible contamination of closely related isoenzyme(s). In the present paper we refer to the active dimer as GST 3–3 and to each subunit as GST 3. We are interested in defining the amino acid residues involved in the

catalytic mechanism of this enzyme. In order to elucidate the involvement of cysteine residue(s) in the catalytic function of GST 3–3, the expressed protein was modified with iodoacetamide, and the S-carbaminomethylated (Cm-)cysteine residue was identified. The cysteine residue thus identified was replaced with serine in the cloned cDNA by site-directed mutagenesis, and the catalytic properties of this mutated enzyme were compared with those of the wild-type and Cm-enzymes.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNA modification enzymes were obtained from New England Biolabs (Beverly, MA, U.S.A.). The oligonucleotide-directed 'in vitro' mutagenesis kit was purchased from Amersham Corp. (Amersham, Bucks., U.K.). The Sequenase kit was obtained from United States Biochemical Corp. (Cleveland, OH, U.S.A.). The [α - 35 S]thio]dATP for sequencing and iodo[14 C]acetamide (6.25 mM solution, specific radioactivity 0.1 mCi/ml) for protein modification were products of New England Nuclear (Wilmington, DE, U.S.A.). *Achromobacter* proteinase I was from Wako (Osaka, Japan). *Staphylococcus aureus* V8 proteinase was from Boehringer Mannheim (Mannheim, Germany). Substrates for enzymic assay were from Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO, U.S.A.). S-(Dinitrophenyl)glutathione (GS-Dnp) was prepared by the method of Lindwall & Boyer [12]. Chemicals used in sequencing were obtained from Applied Biosystems (Foster City, CA, U.S.A.). All other chemicals used were reagent grade or better.

Construction and expression of GST 3 mutants

The Bluescript phagemid (pKs⁺/Yb₁), containing the coding region of GST 3 subunit, has been described previously [11]. The insert was subcloned into the *Bam*H1 site of a M13mp18 vector for single-strand DNA production. An oligonucleotide (19-mer), 5'-CACCACCTGAGTGGAGAGA-3', was synthesized for

Abbreviations used: GST, glutathione S-transferase; Cm, S-carbaminomethylated; GS-Dnp, S-(dinitrophenyl)glutathione.

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mutating the cysteine at position 86 to serine. Mutagenesis was performed by the procedure of Taylor *et al.* [13]. Candidate plaques were screened by dideoxy sequencing [14] with an internal sequencing primer (5'-ATTACCCAGAGCAATGCC-3') located 33 nucleotide residues upstream of the mutation site. One clone containing the cysteine-to-serine mutation was selected and plaque-purified to ensure clonal purity. The *Bam*H1 fragment on this clone was sequenced in its entirety to ascertain that only the desired mutation and no other mutations, re-arrangements or deletions had occurred during the manipulations.

The mutated fragment was excised from the replicative form of the M13mp18 vector, then inserted into the *Bam*H1 site of the baculovirus transfer vector pAcYM1 and designated pAcYM1/C86S for expression in SF9 cells [11]. The SF9 cells were maintained in culture at 26 °C in TNM-FH medium (GIBCO, Grand Island, NY, U.S.A.) as described by Summers & Smith [15]. Plasmid pAcYM1/C86S was cotransfected into SF9 cells with wild-type viral DNA, AcNPV. Transfer of the GST 3 coding sequence from pAcYM1/C86S to the AcNPV genome was achieved by homologous recombination into the baculovirus polyhedrin gene. The recombinant baculovirus was identified in infected insect-cell monolayers by screening the plaques with rabbit antisera raised against wild-type GST 3 subunits. After three cycles of plaque purification, plaques exhibiting no evidence of occlusion bodies (viral polyhedra) by transmission light microscopy were recovered for infection of SF9 monolayer cultures for production of recombinant GST 3 C86S mutants.

Purification of enzymes

The expressed wild-type or mutant proteins were purified by affinity chromatography by the procedure of Mannervik & Guthenberg [16]. GSTs eluted from the column were dialysed against 10 mM-potassium phosphate buffer, pH 7.0, containing 0.5 mM-dithiothreitol. The purity of the enzymes was analysed by SDS/PAGE [17]. Proteins were stored as 1 mg/ml solutions containing 20 % (v/v) glycerol at -70 °C.

Enzyme assay

GST activity was assayed by published methods [18–20] at 25 °C. Protein concentration was determined from absorbance at 280 nm. A protein solution with a concentration of 0.56 mg/ml has 1 A_{280} unit [11]. This value was used for both wild-type and mutant recombinant proteins.

Protein modification

GST 3–3 (0.75 nmol) was modified in the dark (24 h at 25 °C) in a solution (100 μ l) containing Tris/HCl buffer (0.06 M, pH 7.9) and 1 mM-iodo[14 C]acetamide (1.6 μ Ci). The reaction was stopped by passing the solution through an Aquapore RP-300 C₈ reverse-phase column (22 cm $\times$ 0.46 cm). The protein was eluted from the column at a flow rate of 1 ml/min with a linear gradient of 1 %/min of 0.08 % trifluoroacetic acid in acetonitrile. For enzymic assay, GST 3–3 was modified with non-radioactive iodoacetamide under identical conditions and used without purification. The final concentration of iodoacetamide in the assay solution was less than 0.01 mM, depending on the amount of enzyme required in the assay mixture.

Endopeptidase digestion and peptide purification

Modified proteins eluted from the reverse-phase column were dried in a Speed Vac concentrator, then redissolved in 0.075 M- Na_2CO_3 , pH 9.5, containing 2 M-urea. The protein was digested with *Achromobacter* proteinase I (16 h at 30 °C) at a substrate/enzyme ratio of 50:1 (w/w). Resulting peptides were separated on a reverse-phase column under the conditions

outlined above, and 14 C-labelled peptides were identified by liquid-scintillation counting. Fractions of interest were pooled and dried down in a Speed Vac concentrator for *Staphylococcus aureus* V8 proteinase digestion.

Digestion with *Staphylococcus aureus* V8 proteinase was carried out in 0.075 M- NH_4HCO_3 , pH 7.8, containing 2 M-urea. For a typical experiment, 14 C-labelled peptides generated from 320 μ g of GST 3 subunits were digested with 6 μ g of proteinase for 16 h at 37 °C. 14 C-labelled peptides were purified and identified as above.

Protein sequencing and amino acid analysis

Automated cycles of Edman degradation were performed with an ABI gas/liquid-phase model 470A/900A sequencer equipped with an on-line model 120A phenylthiohydantoin analyser [21]. Proteins were hydrolysed in the gas phase (24 h at 110 °C) with 6 M-HCl containing 1 % (w/v) phenol. After hydrolysis, samples were analysed in a Waters PicoTag amino acid analysis system according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Chemical modification of GST 3–3 with iodoacetamide

GST 3–3 was incubated for 24 h with 1 mM-iodoacetamide. The storage buffer for GST 3–3 contains 0.5 mM-dithiothreitol. Assuming that dithiothreitol can react completely with iodoacetamide, then the ratio between the thiol groups on the enzyme and iodoacetamide would be 1:13. The modified protein was purified on a reverse-phase column, then hydrolysed with 6 M-HCl for quantitative amino acid analysis.

Crabb *et al.* [22] appraised the accuracy and precision of amino acid analysis, and pointed out that the recovery of phenylalanine gives the least error. A sample of bovine pancreatic trypsin inhibitor was treated with dithiothreitol and iodoacetamide, then subjected to amino acid analysis. The ratio of the peak area per residue on the chromatogram between Cm-cysteine and phenylalanine was determined to be $1:1.20 \pm 0.02$. This value was used in estimating the number of Cm-cysteine residues in the modified GST 3. With 1 mM-iodoacetamide and 24 h incubation, we identified 0.79 ± 0.15 Cm-cysteine residue per GST 3 subunit. At high iodoacetamide concentration (200 mM) and 5 min incubation, 1.01 ± 0.07 Cm-cysteine residue was recovered per GST 3 subunit. Evidently, only a single cysteine residue is accessible to iodoacetamide in the native protein [23].

The inaccessibility of cysteine residues for iodoacetamide modification probably reflects a substantial amount of ordered structure retained in strong denaturing conditions [24]. In the presence of 6 M-guanidinium chloride, iodoacetamide reacted with 2.4 ± 0.1 cysteine residues per GST 3 subunit. The same result was obtained for enzymes denatured by passage through a reverse-phase column. This value was not affected by pretreating the sample with 10 mM-dithiothreitol at 50 °C for 3 h. Therefore there are no disulphide bonds formed among the cysteine residues. Our finding was in close agreement with that of Carne *et al.* [8]. In their work with ligandin (GST 1–1 and GST 1–2) they reported that out of 4 cysteine residues only 0.9 cysteine residue per subunit can react with 5 mM-iodoacetamide, and 3.1 mol of Cm-cysteine was formed per mol of ligandin in the presence of 8 M-urea.

GS-Dnp is the conjugation product of GST and 1-chloro-2,4-dinitrobenzene. It has been shown that GS-Dnp can protect GST from modification by *S*-(*p*-azidophenacyl)glutathione [25]. *S*-Hexylglutathione is a competitive inhibitor for both rat [26] and human [27] transferases. Presumably these two compounds can interact with the active site of GST. GST 3–3 was modified with

Fig. 1. Effect of inhibitors on iodoacetamide labelling of GST 3-3

GST 3-3 was labelled with 1 mM-iodo[¹⁴C]acetamide in the presence of *S*-hexylglutathione (●) or GS-Dnp (△). The labelled protein was purified by h.p.l.c. The radioactivity and u.v. absorbance at 220 nm of the labelled protein were determined and expressed as a ratio.

Fig. 2. (a) H.p.l.c. profile of *Achromobacter* proteinase 1 digests of iodo[¹⁴C]acetamide-modified GST 3 subunits and (b) radioactivity in the fractions obtained in (a) (measured by liquid-scintillation counting)

—, A_{220} ; ----, concn. of buffer B.

iodo[¹⁴C]acetamide in the presence of increasing amounts of GS-Dnp or *S*-hexylglutathione. The mixture after incubation was separated on a reverse-phase column and the elution profile was monitored at 220 nm. The radioactivity and peak area corresponding to the GST 3 of each sample was determined and expressed as a ratio. A plot of this ratio versus amount of GS-Dnp or *S*-hexylglutathione in the reaction mixture is presented in Fig. 1. The data clearly indicate that neither GS-Dnp nor *S*-hexylglutathione can inhibit the modification of GST 3-3 by iodoacetamide.

Identification of Cm-cysteine in modified GST 3-3

GST 3-3 was treated with 1 mM-iodo[¹⁴C]acetamide (1.6 μ Ci), then digested with *Achromobacter* proteinase I, and the resulting peptides were separated by h.p.l.c. A minor (fractions 64-66) and

Fig. 3. (a) H.p.l.c. profile of *Staphylococcus aureus* V8 proteinase digests of fractions 79-95 from Fig. 1(a) and (b) radioactivity in the fractions obtained in (a)

—, A_{220} ; ----, concn. of buffer B.

a major (fractions 79-95) radioactive peak were identified, which represent approx. 3% and 92% of the total radioactivity (c.p.m.) respectively (Fig. 2b).

Fraction 64 shows u.v. absorption at 220 nm (peak L-1 in Fig. 2a), and was collected for sequence analysis. A 20 μ l sample of amino acid phenylthiohydantoin derivative was removed from each cycle of sequencing for liquid-scintillation counting. L-1 has the amino acid sequence RYAMGDAPDYDRSQWLNEK, which represents residues 31-49 of the GST 3 subunit. The methionine residue of this peptide is radioactive. Therefore Met-34 is a minor target for iodoacetamide modification.

Fraction 82 (peak L-2 in Fig. 2a) has the sequence LGLDFPNLPYLIDGSRK (residues 52-68). Further analysis of fraction 82 on a reverse-phase column with heptafluorobutyric acid providing the counter-ions [28] revealed that L-2 was contaminated with minor radioactive peptides (results not shown).

Fractions 79-95 probably represent peptides of various lengths with similar sequences generated from incomplete digestion of GST 3-3. They were pooled and further digested with *Staphylococcus aureus* V8 proteinase. Most of the ¹⁴C label (60%) was eluted in a sharp peak with 18% acetonitrile (peak G-1 in Fig. 3a and fractions 36-37 in Fig. 3b). G-1 has the sequence HHLCGETEE. The Cm-cysteine phenylthiohydantoin derivative can be identified on the chromatogram of the phenylthiohydantoin analyser (results not shown). Radioactive Cm-cysteine phenylthiohydantoin derivative was also recovered from the sequencer.

G-2 (Fig. 3a) has the sequence HHLCGE and represents 4.7% of the radioactivity recovered from the column. Fractions 72-75 contain 10% of the total recoverable radioactivity. A well-resolved peak from these fractions was selected for sequencing (peak G-3 in Fig. 3a). G-3 has the sequence NQVMDNRM-QLIMLCYNPDFE, but we have not recovered significant

hydroxy group relative to that of a thiol group should ensure a low concentration of active nucleophile in solution at physiological pH. Therefore the replacement of cysteine by serine should cause a marked change in the electronic environment around residue 86. Nonetheless, replacing Cys-86 with serine results in an enzymically active C86S mutant. This report cannot settle the dispute of whether cysteine is involved in the function of class-Mu GST or not, but our results indisputably demonstrate that a thiol group at position 86 is not needed for the enzymic function of GST 3-3.

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